METAL-ION BINDING AND THE MOLECULAR CONFORMATIONAL PROPERTIES OF α LACTALBUMIN

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DEDICATION

For C. P. K. who knows why. For David and Emy who have listened patiently to all of this for some years now and who have provided support and encouragement even though they didn't know it.

I. AN OVERVIEW OF THE PROPERTIES OF α LACTALBUMIN AND ITS ROLE IN THE LACTOSE SYNTHASE REACTION

A. INTRODUCTION

The subject of this review, the milk-specific protein α lactalbumin (α LA), is the regulatory component of the lactose synthase complex which catalyzes the biosynthesis of lactose in the Golgi apparatus of the mammary gland during lactation. Although this protein was identified, perhaps as early as 1885, as a component of milk, its biological activity was not recognized until almost 90 years later. The period subsequent to 1964 has seen extensive study of this protein, not all of which, by any means, has been directed toward its role as a modulator of lactose biosynthesis. The low molecular weight of the protein, 14,200, has made it attractive as a "model" in attempts to formulate generalizations concerning molecular conformation, conformational changes, and stability of proteins. The sequence homology of α LA and hen's egg white lysozyme demonstrated in 1967 has fostered a number of studies which have explored the relationship between molecular conformation and primary structure and the corollary question of the relationship between amino acid sequence and conformational stability. The discovery in 1980 that α LA, as isolated, is a metalloprotein with calcium as its natural ligand, together with the observation that metal-ions are required for lactose synthase activity, has generated extensive study of the binding of such substances to the protein. To date, however, there is no clear-cut indication of how binding of metal-ions to α LA *itself* is related to its regulatory function in the lactose synthase reaction, or whether indeed it has a functional role.

The sequence homology of hen's egg white lysozyme and α LA suggested that their three-dimensional structures must be very similar. While the likely resemblance of the three-dimensional structures for α LA and lysozyme was recognized as early as 1969, it was only

in 1986 that the crystal structure for the former protein became available and established that the overall structures of the two proteins *were* indeed very nearly alike. Unlike lysozyme, which binds to metal-ions very weakly, the structure of α LA shows a well-defined unique calcium-binding site quite different from those found for the EF-hand class of proteins.

The proposed resemblance of the three-dimensional structures of these proteins led to a corollary proposal concerning their mode of action functionally, i.e., since lysozyme binds oligosaccharides prior to cleavage at β-1,4 glycoside linkages, it was thought that α LA might bind monosaccharides prior to *formation* of such linkages during lactose synthase action. However, no evidence has been forthcoming to date to indicate that isolated α LA can bind sugars. Indeed, comparatively little is still known about the molecular basis for its regulatory function in the lactose synthase reaction beyond what has been discussed in earlier reviews[1-3] of the properties of α LA and lactose synthase action.

Although the recent crystallographic studies of the three-dimensional structure of α LA do indeed indicate a great similarity with that of type c lysozymes, in accord with their sequence homology, investigations of their comparative molecular properties indicate significant differences. These proteins have very different stabilities; although complete disruption of lysozyme or α LA requires exposure to harsh conditions, e.g., high concentrations of denaturants, removal of the calcium ion from the native form of the latter protein "melts" the tertiary structure, with little or no change in the secondary structure. The metal-free form of α LA has been shown to be an *unfolding intermediate* comparable to the one found for lysozyme under denaturating conditions.

Lysozyme and α LA also differ markedly in their metal-ion-binding properties. The former protein binds such ions very weakly, if at all (see Section II for an exception to this generalization), while the latter has a number of binding sites for metal-ions including Ca^{+2}, Mn^{+2}, and Zn^{+2} (these observations are outlined later and are discussed in depth in Section II of this review). It is of particular interest that these cations bind to α LA since they also bind to galactosyltransferase (GT) and are required for its activation. The major focus of this review is the complex relationship between the intricate pattern of the transconformational changes and the binding of different metal-ions. The crystal structure of baboon α LA[33,34] and the detailed delineation of the calcium-binding site provide a basis for consideration of such molecular changes and their possible functional significance.

We present in the remainder of this introductory section an overview of the properties of α LA, particularly earlier observations which require reevaluation in light of recent findings. These observations are discussed in depth in subsequent sections of this review. In the final section, we advance some "projections" of a more speculative nature concerning the biological activity of α LA and its relationship to metal-ion binding and molecular conformation.

It has been useful in many studies to correlate properties of α LA with changes in the amino acid sequence of different species of the protein (a discussion of the amino acid sequence of α LA follows). The following abbreviations are used in referring to these α LAs: BLA, bovine; BBLA, baboon; CLA, camel; ELA, equine; GLA, goat; GPLA, guinea pig; HLA, human; RLA, rat; RBLA, rabbit; WLA, wallaby.

B. THE PRIMARY STRUCTURE OF α LACTALBUMIN

The sequence homology of α LA and hen's egg white lysozyme was first demonstrated by Brew et al.[4-6] and subsequently confirmed by the sequences for a number of other species of the protein.[7-14] Figure 1 illustrates the sequences established for the nine species of the protein. There are 35 invariant residues (Table 1), with a considerably larger number of residues which represent conservative replacements. It is of interest that the rat protein differs from the others in having an extension at the C terminus, and WLA has a single additional residue above 95.

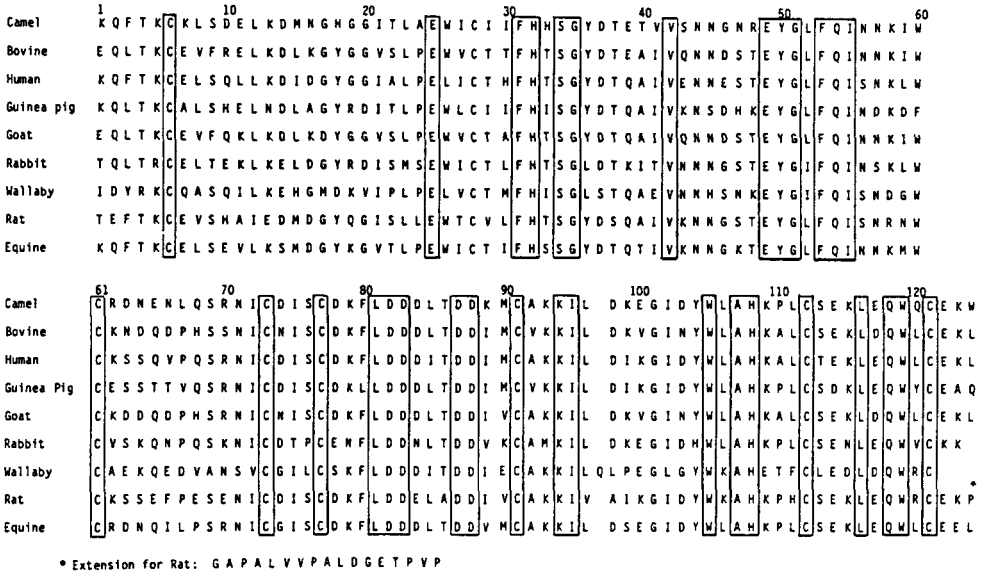


FIGURE 1. Amino acid sequences of camel,[14] bovine,[4,13] human,[9] guinea pig,[8] goat,[12,13] rabbit,[11] wallaby,[13] rat,[10] and equine[7] α LA. The boxed areas indicate residues invarient in the nine proteins.

The close similarity of the nucleotide sequence for RLA, HLA, and GPLA and lysozyme[3,15,16] and the intron-exon pattern for the human gene[15] (Table 2) provides still further evidence for the homology of the milk and egg white proteins and suggests evolution from an common ancestral gene. Shewale et al.[13] discussed the evolution of the α LA at length; we therefore do not consider this aspect of the primary structure further here.

The invariant residues in the sequences (Figure 1) have been arranged in Table 1 according to the classification given by Shewale et al.[13] Residues leu 81, asp 82, 83, 87, and 88 (group IV) are located in the calcium-binding "elbow" seen in the crystal structure[33] (Sections I.D and II.G). These are found in exon III (Table 2). Residues which are probably implicated in the biological activity are also found in group IV and are coded by exons I, II, and IV (Table 2).

C. MOLECULAR PROPERTIES

Preparations of α LA from milk in modern studies have utilized the procedures of Gordon and Semmett[17] or Aschaffenburg and Drewry,[18] or modifications thereof. The former method requires manipulations which are cumbersome and may potentially result in bacterial contamination of the protein; the latter method is more convenient, but necessitates exposure of the protein to elevated temperatures and use of concentrated HCl to isolate the α LA, conditions under which the molecular conformation of the protein is altered (see later). The low pH and thermally induced conformational changes, however, are reversible and α LA prepared by a third procedure,[19] which circumvents the need for exposure of the protein to low pH or to elevated temperature, is indistinguishable from that prepared by the earlier methods[17,18] (a discussion follows of the stability of the metal-free protein).

1. Self-Association of α LA

The molecular weight of monomeric BLA determined from the amino acid sequence[4] is 14,200. The protein has a marked propensity for self-association at low pH, in its isoelectric region (pH 3.5 to 4), and to a lesser, but significant degree, at neutral pH.[20,21] Binding of anions to the low pH form of the protein has a marked effect on its state of aggregation.[19,20]

TABLE 1
Invarient Amino Acids in α LA Sequences

Group*	Residues	Role
I	cys 6, 28, 61, 73, 77, 91, 111, 120	Probably structural role; cys 77 and 91 are of particular interest since they flank the calcium-binding "elbow" and a pair of orthogonal helical segments (Section II.G)
	gly 51	
	gly 100	
	ser 34	
	tyr 50	
	leu 81	
II	asn 44	No function has been demonstrated for α LA; they appear to be implicated in substrate binding in lysozyme
	gln 54	
	ile 55	
	trp 104	
	ala 106	
III	glu 49	No function known for α LA implicated in catalysis and substrate binding in lysozyme
	his 107	
	val 42	
IV	leu 81	Located in calcium-binding "elbow" of α LA
	asp 82	
	asp 83	
	asp 87	
	asp 88	
	glu 25	Function in α LA
	lys 94	
	ile 95	
	phe 31	These residues lie in the contact region with galactosyltransferase in the lactose synthase complex; his 32 and trp 118 may be essential for lactose synthase activity (Section I.F)
	his 32	
	gly 35	
	leu 115	
	gln 117	
	trp 118	

Note: See Figure 1 for sequences.

* The groups as defined by Shewale et al.[13] are based on the variability in sequence for lysozymes and α LA and on the role of these conserved residues in lysozyme: I, structural for lysozyme; II, oligosaccharide binding for lysozyme; III, residues which are invarient for both lysozyme and α LA but differ in the two proteins; IV, residues which are variable in lysozyme but invarient in lactalbumin.

Adapted from Table 12 in Reference 13.

TABLE 2
Exons in the Human α LA Gene

Exon	Residues coded
I	1 to 25
II	26 to 78
III	79 to 104
IV	105 to 123

From Brew, K., Vanaman, T., and Hill, R. L., *J. Biol Chem.*, 242, 3747, 1967.

It seems likely that many of the earlier reports of heterogeneity of α LAs were the result of self-association, although there is probably substance to the reports of heterogeneity of genetic origin for protein prepared from pooled milks.[1] Another type of heterogeneity has been observed by numerous investigators on chromatography of α LA on DEAE-media, where two peaks are commonly observed which are not stable molecular components. Since Fenna[22,23] observed only a single peak when BLA or HLA was chromatographed in the presence of 1 m*M* Ca^{+2}, this apparent heterogeneity likely results from dissociation of the bound calcium ion during, or prior to, this step in the preparation of the protein.

Binding of different cations to metal-free α LA has a marked influence on its association behavior at pHs well above its isoelectric point, e.g., while solutions of Ca^{+2}-BLA in the millimolar concentration range are optically clear near neutral pH, comparable solutions of Zn^{+2}- and Mn^{+2}-BLA are visibly turbid or exhibit precipitates.[40,92] Such phenomena are likely to be significant where the technical requirements of a procedure necessitate the use of protein concentrations approaching, or in excess of, 1 m*M*, e.g., NMR and ESR. While the use of high protein concentrations may be unavoidable in some procedures, some investigators appear to be insensitive to the effect that such self-association may have on their observations. The following illustrates the significance of self-association of α LA on its properties: (1) the association constant for binding of Mn^{+2} to the high affinity site of metal-free BLA decreases 20-fold over the protein concentration range of 4 to 1000 μ*M*;[40] (2) the extent of reaction of a hydrophobic photoreactive probe with native BLA increases 6-fold over a protein concentration range of 35 to 210 μ*M*;[24] (3) histidine 68 of native BLA, while reactive with diethyl pyrocarbonate at a protein concentration of 70 μ*M*, is completely unreactive at a concentration of 3 m*M*.[25] These observations indicate that, while self-association of the calcium form of the protein may be lower than other metal-ion-liganded forms, it may nonetheless have a signficant effect on properties measured at high protein concentrations.

2. Conformational States of α LA

Exposure of native α LA (N state) to pHs below 4 promotes virtually instantaneous formation of a conformer (A state) whose properties are markedly different from those of the native protein. Such differences include a markedly increased propensity for self-association and characteristic changes in spectroscopic properties (Table 3). These and other differences characteristic of N and A states are discussed in detail in Section III of this review.

Changes in the absorption-, fluorescence-, visible-, and near-UV ORD- and the near-UV CD-spectra (Table 3) suggested that the α LA molecule might be unfolded in the A state, thereby exposing "buried" tryptophans to the aqueous medium. The aggregation of α LA observed at pH 2 (References 21 and 210) was also thought to reflect a "denatured-like" molecular state. However, this conclusion is not valid. Solvent perturbation measurements indicate that, in spite of the fact that the spectral changes observed for the N-A transition are comparable to those seen on denaturation of many proteins, there is no increase in the average extent of exposure of tryptophan residues on formation of the A conformer (Table 3). (The decrease in the Yang-Moffitt parameter b_o observed in optical rotation dispersion spectra for N and A conformers results from the abolition of aromatic side chain-Cotton effects in the wavelength range of ca. 250 to 300 nm.) The far-UV ORD- and CD-spectra, however, exhibited none of the changes characteristic of "melting" of the secondary structure of the protein.

The α LA molecule in the A state is currently seen as folded, albeit somewhat less compactly than that in the N state, and of more "fluid-like" character than that of the native protein. The side chains in the A state have increased freedom of movement with no spatial correlation. The characteristics of this state, those of the so-called "molten globule model",[26] are considered at length in Sections III and IV.

TABLE 3
Alteration of the Spectroscopic Properties of BLA in the Low pH N-A Conformational Change

Property	Change in property, N - - - - A	Ref.
UV absorption spectra	Short wavelength shift of spectra; difference spectra characteristic of tryptophan residues	91
Fluorescence tryptophan	Long wavelength shift of spectra; increase in quantum yield	40, 92, 93, 146, 147
Solvent perturbation absorption spectra	Spectra indicate that the number of "exposed" tryptophans is unchanged in the A state	94, 95
ORD, visible near-UV	Decrease in parameter b_o, indicative of helix melting	96
ORD, far-UV	Cotton effects characteristic of helix essentially unchanged	96
CD, near-UV	Complex spectrum characteristic of tryptophan residues in the N state are essentially abolished in the A state	97—99
CD, far-UV	Spectra indicative of secondary structure are essentially unchanged	97—99

Note: N = native conformation; A = low pH conformer (U conformer in the earlier literature); ORD = optical rotation dispersion; CD = circular dichroism.

Molecular states similar to those formed at low pH are also promoted by: (1) heating of the protein above 50°C at neutral pH (Reference 27); (2) exposure of the protein to pHs above 9 (Reference 148); (3) treatment of the protein with low concentrations of guanidine-HCl (Reference 28); and (4) removal of the calcium ion from native protein. The conformational changes which occur at low and high pH and at elevated temperatures all appear to involve dissociation of the calcium ion.

The classification of the molecular states of α LA as "N" or "A" is based on the magnitudes of the parameters of their near-UV CD- or tryptophan emission spectra. Although the values of the spectral parameters reported by different investigators differ somewhat, the following generalizations can be made: *tryptophan emission maxima:* A states ≥ N states; *tryptophan quantum yields:* A states ≥ N states; *amplitudes, near-UV CD-spectra:* A states ≤ N states. Although many investigators refer to N and A states as if *each* were a unique molecular conformation, this does not appear to be the case. The preponderance of evidence favors the view that the character of both N and A states depends on the conditions under which they are formed, including the nature of the liganded metal-ion (see Sections II and III.F). We prefer, therefore, to refer to such states as "N-like" and "A-like", where such names pertain to their "classification" according to the spectral criteria cited earlier.

Perhaps the most striking feature of the N to A transformation is its "global" character, i.e., dissociation of the calcium ion alters the molecular environment of a large number of side chains quite distant from the calcium-binding site (see later). This change completely abolishes a large number of the characteristic side chain-side chain spatial relationships characteristic of the native protein molecule.

D. THE THREE-DIMENSIONAL STRUCTURE OF THE α LACTALBUMIN MOLECULE

The sequence homology of α LA and hen's egg white lysozyme (see Section I.B) demonstrated by Brew et al.[4] led to the conclusion that the three-dimensional structures of these two proteins must be very similar. Subsequently, Browne et al.[29] formulated a detailed "lysozyme-analogy" structure for BLA and constructed a wire-skeletal model based on the X-ray structure for lysozyme and the amino acid sequence of the former protein. Warme et al.[30] computed the three-dimensional structure of BLA by an energy minimization procedure using as a starting point the lysozyme crystal structure and the BLA sequence. This structure, not surprisingly, was very similar to that formulated by Browne et al.,[29] although there were

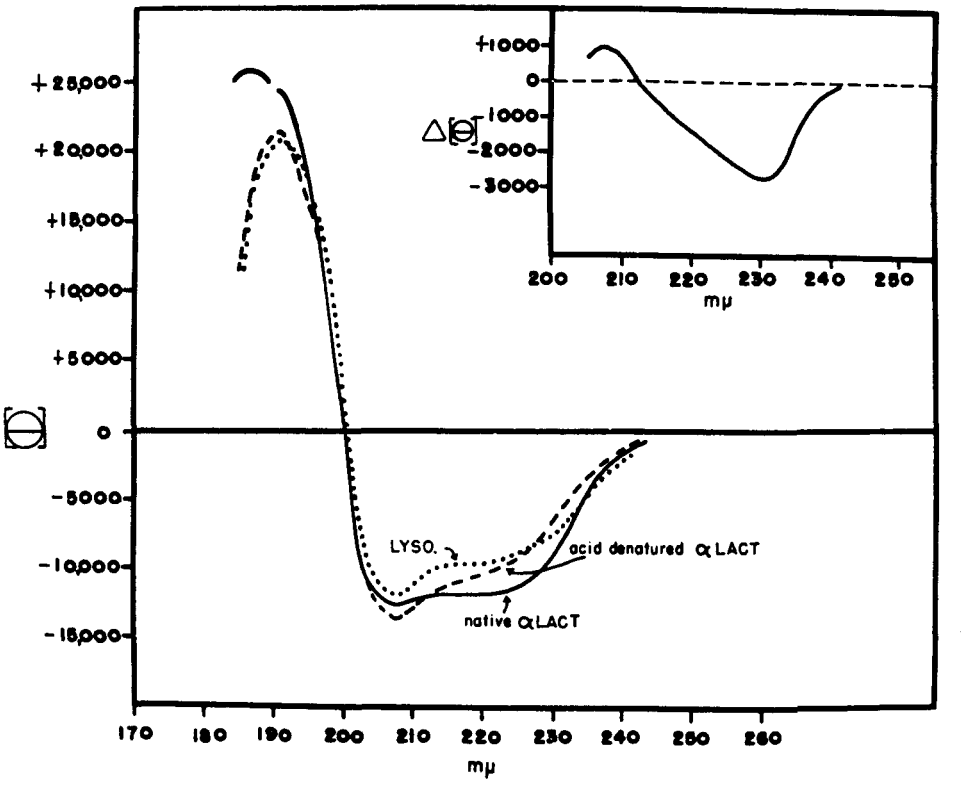


FIGURE 2. Circular dichroism spectra of lysozyme and native and acid "denatured" α LA. Solvent, pH 7, 0.15 *M* KCl or pH 7.5, 0.02 *M* Tris. The "denatured" protein was measured in pH 4, 0.15 *M* KCl. Insert: difference in circular dichroism spectra for native and "denatured" α LA. (From Kronman, M. J., *Biochem. Biophys. Res. Commun.*, 33, 535, 1968. With permission.)

some significant differences in the C-terminal "tail" region. These structures show that the α LA molecule has a cleft-like region, homologous with the active site cleft of lysozyme, dividing the molecule into two lobes. CD measurements in the far-UV demonstrate that the secondary structures of these two proteins are indeed very similar[31,32] and further that *both* the N and A conformers resemble that of lysozyme (Figure 2), demonstrating that the secondary structure of the N and A conformers do not differ markedly.

Although crystallographic studies of α LA began more than 15 years ago, it has only been recently that the technical problems plaguing these studies have been overcome. Stuart et al.[33] recently published a high resolution (1.7 Å) structure for the Ca^{+2}-binding site of BBLA and Smith et al.[34] presented a preliminary low resolution (4.5 and 6 Å) structure for the entire molecule. While the latter resolution is insufficient to give useful information about the molecular environment of individual side chains, the structure shows that the folding of the polypeptide backbone of BBLA is, as predicted, similar to that of hen's egg white lysozyme. The models of Browne et al.[29] and of Warme et al.,[30] therefore, provide a reasonable tentative description of the spatial relationships in this molecule. Because of an absence of a high-resolution structure at present, our subsequent discussions of spatial relationships in the α LA molecule are based on measurements made on a wire-skeletal model constructured according to the procedure of Browne et al. or on distances calculated from coordinates derived from the energy minimization study of Warme et al.

The structure of the high affinity Ca^{+2}-binding site is known in great detail[33] (Section II.G). All of the metal-ion-coordinating atoms are oxygens, five from the protein and two

from water molecules. The binding site consists of an "elbow" comprising ten residues connecting two helical segments. Three of the coordinating residues are aspartate ions. The calcium-binding elbow of α LA is a unique calcium-binding site, with only superficial resemblence to the calcium-binding sites of the "EF-hand" proteins. The structure of the Ca^{+2}-binding site is discussed in detail in Section II.G.

E. THE METAL-BINDING PROPERTIES OF α LACTALBUMIN

Hiraoka et al.[35] were the first to demonstrate that α LA, as isolated, contained a single tightly bound calcium ion and that its abstraction from the protein with EDTA, without removal of the Ca^{+2}- EDTA complex, altered the stability of the protein. Isolation of the metal-free protein (apo-protein) was subsequently carried out by an EDTA-treatment procedure.[36] While complete removal of the chelator or the chelator-Ca^{+2} complex can be demonstrated using [^{14}C]-EDTA, we avoid using chelators in preparing apo-α LA and in other experiments with this protein since the binding of EDTA-Ca^{+2} to apo-BLA has been demonstrated[37] (see Section I.B for a discussion on the effect of binding of EDTA-Ca^{+2} to apo-BLA). We currently isolate apo-α LA by gel filtration of the protein at pH 2 ("Suprapure" HCl), with subsequent removal of the acid by lyophilization and reconstitution in very dilute pH 5 to 6 metal-free Tris buffer.[37] Solutions of apo-α LA are stored in the frozen state at concentrations of 3 to 5 m*M*.

Preparation of apo-BLA has been carried out by treatment of the native protein with 100 μ*M* EDTA in 5 *M* guanidine-HCl with subsequent removal of the denaturant by gel filtration in the presence of 100 μ*M* EDTA;[38] such preparations are "unstable", the authors noting that the aggregation occurred after 5 h of solution of the protein. Desmet et al.,[39] using the same procedure for preparing metal-free protein, have confirmed this time-dependent aggregation. Since neither we, nor other investigators, have observed any indication of instability of their preparations of apo-α LA prepared by either the low pH gel filtration or the EDTA procedures, it seems possible that treatment of the protein with guanidine-HCl during its calcium removal results in its damage.

However, a loss of calcium-binding ability of α LA has been noted by Bratcher and Kronman,[40] who observed that preparations of native BLA after prolonged storage in the refrigerator or freezer would no longer rebind calcium ($^{45}Ca^{+2}$-binding measurements or fluorescence titration) after passage through a column of Chelex 100. Freshly prepared BLA could be demetallized without loss of calcium-binding ability. The origin of this loss in calcium-binding ability is not known.

The sole metal-ion found in isolated native BLA is calcium. Plasma emission spectroscopy for some 26 metals showed them to be below the level of detection (0.02 to 0.2 ppm) with only trace amounts, ca. 0.1 g·a/mol of protein, of manganese and zinc present.[261]

The discovery that α LA is a metalloprotein has generated profound interest in its metal-binding properties since metal-ions are required for the activation of the lactose synthase reaction (Section I.F). The results of a large number of studies summarized in Tables 6 to 8 indicate that apo-α LA binds some 15 different metal ions. These include Ca^{+2}, Mn^{+2}, Zn^{+2}, Co^{+2}, and the monovalent cations Na^+ and K^+. The divalent cations are those which have been found to activate the lactose synthase reaction, while K^+ has been observed to *inhibit* this reaction (Section V). Activation of the catalytic process is discussed in Section I.F.

The binding of these metal-ions and their effect on the conformational state of α LA are exceedingly complex. Some of the salient features of these processes are outlined here (and are discussed in detail in Section II):

1. Binding of Ca^{+2}, Mn^{+2}, Na^+, and K^+ promotes formation of N-like states, while Zn^{+2} and Co^{+2} promote A-like states. The decrease in the association constant for

binding of Ca^{+2} in the pH range 4 to 5 demonstrates that the low pH N-A transition observed with native protein results from dissociation of this cation.

2. There are two binding sites for Ca^{+2} and three, or perhaps four, for Mn^{+2}. Occupancy of the sites of highest affinity for either of these two cations promotes formation of the N conformation.
3. Mn^{+2} and Ca^{+2} do *not* bind competitively and thus appear to bind at *different* high affinity sites, although the binding affinities at these sites appear to be "linked". The *pattern* of the binding of these two cations appears to be kinetically determined, with the extent of binding of each depending on the order of mixing of protein and the two different metal-ions.
4. Terbium, a luminescent ion which can frequently substitute for calcium, binds at three sites. Binding at the site of highest affinity promotes formation of a *N-like state*. Binding at a second site, while maintaining the conformation in an *N state*, decreases the binding affinity at the high affinity site; binding of Tb^{+3} at a third site gives rise to a time-dependent conformational change to an *A-like* state, this state being distinguishable spectroscopically from the metal-free protein.
5. Zn^{+2} binds at two sites on apo-BLA. The conformation promoted on binding at the site of higher affinity is "A-like", but distinguishable spectroscopically from the A state of the metal-free protein. Simultaneous binding of Zn^{+2} and Ca^{+2} is possible until the Zn^{+2} site of lower affinity is occupied, whereupon the calcium ion is dissociated on formation of an "expanded A" state similar to that observed on binding of terbium to its site of lowest affinity.
6. Both potassium and sodium on binding to apo-BLA promote formation of a N-like state. Competitive binding measurements indicate that sodium ion probably binds at the high affinity calcium site.
7. There is considerable evidence that the N-like states of Ca^{+2}-, Mn^{+2}-, and K^{+}-BLA have different molecular character. The difference in the properties of these "N-like" states is discussed in Section III.

We describe in Section II a model for binding of Ca^{+2}, Mn^{+2}, Tb^{+3}, and the monovalent cations Na^{+} and K^{+}, with the central feature being three adjacent interacting sites for these metal-ions, as well as for Co^{+2} and Zn^{+2} whose binding promotes formation of A-like states. This model is based on the crystal structure of the calcium-binding site for BBLA and a ^{13}C-NMR study of Gerken,[41] the latter establishing the position of a manganese-binding site near the N-terminal α amino group, adjacent to the calcium-binding site. Our model provides a plausible explanation of how binding of cations at these sites shifts the conformational equilibria between a large number of molecular states. It also provides a tentative description of the route of propagation of the conformational change through a specific segment of the polypeptide chain from the metal-ion-binding sites to more distant region of the α LA molecule, thereby accounting for the global character of the change.

F. THE ROLE OF α LACTALBUMIN IN LACTOSE SYNTHASE ACTION

1. The Lactose Synthase Reaction

The lactose synthase reaction has been the subject of a number of reviews[40,42-46] since the participation of α LA in this process was discovered. The topology of the GT molecule has been considered by a number of investigators,[44,47-49] with particular reference to the proximity of its substrate-binding sites to the α LA moiety in the lactose synthase complex. Although a number of studies[46,51-55] have been directed toward identifying these moieties of the GT molecule involved in the interaction with substrates and with LA, very little is known about those aspects of lactose synthase action. This deficit will probably be remedied shortly with the recent cloning of the C DNA for the enzyme.[56,57] Those aspects of lactose synthase

TABLE 4
Association Constants for Formation of Complexes of α LA and GT in the Presence of Metal-Ions and Substrates

Complex	K_a
E;α LA	0
E;Mn_I;α LA	4.1×10^3
E;Mn_I;Mn_{II};α LA	4.7×10^3
E;Mn_I;Ca_{II};α LA	1×10^3
E;Mn_I;Mn_{II}; UDP-galactose;α LA	1.1×10^5
E;Mn_I;Mn_{II}; UDP-glucose;α LA	0.8×10^5
E;Mn_I;Mn_{II}; UDP-glucose, glucose;α LA	4.8×10^5
E;Mn_I;Mn_{II};GlcNac;α LA	7.4×10^5

Note: Association constants were determined by ultracentrifugation.[66] The subscripts I and II indicate the site of binding of the metal-ion on GT.

action are not considered in depth here, except to emphasize observations which shed light on the role of α LA in the modulation of its catalytic activity.

Brodbeck and Ebner[58] first showed that lactose synthase could be resolved into two components, one which proved to be α LA[59] and the other a GT. Both components were required to catalyze the reaction:

$$\text{UDP-galactose} + \text{glucose} \xrightarrow{\text{metal ion}} \text{lactose} + \text{UDP} \qquad (1)$$

Brew et al.[60] subsequently showed that the GT could utilize glucose *in the absence of α LA* as a galactose acceptor, albeit poorly, and that catalysis with *N*-acetyl glycosamine as acceptor was far more efficient. The metal-ion requirements for catalysis in the presence and absence of α LA are considered later.

GT is ubiquitous in cells including those of the mammary gland, constituting as it does an integral trans-Golgi enzyme involved in the biosynthesis of glycoproteins. In the mammary gland during lactation, however, α LA is synthesized under hormonal control[50] and passes through the Golgi where it interacts with the GT thereby (1) inhibiting the transfer of galactose to *N*-acetyl glycosamine moieties on glycoproteins and (2) increasing the affinity of the enzyme for glucose thus permitting its use in the synthesis of lactose as indicated in Equation 1. The kinetics of reaction[61,62] indicate a decrease in the K_m for glucose of ca. three orders of magnitude.

Formation of a complex of α LA and GT has been demonstrated *in vitro* kinetically[61-64] and by direct physical techniques.[64-68] The relative magnitudes of the association constants given in Table 4 permit the following generalizations concerning the effect of metal-ions, substrates, and substrate analogs on complexation of α LA and GT:

1. Complex does not form in the absence of metal ions.
2. Binding of metal-ion at site I promotes complex formation, but neither occupancy of site II by Mn^{+2} or Ca^{+2} nor binding of UDP-gal have any additional effect on stabilization of the complex.
3. UDP-glucose, which is not a substrate for GT, is equally effective as UDP-galactose in stabilizing the complex.
4. Binding of monosaccharide, however, results in a five- to sevenfold increase in stability of the complex compared with that promoted by Mn^{+2} and UDP-gal. As might be expected, the binding of α LA enhances that of UDP-gal and substrates.

TABLE 5
Binding of Metal-Ions to GT

Site	Metal-ions bound
I	Mn^{+2} (a,b), Zn^{+2} (a,b), Co^{+2}
II	Mn^{+2} (a,b), Zn^{+2} (a,b), Co^{+2} (a,b), Ca^{+2} (a,b), Sr^{+2} (a), Cd^{+2} (a), Fe^{+2} (a), Al^{+3} (b), Pr^{+3} (a), Eu^{+3} (b), Tb^{+3} (b)

Note: (a) refers to data found in Reference 69 and (b) refers to data found in Reference 70.

Complex formation leads to an enhanced binding of monosaccharides reflected as a decrease in the kinetic parameter K_m. This effect extends to substrates such as GlcNAc, but the net effect is inhibition of the reaction using this substrate since there is also a decrease in the rate of product release (decrease in V_m).[45] The amino acid sequence homology of α LA and lysozyme has favored the view that the two proteins also share an ability to bind monosaccharides, although all attempts to demonstrate the existence of such sites in α LA have been unsuccessful. Sites for binding of saccharides to lysozyme are part of the active site cleft, and it has been proposed by Brew and Sinha[45] and others that a homologous region of the α LA molecule should likewise have comparable significance. This question is pursued later in considering the GT-α LA contact region in the lactose synthase complex.

2. Metal-Ion Activation of Galactosyltransferase

GT has an absolute requirement for divalent cations for its activity. Kinetic studies carried out by Powell and Brew,[69] using *N*-acetyl glucosamine as substrate, were consistent with a *minimum* of two binding sites for metal-ions, sites I and II with association constants of ca. 10^6 and 10^3, respectively. These observations were subsequently confirmed by O'Keeffe et al.[70] The metal-ion specificities at the two sites are quite different (Table 5), site I having an absolute requirement for Mn^{+2}, Zn^{+2}, or Co^{+2}, while site II binds a wide range of cations. It is noteworthy that all of the metal-ions which activate GT also bind to α LA. Activation with manganous ion leads to maximal activity, while those with zinc and cobalt are only 25 and 10%, respectively, of this. Activation of the enzyme by binding at site I is thought to be the result of alteration of its molecular conformation, although little is known about this process. Binding of metal-ions at site II is associated with increased affinity of the enzyme for UDP-gal, which has generally been assumed to be the result of a metal-ion-enzyme-UDP-gal bridge. However, the following observations introduce the possibility that occupancy of site II may bring about a conformational change which makes for tighter binding of UDP-gal.

A recent study by Navaratnam et al.[71] raises the question of whether activation of GT by the binding of *metal-ions at site II per se* is obligatory. This investigation showed that a wide range of polycationic substances, including polyamines, basic proteins, and peptides, could also activate GT when only site I was occupied by Mn^{+2}, the observed activation being comparable with that found when both sites I and II were occupied by this metal-ion. The authors have concluded that the natural activator *in vivo* is a secretory protein rather than metal-ion. These findings, however, need to be substantiated.

It has been suggested[69] that activation of GT *in vivo* occurs by binding of Mn^{+2} and Ca^{+2} at sites I and II, respectively. As indicated earlier, activation may not require binding of metal-ions at site II. It is of interest to note also that Witsell et al.[267] have observed with mouse mammary vesicular GT that high concentrations of calcium ion, instead of activating GT, actually inhibit its action.

The metal-ion studies referred to here have been mostly concerned with the reactions catalyzed by GT in the *absence* of α LA. Powell and Brew[69] compared the effect of manganous ion on the enzymatic synthesis of *N*-acetyl-lactosamine and lactose and found comparable Mn^{+2} activation curves, suggesting that there are at least two metal-ion-binding sites for activation of *both* reactions. However, there do not appear to have been any in-depth studies of the effects of other metal-ions in promoting lactose synthase activity. It should be emphasized that our knowledge of the metal-ion-binding properties of GT comes almost exclusively from kinetic measurements. Detailed studies of binding using direct physical measurement would be highly desirable. Such measurements have proven to be very difficult because of the marked propensity to the enzyme to aggregate even at moderate concentrations.[69,261] A study of the binding of Mn^{+2} to GT by Andree and Berliner,[73] using ESR, gave a stoichiometry of 2:1. The analysis of the data, however, did not permit a distinction to be made between two models: (1) equivalent manganese-binding sites and *two* UDP-gal sites; (2) nonequivalent manganese-binding sites and a single UDP site. It should be noted also that these experiments required the use of an enzyme concentration of ca. 0.1 m*M*, where extensive self-association is expected.

3. What Structural Moieties of the α Lactalbumin Molecule Are Significant for Its Biological Activity?

Although there has been considerable interest in how α LA modulates the catalytic activity of GT, there is still comparatively little known about which of its amino acid residues are involved in forming the lactose synthase complex with GT, substrate, and metal-ions and in subsequent molecular events such as binding of substrates. Attempts to characterize a possible site for binding of monosaccharides on α LA alone have been thwarted by the inability to demonstrate such binding in the isolated protein.

Classical chemical modification techniques, with one notable exception (see later), have provided little in the way of unambiguous information about involvement of specific amino acid residues of α LA in lactose synthase action. Reaction of its amino groups with maleic[74] or acetic anhydride[75] had little effect on its lactose synthase activity, except in the latter case where a progressive loss of activity was observed at high degrees of reaction, most likely the result of a conformational change in the protein. Modification of carboxyl groups by carbodimide-mediated coupling with amines resulted in loss of activity,[43,76] but the former authors showed that when tourine, an amine with a negatively charged group, was substituted for methylamine or glucosamine in the coupling reaction, no loss of activity occurred.

These authors concluded that carboxyl groups *per se* were not essential for activity, although it appeared that maintenance of a local charge was required for formation of the lactose synthase complex.

Denton and Ebner[78] found that all four tyrosyl groups of BLA reacted readily with tetranitromethane with significant loss of activity. However, Prieels et al.[79] subsequently demonstrated that treatment of the protein with this reagent under milder reaction conditions gave selective reaction of the tyrosyl residues of HLA with virtually no loss of lactose synthase activity (see Section III.D for a more detailed discussion of tyrosyl reactivity). Substantial loss of lactose synthase activity observed at relatively high concentrations of tetranitromethane was shown to result from reaction with trp 118. However, Prieels and co-workers propose, based on a change in the far-UV CD-spectrum, that the loss of activity resulted from a conformational change occuring on reaction with trp 118. The spectrum of the modified protein, however, is not characteristic of a completely unfolded protein and it seems likely, therefore, that such a conformational change must be local in nature. It should be noted also that trp 118 is invariant in the amino acid sequence of α LA (see earlier discussion).

There is considerable evidence to suggest that a tryptophan residue(s) of α LA may be

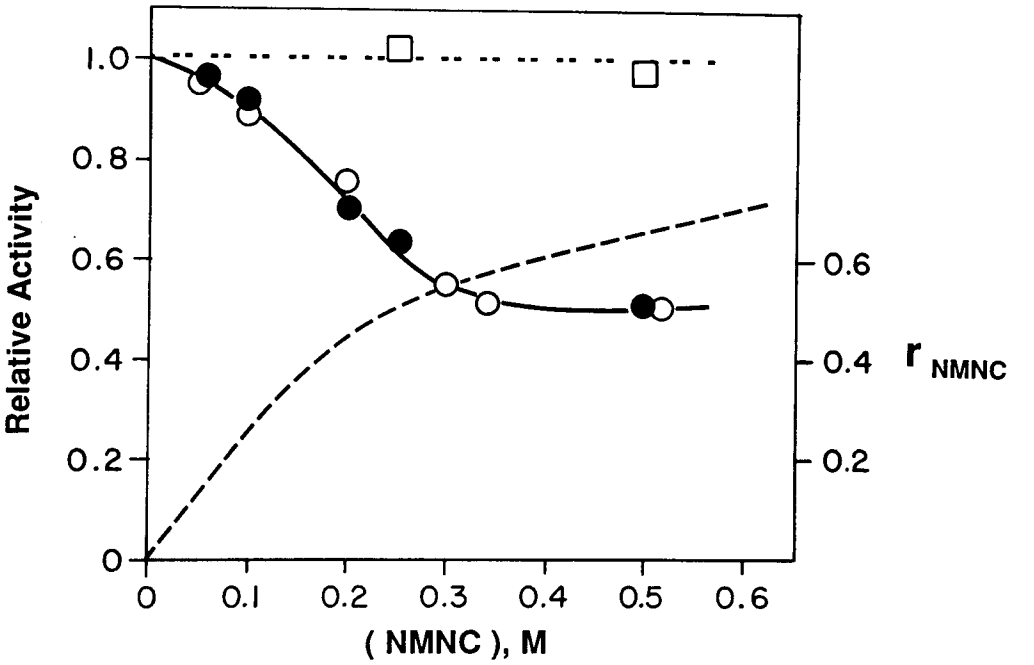


FIGURE 3. Inhibition of lactose synthase action on binding of *N*-methyl nicotinamide chloride. Assay conditions: 37°C; time of reaction, 15 min; buffer, pH 7.4, 0.03 *M* cacodylate; (Mn^{+2}), 10 m*M*; (UDP-gal), 0.05 m*M*; *N*-acetyl glucosamine (upper curve in absence of α LA) *or* glucose (lower curve in the presence of native BLA, ● or apo-BLA, ○), 10 m*M*; (- - - - - - -), binding of inhibitor as calculated from the data of Robbins and Holmes.[82] (Data from Reference 264.)

essential for lactose synthase activity, although definitive proof for involvement of a *specific* residue is lacking at present. Comparison of the spectral properties of the lactose synthase complex formed with BLA, HLA, and GPLA lead Ebner et al.[80] and Takase et al.[81] to conclude that trp 60, and possibly trp 26, is perturbed by interaction of α LA with GT (see Figure 1 for a comparison of the sequence homology of tryptophan residues in α LA).

The observations described in Figure 3 likewise demonstrate the involvement of a tryptophan residue in lactose synthase action. Robbins and Holmes[82] have shown that a tryptophan residue of BLA in the N state forms a 1:1 charge transfer complex with *N*-methylnicotinamide chloride (NMNC). The data of Figure 3[261] indicate that NMNC inhibits lactose synthase action, but has no effect on the catalysis by GT in the absence of α LA. The inhibition curve roughly parallels the binding curve calculated from the association constant. Robbins and Holmes proposed that NMNC complexes with trp 118, based on their assessment that this residue is most accessible to the medium. However, as is discussed in Section III.C, the latter conclusion may not be warranted.

Bell et al.[83] concluded from a study of the modification of the tryptophans of BLA with *N*-bromosuccinimide that trp 118, and possibly trp 26, of α LA is essential for incorporation of α LA into the lactose synthase complex (see Section III.C for a discussion of the reaction of *N*-bromosuccinimide with α LA and its effect on the conformation of the protein). If trp 26 were implicated in formation of the lactose synthase complex, its requirement cannot be absolute since it is not conserved in the amino acid sequence (Figure 1). Barman[84] observed that modification of trp 60, 104, and 118 of BLA and HLA by 2-hydroxy-5-nitrobenzyl bromide leads to loss of lactose synthase activity. While trp 104 and 118 are invariant in the α LA sequence, trp 60 is not and its requirement cannot be absolute. All of these chemical modification studies suffer from the inability to ascertain if loss of activity results from a

conformational change occurring on reaction or if it is the consequence of the alteration of the residue *per se*. Modification of such residues by a technique such as site-directed mutagenesis may be a more fruitful way to establish *which* tryptophans are essential for activity.

His 32, which is invariant in the α LA sequence (Figure 1), has been shown to be essential for lactose synthase activity. Carboxymethylation of this residue, which occurs at nitrogen N-3, only *partially* inactivates the protein. Ethoxyformylation of this residue with diethylpyrocarbonate, specific for reaction with *N-1* nitrogen, however, leads to total inactivation.[25,85] Inactivation of the ethoxyformylated protein is reversed on treatment with hydroxylamine, characteristic of histidine-modified derivatives.

The difference in the effect of reaction at nitrogens N-1 and N-2 is quite remarkable and suggests a highly specific interaction with another moiety critical for the biological activity of α LA. The nature of this interaction has not been established. We consider in Section V a possible role for this residue in forming the lactose synthase complex.

4. The Site of Interaction of α Lactalbumin with Galactosyltransferase

Studies by Richardson and Brew[86] and by Sinha and Brew[87] have established the region of the α LA molecule comprising the site of interaction with GT on formation of the lactose synthase complex. The former study made use of the differential kinetic-labeling technique to determine *changes* in the rates of reaction of the 13 amino groups of BLA with acetic anhydride. Unlike the "classical" chemical modification procedures employed in the studies referred to earlier, the differential kinetic-labeling technique is inherently immune from the complications of conformational changes resulting from multiple sites of reaction. Of the 13 amino groups in BLA, only 2, those of lys 5 and 114, gave evidence of *changes* in reaction rate on incorporation of BLA into the lactose synthase complex, the former lysine exhibiting a threefold *reduction* in rate and the latter a twofold *increase*.

The increase in reaction rate seen with lys 114 could be due either to greater accessibility of the amino group in the complex or to alteration of the charge distribution in that region of the molecule reflected as a decrease in the ionization constant for that group. The former connotes the occurrence of a conformational change in the BLA molecule on its incorporation into the complex. The change in charge distribution could also reflect such a conformational change in the α LA molecule which changed the spatial relationship of the amino group with a positive moiety in the same molecule. However, the positively charged group might equally well be from the GT molecule in the complex. The decrease in reaction rate of the amino group of lys 5 could be explained in a similar manner, i.e., as being the result of a conformational difference between the complexed and uncomplexed BLA molecule or the proximity of this residue to charges in the GT moiety of the complex. Further discussion of the significance of changes in molecular conformation of α LA as it relates to its biological activity is given in Section V.

Before considering the location of lys 5 and 114 on the α LA molecule and relevance of their location in establishing the GT interaction site, we consider here the studies of Sinha and Brew,[87] which provide additional information about that region of the α LA molecule. This study, employing a label-selection procedure with reversibly cross-linked BLA and GT,[54] in addition to confirming the observations of Richardson and Brew of changes in reactivity of lys 5 and 114, established the primary site of cross-linking as the amino group of lys 108 located at a distance of ca. 6 to 7 Å from an amino group on the GT molecule.

Sinha and Brew[87] have defined a contact region on the α LA molecule containing the site for interaction with GT in the lactose synthase complex, based on the locations of lys 5, 114, and 118. This region represented schematically in Figure 4, an abbreviated Y-Z coordinate plot, is bounded by dashed lines. Shown also are calcium-binding site, site I, and two putative metal-binding sites which are described in more detail in Sections II and

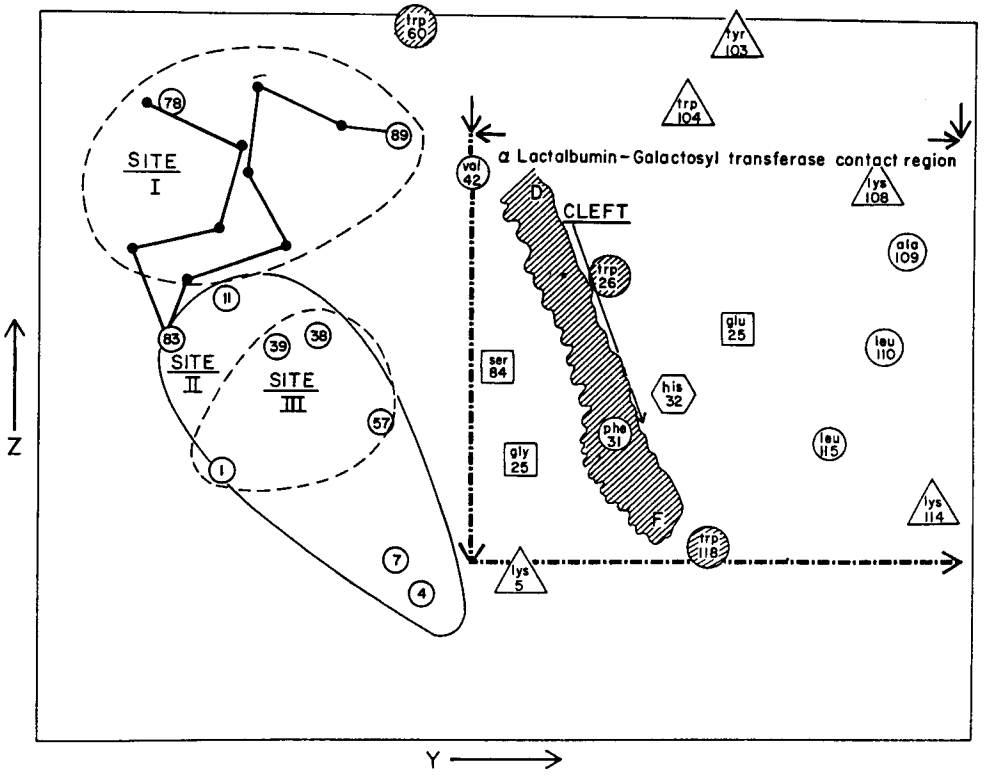


FIGURE 4. Schematic representation of the major metal-ion-binding sites on the α LA molecule and its region of contact with galactosyltransferase in the lactose synthase complex (see text for explanation). This scheme was constructed from the Z-Y coordinates derived from the study of Warme et al.[30] The metal-ion-binding sites are those which control its molecular conformation (see Section II); site I, the calcium-binding "elbow" (Reference 33); site II, the putative high affinity Mn^{+2} site as deduced from ^{13}C-NMR measurements;[41] site III, the putative site whose occupancy by Zn^{+2} or Tb^{+3} promotes formation of a unique "A-like" state from native or metal-free protein (Section IV). The two lysyl residues, 5 and 114, whose reactivity is altered on formation of the lactose synthase complex[86] are indicated as △ and the site of cross-linking with galactosyltransferase[87] is outlined by -·-·- Other residues represented are described in the text.

IV. Suffice it to say here that these three sites are spatially too distant from the α LA-GT contact region for their bound metal-ions to participate directly in lactose synthase action. As pointed out by Sinha and Brew, although the amino groups of lys 5 and 114 are sufficiently close to the interaction site to be perturbed on formation of the complex, their acetylation does not inactivate α LA. These residues, therefore, although not *within* the interaction site itself, can be used to indicate its boundaries. Similarly, the cross-link at lys 108 can be used to roughly define the upper limit of the site, to include residues whose distance from this residue is greater than 7 Å.

This interaction region of the α LA molecule includes the cleft area, homologous with the active site of hen's egg white lysozyme, including the binding subsites D-F for saccharide substrates and inhibitors,[88] as well as his 32 which has been shown to be essential for lactose synthase activity. It also contains a large number of hydrophobic residues, some of which may contribute to the energy of stabilization of the lactose synthase complex through interactions with similar residues on the GT molecule. It is of interest that a secondary reaction of 2-hydroxy-5-nitrobenzyl bromide has been found with his 32 (Reference 89). Enhanced reactivity of this reagent has often been observed to be promoted by its absorption into hydrophobic regions of protein molecules. It is of further interest that binding of the flu-

orescent dye anilino-naphthalene sulfonate, presumably at a hydrophobic site, *protects* his 32 against reaction.[89]

The hydrophobic residues in the contact region include the invariant residues val 42, phe 31, leu 115, trp 118, and perhaps trp 104 (Figure 4). Other hydrophobic residues such as trp 26, ala 109, and leu 110, while not invariant, are frequently replaced by amino acids of similar character, which could perhaps be accommodated in the interaction site. Identification of those residues involved in the interaction with GT is likely to prove difficult, particularly since chemical reactivity determinations are precluded with most hydrophobic residues and the large number of residues to be examined makes the use of the site-directed mutagenesis technique a rather formidable undertaking. An approach which may prove to be of more general applicability would be to use photoaffinity probes for hydrophobic sites such as done by Van Ceunebroeck et al.[24] (see also Section III.G for further discussion of hydrophobic sites of α LA).

As considered previously, the cleft area of the α LA molecule has been regarded as an attractive site for moieties forming part of the monosaccharide-binding site in the complex, consistent with the location of the α LA-GT contact region (Figure 4). O'Keefe et al.[90] reject this idea, however, maintaining that the distance from a dansyl group coupled to the amino group of glu 1 of α LA and a lanthanide ion bound at site I of GT is too large. This distance, as determined by energy transfer measurements, was 32 Å. Inspection of a molecular model of α LA indicates that glu 1 lies 15 to 16 Å from his 32 of α LA, a residue which lies in the cleft region and is essential for lactose synthase activity. Since the spatial relationship between site I and the molecular region of GT in proximity to his 32 of α LA is not known, a distance of 32 Å between site I of GT and glu 1 of α LA may be completely reasonable.

II. BINDING OF METAL-IONS TO α LACTALBUMIN AND THEIR EFFECT ON ITS CONFORMATIONAL STATE

A. INTRODUCTION

Subsequent to the observations of Hiraoka and Sugai[107] that BLA was a metalloprotein containing a single strongly bound calcium ion, Kronman et al.[36] prepared the apo-protein and demonstrated that its native molecular conformation could be restored by addition of Ca^{+2} or Mn^{+2}. The metal-free state of the protein, the A state, was found to be essentially identical to the low pH form of the protein, and it was proposed that the latter conformer was formed on dissociation of Ca^{+2} below pH 4. With the availability of simple procedures for preparing apo-BLA by treatment of α LA with EDTA[36] or by gel filtration of the protein at pH 2 (Reference 37), there has been a flood of publications describing its binding of a variety of metal-ions (Tables 6 to 8). As these data indicate, a wide range of cations bind to α LA, most of which promote formation of N-like states. Such cations include substances such as Tris buffer, histidine, and the methyl esters of basic amino acids such as lysine and arginine (Table 8). In a few instances, however, binding of metal-ions promotes formation of A-like states (Tables 7 and 8).

As discussed earlier, the terms "A-like" and "N-like" are used to indicate *similarity* of conformation based on spectroscopic criteria reflecting the molecular environment of tryptophan residues in these forms of the protein. However, as will be evident from the discussion in the following sections of this review, neither the N nor the A state are unique conformations.

It has generally been assumed that formation of unique N or A states required binding of cations at particular sites. Many investigators thus maintain that since binding of Ca^{+2}, Mn^{+2}, Na^{+}, and K^{+} promotes formation of the N state, they must all bind at the same site. As is considered later, however, neither the characteristics of the binding nor the properties of the molecular states formed are consistent with this assumption.

TABLE 6
Binding of Ca^{+2} to α LA[a]

Sites	pK_a	Technique[b]	Comments	Ref.
2	6.43[a], 4.49	D	K^+ absent	36
1	4.20 to 6.40[d]	D	+ 0.1 *M* K^+	40
1	6.43	F	+ 0.1 *M* K^+	40
2		D	+ 0.1 *M* K^+, after preequilibration with Mn^{+2}	40
1	6.40	MC		100
1	6.5	D	Measured in the presence of Mn^{+2}; value obtained as $(Mn^{+2}) \rightarrow 0$	101
1	7.34	PT	Measured at 37°C	102
1	8.31	PT	Measured as a function of temperature and extrapolated to 25°C	
1	8.31	MC		39
2	[c]	$^{43}Ca^{+2}$-NMR		75
2	[c]	Proton NMR		41
1	8.5	D	Assumes competative binding with Mn^{+2}	101
1	8.60	F	Measured at 20°C, EGTA present	103
1	7.31	F	Measured at 37°C, EGTA present	103
1	8.65	F	EGTA present	28
1	8.4	CD	EDTA present	38
1	8.7	CD(T)	+ 0.01 *M* Na^+	104
1	6.9	CD(T)	+ 0.1 *M* Na^+	104
1	9.0	D	EDTA present	101
1	9.7	F	EGTA present	105

[a] pK_a defined as log (K_a).

[b] D, direct binding measurements, e.g., Hummel Dryer procedure, using $^{45}Ca^{+2}$ or measurement of (Ca^{+2}) by another procedure; F, fluorescence (tryptophan emission) titration; CD, determined from changes in the near-UV circular dichroism spectrum. CD(T), binding determined from the effect of Ca^{+2} on the thermal transition curve using CD to measure the conformational change; MC, microcalorimetry; PT, potentiometric titration with a calcium-sensitive electrode.

[c] Values of pK_a not determined.

[d] pK_a is dependent on pH below 6; pK_a above pH 6 is 6.40 ± 0.01.

The recent publication of the crystal structure for the calcium-binding site of α LA[12] and the observations made in a ^{13}C-NMR study[41] of its binding of Ca^{+2}, Mn^{+2}, and Zn^{+2} now permit formulation of a plausible model for binding of metal-ions to this protein. This model, which has as its central feature three adjacent interacting-binding sites and multiple conformational states of the protein, resolves in large part the complexity of metal-ion binding to α LA.

The question as to the magnitude of the association constant for binding of Ca^{+2} to its high-affinity site has been a vexing one. The range of values of the association constants, obtained by a wide variety of direct and *less* direct procedures, spans more than three orders of magnitude. The disagreement seems to be primarily between the studies reported from the two laboratories of Kronman and of Brew, who found a value of pK_a (log K_a) of 6.43 for binding at the high affinity site and those of other investigators whose values ranged from 8.3 to 9.7. Possible sources of these differences are discussed in the following sections.

B. WHAT IS THE MAGNITUDE OF THE ASSOCIATION CONSTANT FOR BINDING OF CALCIUM ION?

The marked difference in the magnitudes of the association constants reported by different investigators for binding of calcium ion to α LA invites serious inquiry into their source. A paper by Permyakov et al.[112] published recently considers one aspect of this problem, i.e., the effect of chelators on the magnitude of the association constant for binding of Ca^{+2}

TABLE 7
Binding of Mn^{+2}, Zn^{+2}, and Co^{+2} to α LA

Cation	Sites	pK_a	State	Technique[a]	Ref.
Mn^{+2}	4	4.5, 3.0, 2.3, 2.3	N	ESR	105
Mn^{+2}	3	(4.5-5.8[b], ca. 4, ca. 3)	N	D,F	40
Mn^{+2}	2[c]		N	D,F	40
Mn^{+2}	1	4.7	N	ESR	41
Mn^{+2}	1	4.5[d]	N	F	105
Zn^{+2}	1	5.25	A	F, ESR	105
Zn^{+2}	2	5.05, 2.78[e]	A	D,F	40
Co^{+2}	1	3.9	A	F	106

[a] ESR, electron spin resonance (see footnotes for Table 6 for other abbreviations).
[b] The range of values given for pK_a for the site of highest affinity reflects its dependence on self-association of the protein (see text). The values given for the other two sites are estimates since no multisite analysis was possible (see text).
[c] Preequilibration with Mn^{+2} prior to addition of 8.5 μ*M* Ca^{+2} (see Table 6 and text).
[d] Assumes competition with Mn^{+2}.
[e] Binding of Zn^{+2} at the site of higher affinity gives a small increase in tryptophan fluorescence relative to apo-BLA. Binding at the second site gives rise to time-dependent increase in fluorescence reflecting formation as an "expanded A" state.

to α LA. However, it provides very little help in the resolution of the discrepancy in the binding affinities. These authors report still another value for pK_a, 8.4 ± 0.1 to 8.9 ± 0.1. While their equilibrium dialysis procedure is described, *no data* are presented to support the reported values. Their observations and conclusions concerning the effect of chelators on binding of calcium ion to α LA are considered as part of a more general discussion on errors in these determinations.

1. The Studies of Kronman et al. and Bratcher and Kronman

Values of pK_a obtained at 25.0°C with $^{45}Ca^{+2}$ using the Hummel-Dryer method were 6.43 ± 0.01 (buffered 0.1 *M* KCl)[36] and 6.40 ± 0.03 (buffer alone).[40] Although these values were reported in publications from the same two laboratories, the earlier measurements[36] were carried out in Brew's laboratory using a conventional Hummel-Dryer procedure; the later more detailed ones,[40] using a modified Hummel-Dryer method, were carried out in Kronman's laboratory. The earlier studies employed apo-BLA prepared by treatment with chelators,[36] while in the later ones metal-free protein was obtained by low pH gel filtration.[37] No chelators were used in any of these measurements. Determination of calcium ion binding was carried out with more than 25 different preparations of apo-BLA in Tris, PIPES, and acetate buffers at a number of different pHs.[40] The value of pK_a obtained ranged from 6.2 to 6.4 in the pH range of ca. 8.5 to 5.5; a marked decrease in binding affinity occurs below pH 5 (Figure 8).

Fluorescence titration of apo-BLA with calcium ion yielded a curve characteristic of strong binding of a single cation (Figure 5). Analysis of the titration data yielded a value of 6.43 ± 0.01, *identical* to the value obtained by the Hummel-Dryer procedure.

It is apparent that the measurements of Kronman et al.[36] and Bratcher and Kronman[40] are *highly* reproducible, yielding identical values of the calcium-binding constant by two independent kinds of measurement under a range of experimental conditions.

TABLE 8
Binding of Miscellaneous Ligands to α LA[a]

Ligand	Sites[b]	pK_a	State	Technique	Ref.
Na^+	1	2.1	N	CD	107
Na^+	1	1.6	N	F	103
Na^+	1	2.0	N	F	103
Na^+	1	2.3	N	MC[e]	39
Na^+	1	2.0[e]	—	PT[f]	102
K^+	1	1.3	N	CD	107
K^+	1	1.2	N	F	40
K^+	1	0.78	N	F	103
K^+	1	0.90	N	F	103
K^+	1	0.60	N	F	39
Mg^{+2}	1	3.2, 3.1[d]	N	F, ESR[e]	105
Mg^{+2}	1	3.3	N	F	108
Mg^{+2}	2	3.3, 2.3	N	F	103
Sr^{+2}	1	5.7	MC	D	100
Cd^{+2}	1	5.6	N	F	106
Al^{+2}	1	5.4	N	F	106
Cu^{+2}	1	5.2	N	F	106
Tb^{+3}	3[c]	6.5—8.8	N	D,F[c]	40, 92
		ca. 6	N	D,F[c]	40, 92
		ca. 3	A	F[c]	92
Tb^{+3}, Dy^{+3} Y^{+3}, Eu^{+3}, Gd^{+3}	1	10—12	N	F, ESR	91, 256
Vo^{+2}			N	ESR	256
EDTA, EGTA			N	F	37
Histidine			N	F	37
Lysine methyl ester			N	F	37
Arginine methyl ester			N	F	37
Tris buffer			N	CD	107
Tris buffer			—	E	109, 223

[a] See Tables 6 and 7 for abbreviations. E, electrophoresis.
[b] With the exception of measurements made for Tb^{+3} (References 40 and 92), a single site was *assumed* for the other ligands.
[c] The presence of three sites for binding of Tb^{+3} was deduced from studies of competative binding with $^{45}Ca^{+2}$, Tb^{+3} luminescence and tryptophan fluorescence. Binding of Tb^{+3} to the site of lowest affinity gives rise to a time-dependent conformational change from an N to an "expanded A" state (see text).
[d] Assumes competition for binding with Mn^{+2}.
[e] Assumes competition with Ca^{+2}.
[f] Values determined at 37°C, protein concentration 0.8 to 0.9 m*M*, assuming binding competition with Ca^{+2}.

2. The Effect of the Method of Preparation of BLA and apo-BLA on Its Ca^{+2}-Binding Properties

A variety of procedures[17-19,107] have been used to prepare α LA, all of which appear to yield satisfactory preparations of the protein. One of the more convenient and widely used methods, that of Aschaffenburg and Drewry,[18] necessitates exposure of the protein to elevated temperatures to precipitate caseins and addition of HCl to bring the whey solution to pH 2 to precipitate α LA. It is arguable that such potentially harsh treatment might irreversibly alter the protein. However, preparation and purification of α LA by an alternative method[107] not requiring low pH precipitation of α LA or the use of elevated temperature to remove caseins yielded protein which was indistinguishable from that obtained by other methods.

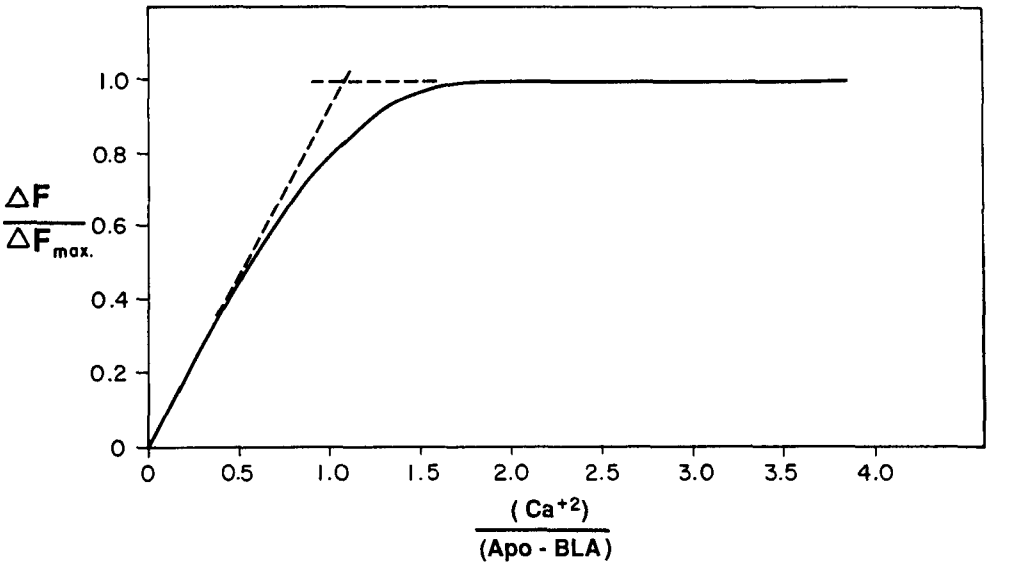


FIGURE 5. Fluorescence titration of apo-BLA with Ca^{+2} at 25°C. Buffer: 0.02 *M* Tris, pH 7.5, protein concentration 16.4 μ*M*. The lines correspond to smoothed data from 3 independent titrations comprising upwards of 60 data points.[36]

Furthermore, it has been shown that BLA maintained at pH 2 at room temperature for as long as 24 h regained its native properties on neutralization.[262] It is apparent from these observations that exposure of the protein to the conditions of the standard methods of preparation or to low pH does not irreversibly alter its properties, including its ability to bind calcium ion.

While it is possible to demonstrate, using [^{14}C]-EDTA or $^{45}Ca^{+2}$, that EDTA or Ca^{+2}-EDTA can be effectively removed from apo-BLA by gel filtration (see later), we believe it wise to avoid the use of chelators in investigations with α LA in view of the observations that Ca^{+2}-chelator complexes bind to the protein (Section II.B.5). Gel filtration of a pH 2 ("Suprapure" HCl) solution of α LA with subsequent removal of the acid by lyophilization[37] is a procedure currently used by a number of investigators for preparing the metal-free protein. The protein can be stored in the frozen state at concentrations of 3 to 5 m*M* (dilute metal-free Tris buffer, pH 6) for at least 3 months with no indication of any changes in its properties.[262]

In contrast, Segawa and Sugai[38] report that apo-BLA is "unstable", with aggregation occurring after 5 h of solution of the protein. Their preparative procedure appears to be somewhat harsh, however, involving as it does treatment of native BLA with 100 μ*M* EDTA in 5 *M* guanidine-HCl, followed by gel filtration in a medium containing 100 μ*M* EDTA. Desmet et al.,[39] who prepared apo-BLA by the procedure of Segawa and Sugai, confirmed this "instability" of the protein.

Complete loss of calcium-binding ability of BLA has been observed by Bratcher and Kronman[40] for preparations of the protein which had been repeatedly brought to room temperature from the freezer or refrigerator. The spectral properties of such preparations were indistinguishable from those of native protein, but after removal of calcium by passage through a column of Chelex 100, they lost their ability to bind calcium ($^{45}Ca^{+2}$-binding measurements or fluorescence titration). In contrast, however, freshly prepared BLA can be demetallized without loss of its calcium-binding ability. The origin of this loss in calcium-binding ability is not known. These observations underscore the need to use preparations of both native and metal-free protein of known history. In our opinion, since prolonged storage

TABLE 9
Binding of Mn^{+2} to Its High Affinity Site on Apo-BLA: Effect of Protein Concentration on the Magnitude of Its Association Constant

pK_a	KCl (*M*)	apo-BLA (μ*M*)	Technique	Ref.
5.8	0	4	FT	40
5.6	0	16	FT	36
5.2	0	53	FT	40
5.2	0	30—50	HD	40
4.6	0.1	30—50	HD	40
4.7	0.1	200	ESR	41
4.5	0	400—10,000	ESR	105

Note: FT = fluorescence titration; HD = Hummel-Dryer gel filtration using $^{54}Mn^{+2}$; ESR = electron spin resonance.

of BLA may ultimately alter its properties, the use of commercially prepared BLA, whose history of preparation and storage is unknown, is probably undesirable. This conclusion is supported by the following observations: Van Cauwelaert and co-workers,[113] who reported *high* values of pK_a for binding of calcium ion (Table 6), employed commercial preparations of BLA in their studies. More recently, however, in using protein prepared in their laboratory by the Aschaffenburg procedure, they obtained values of pK_a comparable in magnitude, or even *lower*, than those observed by Kronman et al.[36] and Bratcher and Kronman.[40,263]

3. Self-Association of α LA and Calcium-Binding Properties

α LA is known to exhibit a marked propensity for self-association, even in its N conformational state. This was discussed in Section I. While the effect of self-association on binding of calcium ion to apo-α LA has not been investigated, Bratcher and Kronman[40] have shown that its binding affinity for Mn^{+2} *decreases* 20-fold over the protein concentration range 4 μ*M* to 1 m*M* (Table 9). Techniques such as ESR and NMR, requiring the use of protein concentrations approaching the millimolar range, would be particularly vulnerable to the effects of self-association. Investigators who have employed high concentrations of α LA[41,75,100] in studies of this protein do not appear to have addressed this question.

4. Effect of Monovalent Cations on the Binding of Ca^{+2}

Prior to the discovery by Segawa and Sugai[38] that monovalent cations bind to apo-BLA and promote formation of the N-like conformer, it was common to use sodium or potassium salts as the supporting electrolyte in buffers. As is discussed later, the use of EDTA or EGTA also entails the "contamination" of solutions of α LA with sodium ion since these chelators are commonly supplied as the sodium salt. The observation of Mitani et al.[104] that the affinity of binding of Ca^{+2} for apo-BLA is decreased in the presence of sodium ion suggests that these two ions compete for the same binding site; the observations with potassium ion are less clear-cut (see Section II.F for further discussion).

The decrease in binding affinity of Ca^{+2} in the presence of sodium ion is of particular concern in those studies where buffers have been treated with the chelating resin, Chelex 100, to remove divalent and heavy metal-ions and particularly critical where Chelex is used for demetallization of α LA with subsequent concentration and use of the protein at millimolar concentrations.[114,139] The resin is generally treated with NaOH as part of the regeneration

procedure, and unless great care is used to insure its complete removal, sodium ion will "bleed" in significant amounts into the solution being demetallized. Desmet et al.,[39] for example, have shown that preparation of apo-BLA by chromatography on columns of Chelex resulted in the introduction of 20 g-atoms of sodium ion per mole of protein. Such contamination of apo-BLA with sodium proved to be the origin of the apparent dependence on protein concentration of the enthalpy change on binding of calcium ion to apo-BLA.[113]

The following Chelex-treatment procedure employed in our laboratory for the demetallization of buffers maintains their sodium concentration below 0.1 m*M*, sufficiently low to be inconsequential in competing for binding with divalent cations:[264] (1) a suspension of Chelex 100 after regeneration is adjusted to pH 9.5 to 10 with HCl; (2) prior to its use, a 10 to 20 ml column of the resin is washed with 20 column-volumes of a 2 *M* buffer of the same pH and composition as the buffer to be demetallized; (3) the column is then washed with 20 column-volumes of the buffer to be demetallized prior to its collection for subsequent use. Since the buffers used in the metal-ion-binding studies reported by Kronman et al.[36] and by Bratcher and Kronman[40] were demetallized by this procedure, contamination with sodium could not have had a significant effect on calcium binding to apo-BLA.

Although binding of sodium, potassium, or calcium ions promotes formation of N-like states, these have somewhat different properties (Section III.F). It seems imperative, therefore, that monovalent cations be scrupulously excluded in experiments where characterization of the calcium-mediated conformation is the goal. If a supporting electrolyte is required, use of an ammonium ion salt may be preferable since this cation does not appear to bind to α LA.

5. Effect of Chelators on the Binding of Ca^{+2} to α LA

Chelators such as EDTA or EGTA might be expected to alter the apparent binding constant for calcium in two ways: (1) by competitive binding of the Ca^{+2} and the sodium counter-ions of the chelator when high concentration of EDTA or EGTA is employed; (2) by binding of the Ca^{+2}-chelator complex to apo-α LA. Since this process alters the tryptophan fluorescence, such spectral changes would "mimic" those occurring on binding of the divalent metal-ion alone. Since the binding of the metal-ion-chelator complex is relatively strong, this effect manifests itself at EDTA or EGTA concentrations where binding of the sodium counter-ions is negligible.

Mitani et al.[104] demonstrated that if binding of EDTA or EGTA to apo-BLA occurs at all, it is sufficiently weak to be undetectable at 3-m*M* concentrations of the chelator. It seems likely, therefore, that the changes in fluorescence observed at high concentrations of EDTA and EGTA by Kronman and Bratcher[37] were due to the binding of sodium ions rather then binding of the chelator. Similarly, the changes in the fluorescence of Ca^{+2}-BLA observed by Permyakov et al.[112] at chelator concentrations above 1 m*M* and attributed by them to binding of EDTA or to an effect of sodium ion on the activity coefficient of calcium ion are almost certainly due to binding of the monovalent cation. Permyakov et al. seem unaware of the demonstration by Hiraoka and Sugai[107] of the *binding* of monovalent cations to apo-BLA, although this is discussed by Mitani et al.,[104] whom they cite.

The changes in fluorescence properties observed by Kronman and Bratcher[37] at micromolar concentrations of EDTA cannot be readily dismissed since binding of sodium ions at such concentrations is negligible. Fluorescence titration of apo-BLA with calcium ion *in the presence of EDTA* strongly suggests that the binding of a Ca^{+2}-EDTA complex to the protein gives rise to spectral changes similar to those observed on titration of the protein with the divalent cations itself, i.e., a decrease in quantum yield and a short wavelength shift of the emission spectrum. The biphasic character of the fluorescence titration curve in the presence of 10 μ*M* EDTA (Figure 6) is particularly striking. Significant spectral changes are evident just above a calcium ion concentration of 1 n*M* (free and protein-bound Ca^{+2}),

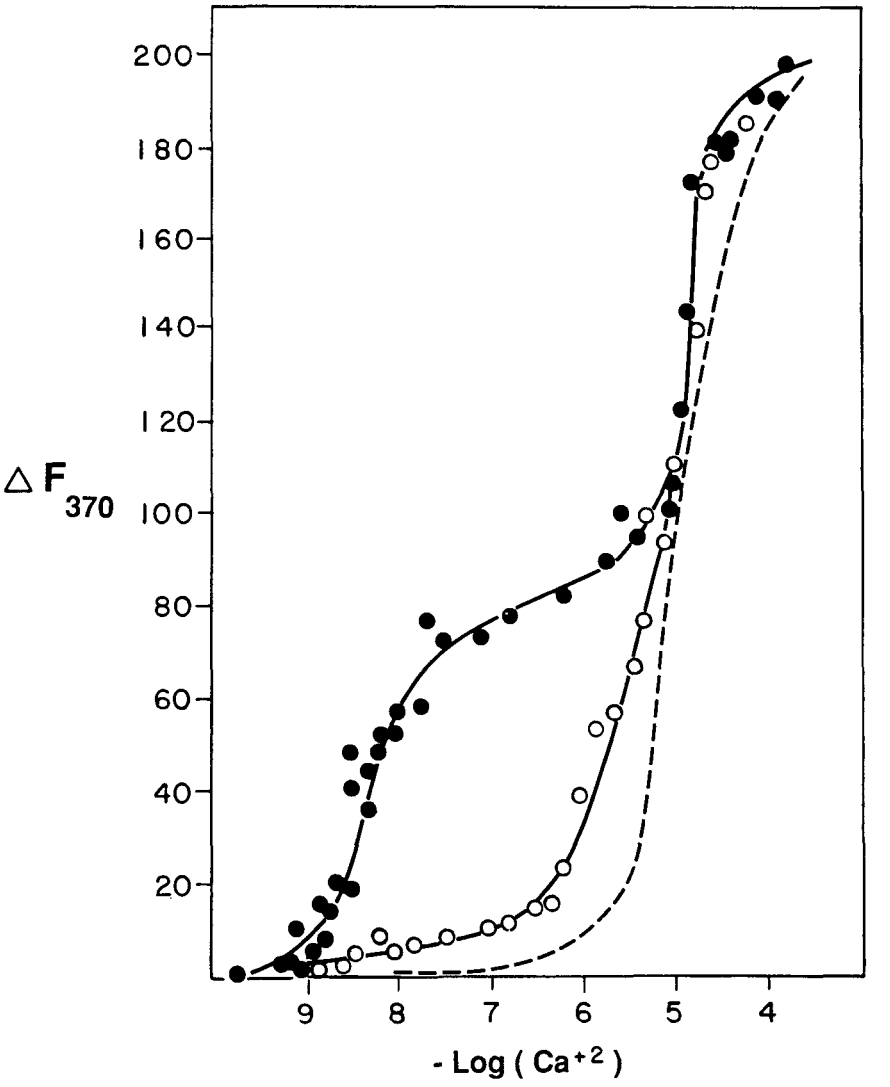


FIGURE 6. Fluorescence titration of apo-BLA with Ca^{+2} in the presence and absence of EDTA at 25°C. Buffer, 0.02 *M* Tris, pH 7.5; protein concentration, 16.1 μ*M*; wavelength of emission, 370 nm; wavelength of excitation, 295 nm; EDTA concentrations: ○, 1 μ*M*; ●, 10 μ*M*; - - - - -, 0 *M*. (Adapted from the data of Kronman and Bratcher.[37])

much too low to be due to binding of calcium (compare the curves for 0 and 10 μ*M* EDTA in Figure 6). These observations strongly suggest that the binding of Ca^{+2}-EDTA to apo-BLA occurs even at very low concentrations of chelator and that the concomitant spectral changes can have the *appearance* of the binding of calcium ion.

Permyakov et al.[112] have criticized these experiments on the grounds that competition of binding of Ca^{+2} with apo-BLA and EDTA had not been taken into consideration in calculating the calcium concentration in solutions containing EDTA. Since the association constants for formation of the Ca^{+2}-EDTA and Ca^{+2}-BLA complexes are of the order of 1×10^8 and 2×10^6, respectively, at an EDTA concentration of 10 μ*M*, ca. 98% of the bound calcium ion will be chelated to EDTA.

The chromatograms shown in Figure 7 are further evidence for the binding of Ca^{+2}-EDTA to apo-BLA. When EDTA was added to Ca^{+2}-BLA and the mixture gel filtered, the

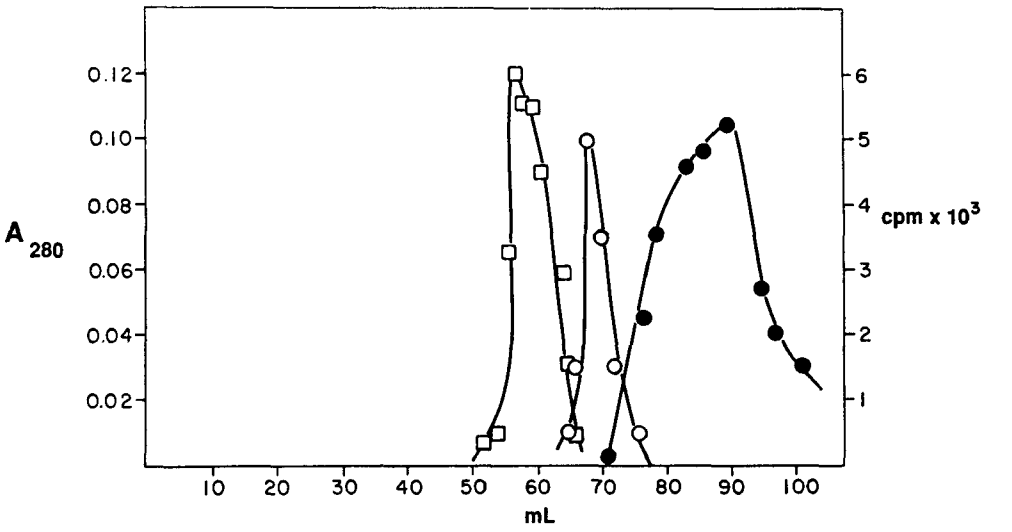


FIGURE 7. Gel filtration of the Ca^{+2}-EDTA complex in the presence and absence of apo-BLA. Apo-BLA was equilibrated with $^{45}Ca^{+2}$ after which the solution was made with 10 m*M* in EDTA and allowed to stand for 3 h before application to a 1.5 × 95 cm column of Sephadex G-15. In a control experiment, $^{45}Ca^{+2}$ was added to EDTA and the complex gel filtered through the same column. □, absorbance at 280 nm. ○, ●, cpm; filled symbols, control experiment (EDTA + $^{45}Ca^{+2}$, no protein); note, different aliquots of solutions were taken for counting in the two experiments. Prior to its use, the column was washed with 10 m*M* EDTA, 10 m*M* $^{45}Ca^{+2}$, and then with metal-free buffer (pH 7.6, 0.02 *M* Tris) until no radioactivity could be detected.[262]

radioactive peak corresponding to the chelator-metal-ion complex was retarded relative to the position of the complex formed in the absence of protein.

The observations cited here indicate that Ca^{+2}-EDTA binds to apo-BLA even at low chelator concentrations and gives rise to changes in fluorescence comparable to those seen on binding of calcium ion alone. Fluorescence titrations of apo-BLA with calcium ion carried out in the presence of chelators, therefore, are likely to lead to erroneous results.

6. Determination of the Association Constant for Binding of Ca^{+2} Assuming Competition with Mn^{+2}

Determination of the association constant for binding of calcium ion to apo-BLA through its competition with Mn^{+2} is not a valid procedure. As is discussed later in Section II.D, binding of these two cations is *not* competitive and, furthermore, the binding pattern in solutions containing both depends on the order of mixing. Bryant and Andrews[101] used this approach and $^{45}Ca^{+2}$-gel filtration to obtain a value of 8.5 for pK_a for calcium binding. It is interesting to note, nonetheless, that their value of pK_a approached 6.3 as the (Mn^{+2}) concentration approached zero, in very good agreement with values obtained by Kronman et al.[36] and Bratcher and Kronman.[40]

7. Conclusions

We have identified a number of factors which might have a significant influence on the magnitude of the association constant for binding of Ca^{+2} to apo-α LA. These include (1) the method of preparation of the protein and its demetallization; (2) self-association of the protein; (3) binding of monovalent cations in the medium; (4) binding of a Ca^{+2}-chelator complex to apo-BLA; and (5) assumption of competition of binding of Mn^{+2} and Ca^{+2}.

The investigations which led to the higher association constants are in relatively poor agreement with each other, spanning as they do a 25-fold range in magnitude. These studies can also be generally faulted in not considering the problems outlined previously. In contrast,

the measurements of the association constant for binding of Ca^{+2} to apo-BLA by Kronman et al.[36] and Bratcher et al.[40] are very reproducible, the same value, 6.43, having been obtained by both the Hummel-Dryer procedure and fluorescence titration. Further discussion of calcium binding and its relationship to binding of other cations in this review consequently are given with reference to the lower reported value.

Nonetheless, the source of the difference between the latter value and values of $pK_a >$ 8 may still not be identified. It would be desirable if investigators concerned with this problem might exchange samples of BLA or apo-BLA for independent determinations of calcium-binding affinity. Furthermore, one would hope to see more use of $^{45}Ca^{+2}$ and the Hummel-Dryer technique in such determinations since the demands of this procedure are free of many of the uncertainties and assumptions cited in the earlier sections.

C. BINDING OF Ca^{+2} AND Tb^{+3}

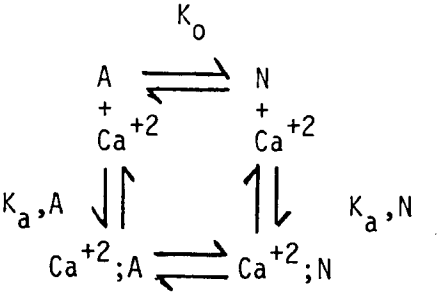
1. Binding of Ca^{+2}

Kronman et al.[36] demonstrated that there were two binding sites of different affinity for binding of Ca^{+2} (pK_a, 6.43 ± 0.03 and 4.49 ± 0.03; pH 7.5, 0.02 *M* Tris, no KCl). Binding of Ca^{+2} at low affinity site has been confirmed by $^{43}Ca^{+2}$-NMR[75] and ^{13}C-NMR[41] measurements. Changes in tryptophan fluorescence properties occurring on removal of the calcium ion were comparable to those observed in the N-A transition observed below pH 4. No change in tryptophan fluorescence was observed on binding at the site of lower affinity. Kronman et al.[36] concluded that the pH-dependent N-A transition of α LA results from dissociation of the calcium ion from its high affinity site. Similar changes in fluorescence[28,103,105] and in CD properties[38] were subsequently reported by other investigators, who likewise related the low pH conformational change to dissociation of Ca^{+2}.

Kronman and Bratcher,[40] in a more detailed study of metal-ion binding to BLA, obtained a pK_a of 6.40 ± 0.01 for binding of Ca^{+2} at its high affinity site. These measurements, made in buffers containing 0.1 *M* KCl did not detect binding of Ca^{+2} at the low affinity site described earlier, even though they were carried out over a range of calcium ion concentrations of 0.1 μ*M* to 0.6 m*M*. It is apparent from more recent studies,[102] which demonstrated binding of Na^+ and K^+ to BLA, that the absence of binding of Ca^{+2} at the low affinity site results from binding of potassium ion. The relationship between Ca^{+2} and K^+ binding is discussed in Section II.G as part of a more general discussion of a model of multiple metal-ion-binding sites and conformational states for α LA.

2. Does Ca^{+2} Bind to Both N and A Conformational States?

Since binding of Ca^{+2} at its high affinity site alters the conformational equilibrium between N and A conformers of α LA, the association constant *in principle* reflects binding to sites in *both* states of the protein and is thus an *apparent* value (Scheme I):



Scheme I

Analysis of this equilibrium scheme leads to the following relationship for binding of Ca^{+2} to the two conformers:

$$r = \frac{K_{app}\,(Ca^{+2})}{1 + K_{app}\,(Ca^{+2})} \tag{2}$$

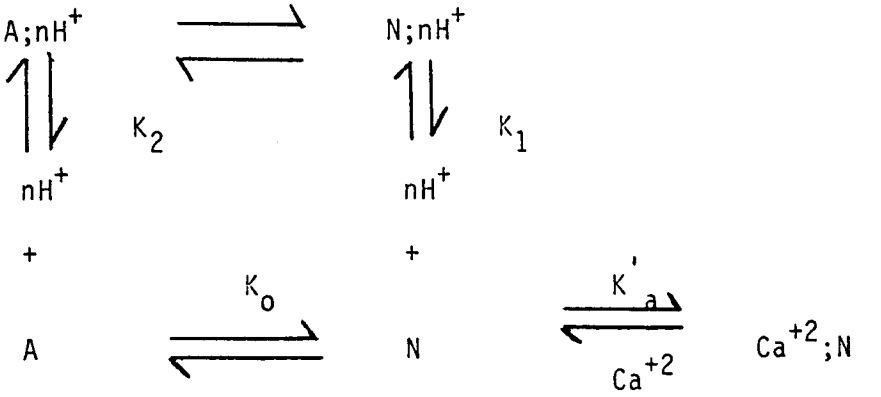
where, K_{app}, the measured association constant, is approximated by:

$$K_{app} = K_{a,A} + K_o K_{aN} \tag{3}$$

$K_{a,A}$, K_o, and $K_o K_{aN}$, determined from a nonlinear regression analysis of the dependence of tryptophan fluorescence on calcium ion concentration, were 0.034 ± 0.020, −0.33 ± 227, and 2.68 ± 0.1 × 10^6, respectively.[40] These values indicate that within experimental error calcium ion does not bind to the A conformer and that the *intrinsic* association constant, $K_{a,N}$, is equal to ca. 1 × 10^8. The value of K_o obtained in this analysis is somewhat higher than determined by other procedures. The *intrinsic* association constant, therefore, may be as high as 1 × 10^{10}, indicating that binding of Ca^{+2} to the *N conformer* is very tight indeed.

3. The pH Dependence of the Binding of Ca^{+2}

Analysis of the pH dependence of the binding of Ca^{+2} to BLA has provided information about the side chains which coordinate to the metal-ion. While the association constant is essentially unchanged in the pH range 6 to 8, it exhibits a sharp decrease below pH 5 (Figure 8).[40] Since the three histidines of BLA titrate with an average pK_{diss} of 6.13 (Reference 115), the constancy of pK_a observed between pH 6 and 8 rules out the presence of a histidine residue in the binding site. The marked decrease in the association constant below pH 5 is evidence for the presence of carboxylate ions in the calcium-binding site and strongly supports the conclusion that the N- -A conformational change below pH 4 results from dissociation of this cation. The data of Figure 8 can be analyzed according to Scheme II:



Scheme II

In analyzing this equilibrium scheme, it is assumed that there are *n* carboxylate ions in the binding site and that binding occurs solely with the unprotonated N conformer. A pH-independent association constant, K'_a, is also defined for binding at the site when all *n* carboxyl groups are unprotonated. K'_a is taken to be the constant value of K_{app}, 2.51 × 10^6, observed in the pH range 7 to 8 (Figure 8). This equilibrium scheme yields the following expression which relates K_{app}, the measured pH-dependent association constant, to K'_a:

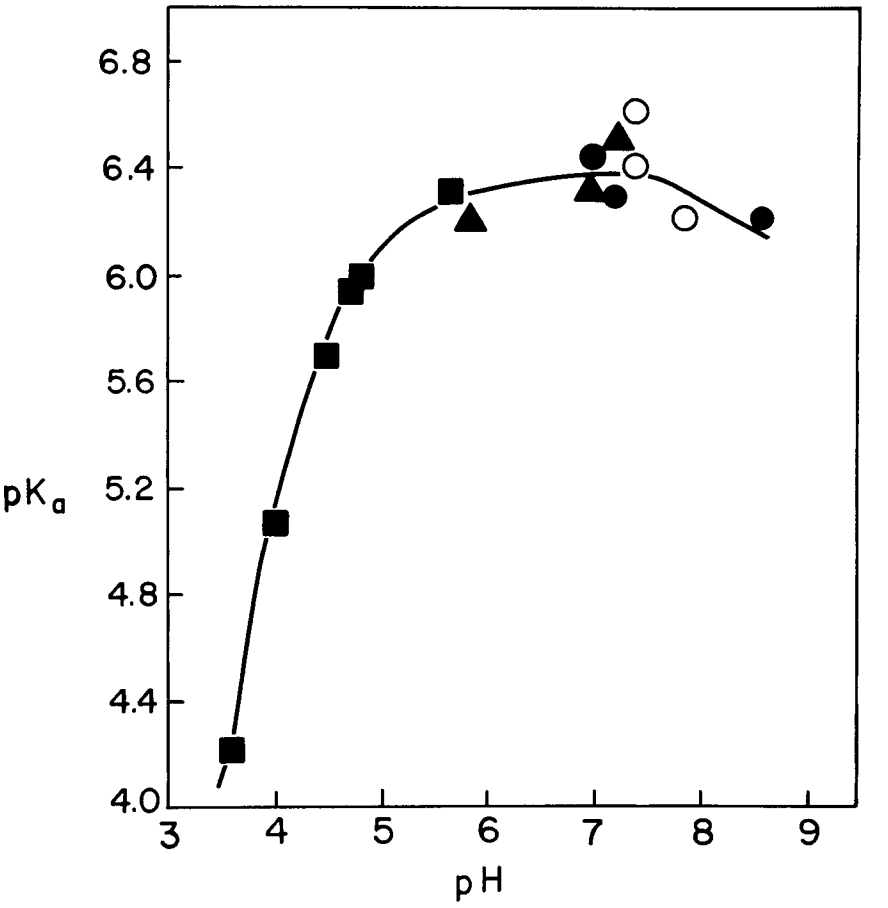


FIGURE 8. pH dependence of the binding of Ca^{+2} to apo-BLA. Buffers (0.02 *M*): ▲, PIPES, 0.1 *M* KCl; ■, acetate, 0.1 *M* KCl; ○, Tris, 0.1 *M* KCl; ●, Tris. (From Bratcher, S. C. and Kronman, M. J., *J. Biol. Chem.*, 259, 10875, 1984. With permission.)

$$[(K_a'/K_{app}) - 1] = (H^+)^n[1/K_1 - 1/(K_2K_o)] \quad (4)$$

A log plot of the data in the form of Equation 4 yields a value of n equal to 1.85, indicating there are two carboxylate ions in the Ca^{+2}-binding site. This is one less than the number of aspartate ions coordinated to the Ca^{+2} ion as seen in the X-ray crystallographic structure (see Section II.F for a detailed discussion of the structure of the calcium ion-binding site). Analysis of the pH dependence of the equilibrium constant for the low pH N-A equilibrium indicated that there were *three* carboxyl groups whose pK_{diss} were abnormally low in the N state.[27] A similar analysis of the pH dependence of the equilibrium constant for the N-A equilibrium observed on denaturation of BLA by guanidine-hydrochloride likewise demonstrated the presence of three abnormally dissociating carboxyl groups in the N state of the protein.[116,117] Coordination of these three carboxylate ions to Ca^{+2} would tend to reduce the magnitudes of their pK_{diss}.

Computation of n from the pH dependence of K_{app} for binding of Ca^{+2} assumed that all of the carboxyls in the binding site had the same proton dissociation constants. The value of 2 for n obtained from this analysis may reflect binding of Ca^{+2} at this site even when all three aspartates are not ionized, but more likely reflects experimental error.

4. Binding of Tb^{+3}

Tb^{+3} has proven to be very useful as an analog of Ca^{+2} in biological systems, particularly because of its luminescent properties. Like calcium ion, this cation prefers coordination to oxygen atoms of proteins[242] and thus might be expected to bind at the calcium site(s) of α LA. Binding of lanthanide ions to apo-BLA and their stabilization of N-like conformational states has been demonstrated by a number of investigators (Table 8), but detailed characterization of their binding and the concomitant conformational changes has been carried out only with terbium.[40,92]

Binding of Tb^{+3} has been shown to occur at *three* distinct sites on the BLA molecule.[40,92] Binding of the lanthanide at low concentrations of the ion gives rise to changes in tryptophan fluorescence which are *qualitatively* similar to those observed on binding of calcium ion to its high affinity site, i.e., a decrease in quantum yield (emission intensity) and a short wavelength shift of the emission spectrum. Such spectral changes, however, are not *quantitatively* identical to those observed with binding of Ca^{+2} and therefore are not suitable for determining the association constant by fluorescence titration. Since the procedure of Barela and Sherry[118] for determining terbium concentrations is too imprecise to use at the concentrations of lanthanide ion and protein employed, Bratcher and Kronman[40] determined binding of this ion using a Hummel-Dryer procedure, assuming its competition for binding with $^{45}Ca^{+2}$. The binding profile obtained by this method was highly anomalous (Figure 9A). The shape of this curve was accounted for by assuming two sites for binding of Tb^{+2} and that binding at the site of lower affinity *decreased* that at the one of higher affinity (Figure 9B).

Spectral data provide evidence for this second site (Figure 10). Plots of both tryptophan fluorescence and terbium luminescence intensity show discontinuities at (Tb^{+3})/(apo-BLA) ratios of ca. 0.5; this indicates the presence of a spectral change underlying the major one which occurs on binding of the cation to the high affinity site. In contrast, fluorescence titration of apo-BLA with calcium ions gives a sharp break at a (Ca^{+2})/(apo-BLA) ratio of 1.0 (Figure 5). The secondary spectral change, corresponding to binding of Tb^{+3} at the site of lower affinity, is seen as well as a small increase in both Tb^{+3} luminescence and tryptophan fluorescence at (Tb^{+3})/(apo-BLA) ratios >1 (Figure 10).

Binding of Tb^{+3} at a third site (pK_a estimated to be ca. 3) gives rise to time-dependent increases in both terbium luminescence and tryptophan fluorescence; the fluorescence properties after the spectral changes are complete are characteristic of an A-like molecular conformation. This pH-dependent change occurs with binding of Tb^{+3} to both apo- and Ca^{+2}-BLA (Figure 11). However, this spectral change does not merely reflect displacement of Ca^{+2} from BLA with formation of the "normal" A state of the protein. Addition of Tb^{+3} to *apo-BLA* below pH 7 (Figure 11A) results in binding at the vacant Ca^{+2} site and stabilization of the N conformation; but with increasing pH the Tb^{+3}-liganded protein takes on increasingly "A-like character". It should be emphasized that the pH dependence observed does not reflect that for binding of Ca^{+2} since pK_a for binding of the latter cation is almost constant from pH 6 to 8 (Figure 8). Note also that the quantum yield for tryptophan fluorescence of the A-like state promoted by binding of high concentrations of terbium ion is markedly *larger* than that determined for the metal-free state.[92] The characteristics of this "expanded A state" and a similar one promoted by the binding of Zn^{+2} (Section II.E) together with consideration of the origin of the pH dependence observed for the formation of this Tb^{+3}-mediated conformer of the protein are discussed in Section IV.

D. BINDING OF MANGANOUS ION; DO CALCIUM AND MANGANOUS IONS BIND AT THE SAME SITE?

There are three (Reference 40) or four (Reference 105) binding sites for Mn^{+2} on the BLA molecule. Binding at the site of highest affinity stabilizes an N-like state, as deduced

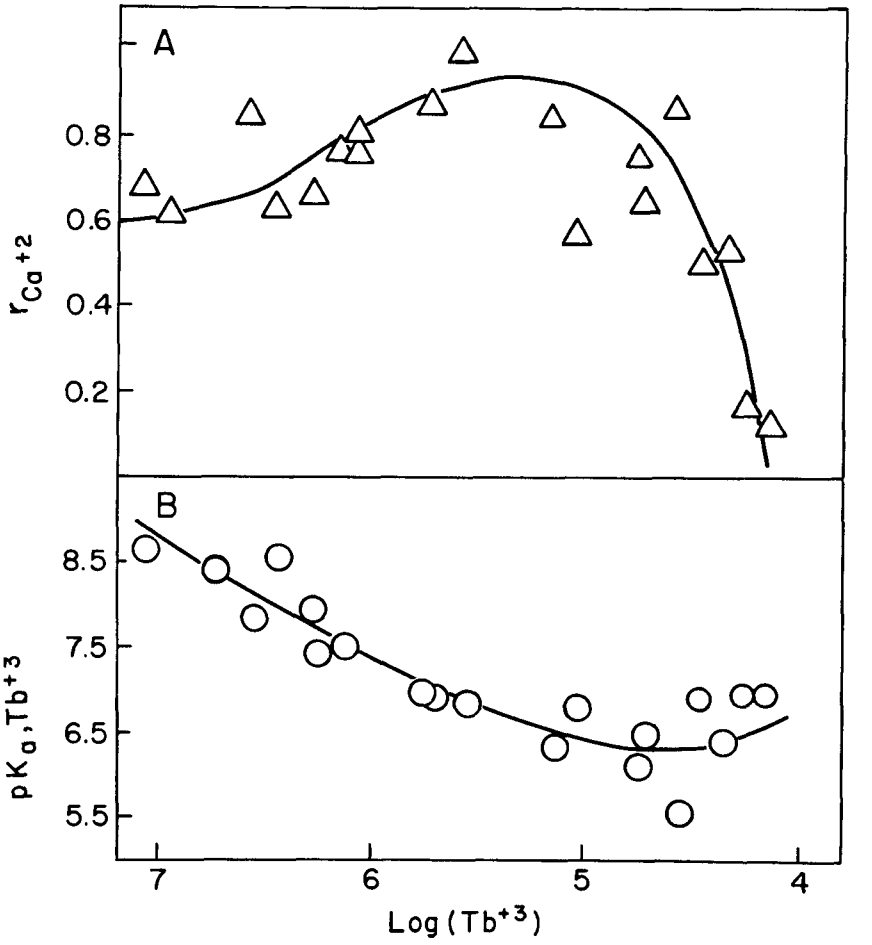


FIGURE 9. Binding of $^{45}Ca^{+2}$ to apo-BLA as a function of (Tb^{+3}). Buffer, pH 6.8, 0.02 *M* PIPES; (Ca^{+2}), 30 μ*M*. (A) Binding profile determined by a column-centrifuge technique. (B) Apparent dependence of pK_a on (Tb^{+3}), assuming competition of Ca^{+2} and Tb^{+3} for binding at a common site. (From Bratcher, S. C. and Kronman, M. J., *J. Biol. Chem.*, 259, 10875, 1984. With permission.)

from changes in tryptophan fluorescence[36,40,105] or in near-UV CD-spectra. Although comparable changes in these physical properties are observed on binding of Ca^{+2} at its high affinity site, as is discussed later, it is unlikely that the two cations bind at the same site.

1. Self-Association of Mn^{+2}-Liganded α LA

Characterization of the binding of Mn^{+2} to BLA is complicated by significant self-association of the Mn^{+2}-liganded protein. While solutions of Ca^{+2}-BLA or of apo-BLA of millimolar concentrations are visually transparent, solutions of Mn^{+2}-BLA greater than ca. 80 μ*M* (no KCl in buffer) or 60 μ*M* (0.1 *M* KCl) are very turbid.[40] Self-association of Mn^{+2}-BLA has been demonstrated even below 80 μ*M* in experiments where binding of the metal-ion was determined by fluorescence titration and by the Hummel-Dryer method using $^{54}Mn^{+2}$ (Reference 40). The results summarized in Table 9 indicate that pK_a for binding at the site of highest affinity decreases systematically with increase in protein concentration.

2. Binding of Mn^{+2} to BLA

Because of the self-association of the manganese-liganded protein, even at the moderate

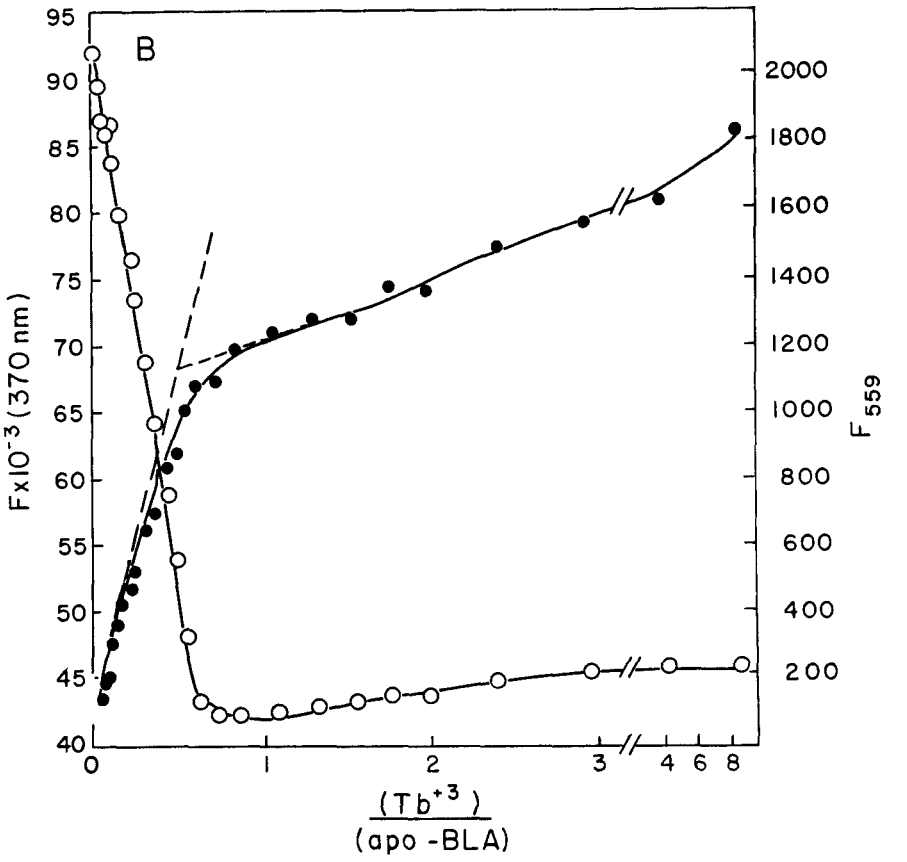


FIGURE 10. Fluorescence titration of apo-BLA with Tb^{+3}. Buffer, pH 6.4, 0.02 *M* Hepes. UV fluorescence (370 nm) and Tb^{+3} luminescence (559 nm) determined on the same solution after addition of each aliquot of terbium stock solution. ○, UV fluorescence; ●, Tb^{+3} luminescence. Excitation wavelength, 280 nm. [Tb^{+3}], 0.12 to 125 μ*M*. (From Bratcher, S. C. and Kronman, M. J., *J. Biol. Chem.*, 259, 10875, 1984. With permission.)

concentrations required for direct measurement of metal ion binding using $^{54}Mn^{+2}$, the measured binding profiles [*r* vs. $-\log(Mn^{+2})$], as well as the corresponding Scatchard plots, were anomalous and could not be analyzed for individual pK_a values for binding at the three sites.[40] The limiting value of pK_a for binding of Mn^{+2} to its high affinity site as the protein concentration approaches zero is ca. 5.8. The following discussion is restricted to binding of Mn^{+2} at this site.

3. Do Ca^{+2} and Mn^{+2} Bind at the Same Site on the BLA Molecule?

The fact that both Mn^{+2} and Ca^{+2} give rise to comparable changes in tryptophan fluorescence and in near-UV CD-spectra on binding to apo-BLA led Murakami et al.[105] to conclude that the two cations bind at the same site and stabilize the same conformational state. Competitive binding measurements carried out by Bratcher and Kronman,[40] however, have cast doubt on this conclusion. Scheme III describes the competitive binding of Mn^{+2} and Ca^{+2} to the N conformer of BLA.

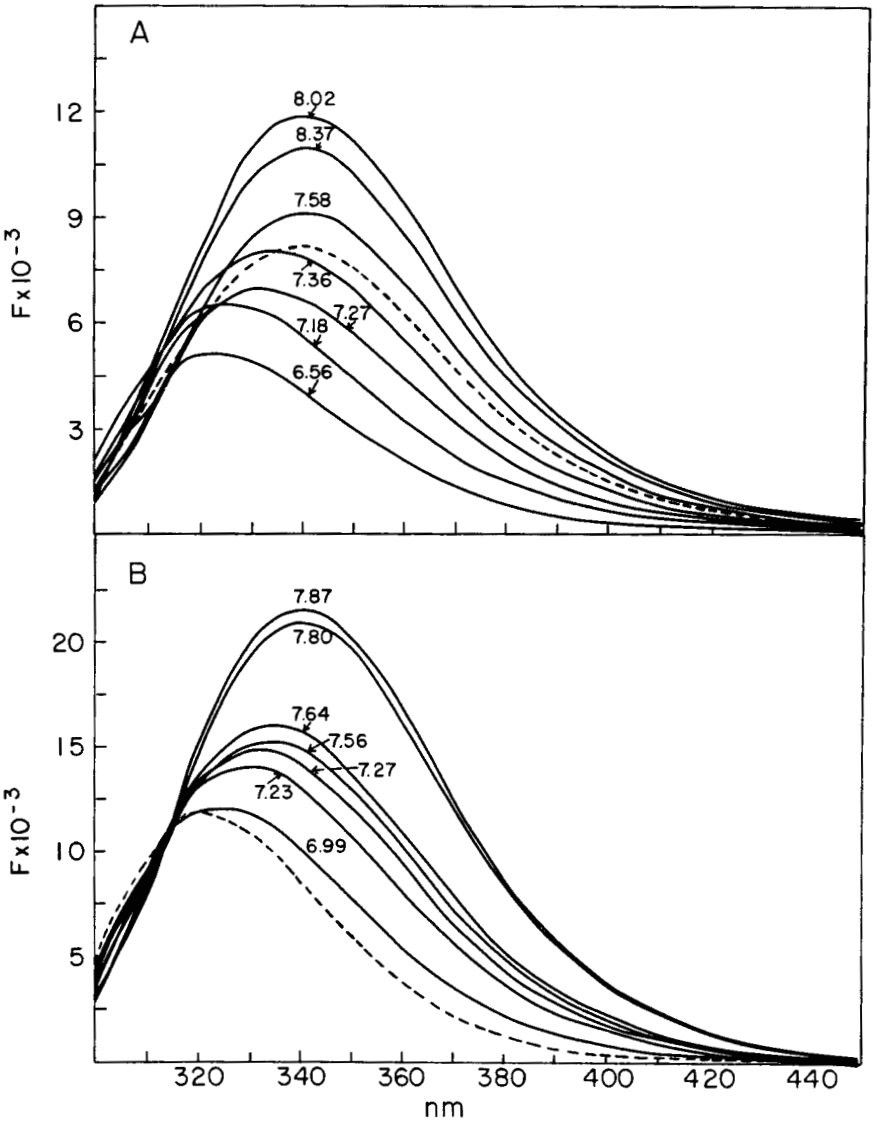


FIGURE 11. pH dependence of tryptophan emission spectra of apo-BLA (A) and BLA (B) on addition of 2.3 m*M* Tb^{+3}. The dashed spectra are those of apo-BLA (A) and BLA (B) prior to addition of Tb^{+3}. The numbers on the spectra indicate the pH of the buffered solution. Spectra were obtained after the fluorescence had attained a maximum value. (From Kronman, M. J. and Bratcher, S. C., *J. Biol. Chem.*, 259, 10887, 1984. With permission.)

$$(Mn^{+2};N) \rightleftharpoons Mn^{+2} + N \rightleftharpoons A \rightleftharpoons N + Ca^{+2} \rightleftharpoons (Ca^{+2};N)$$

$$K_{a,Mn+2} \qquad K_o \qquad K_o \qquad K_{a,Ca+2}$$

Scheme III

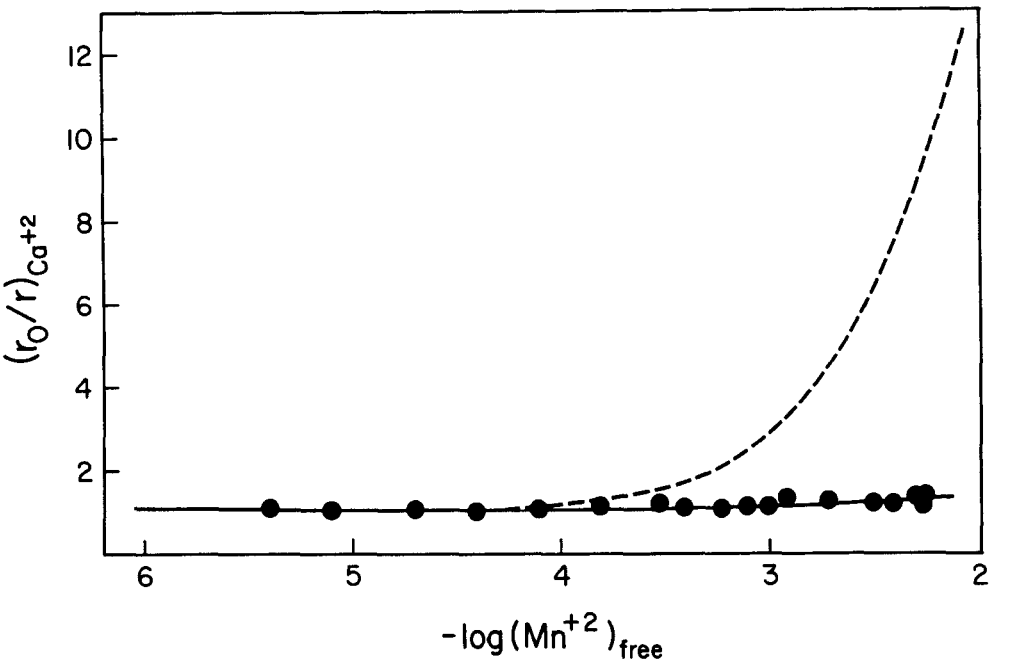


FIGURE 12. Binding of Ca^{+2} to apo-BLA as a function of (Mn^{+2}). Buffer, pH 6.9, 0.02 *M* Tris, 0.1 *M* KCl. (Ca^{+2}), 8.5 μM, - - -, concentration dependence of binding predicted for binding of Ca^{+2} and Mn^{+2} at common site. (From Bratcher, S. C. and Kronman, M. J., *J. Biol. Chem.*, 259, 10875, 1984. With permission.)

The binding of Mn^{+2} to the N conformer of BLA in the presence of Ca^{+2} is described by:

$$r = \frac{K_{app}\,(Ca^{+2})}{1 + K_{app}\,(Ca^{+2})} \tag{5}$$

where

$$K_{app,Ca^{+2}} = \frac{K_o K_{a,Ca^{+2}}}{1 + K_o K_{a,Mn^{+2}}\,(Mn^{+2})} \tag{6}$$

$(K_o\ K_{a,Ca+2})$ and $(K_o\ K_{a,Mn+2})$ are the measured association constants for binding of the individual cations. A similar equation describes the binding of manganese in the presence of calcium. Equation 6 can be transformed to Equation 7 where binding of calcium is measured as a function of (Mn^{+2}) at a fixed (Ca^{+2}).

$$(r_o/r) = 1 + \frac{(K_o K_{a,Mn^{+2}})(Mn^{+2})}{[1 + (K_o K_{a,Ca^{+2}})(Ca^{+2})]} \tag{7}$$

where r_o is the binding of calcium ion in the absence of manganese. Binding of Ca^{+2} to apo-BLA is almost completely unaffected by Mn^{+2} even at $(Mn^{+2})/(Ca^{+2})$ ratios as high as 700:1, although competitive binding of the two ions should have resulted in a nine- to tenfold decrease in r_o/r (compare ● and - - -, Figure 12). Binding of Ca^{+2} was relatively insensitive to the presence of Mn^{+2} (Reference 40); when binding of $^{45}Ca^{+2}$ was determined in the presence of 1 m*M* Mn^{+2} over a (Ca^{+2}) range of 0.1 to ca. 100 μM, a small reduction in binding of calcium ion was observed, corresponding to a decrease in pK_a of less than 0.4

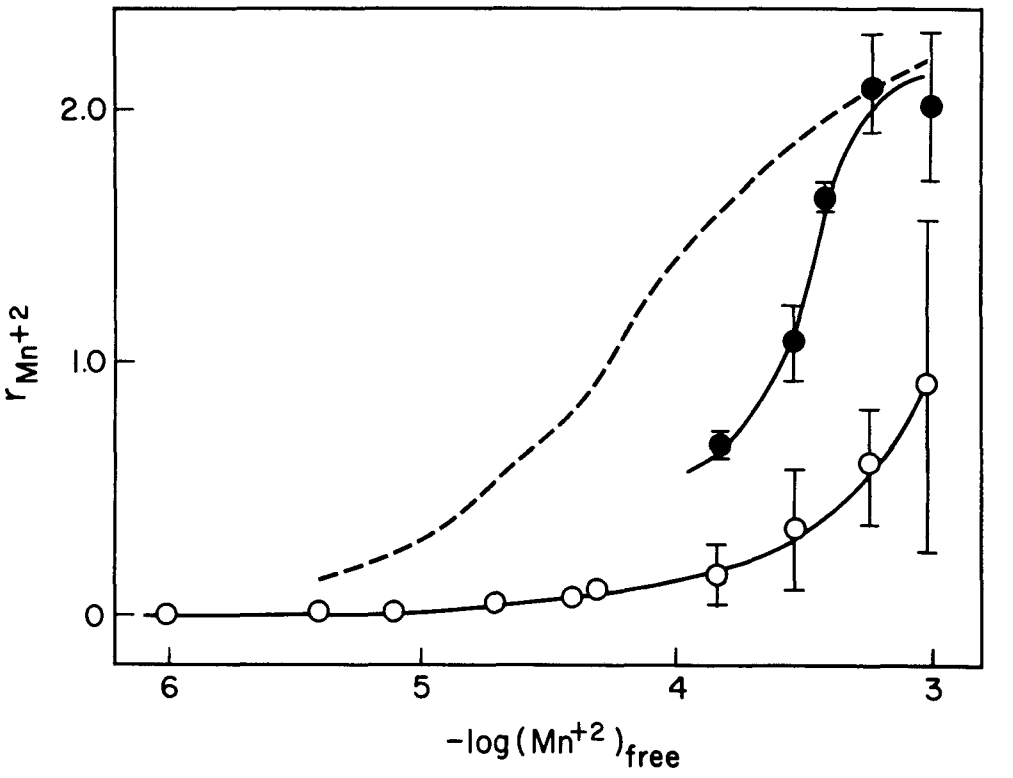


FIGURE 13. Binding of Mn^{+2} to apo-BLA in the presence of 8.5 μM Ca^{+2}. Buffer, pH 6.9, 0.02 *M* Tris, 0.1 *M* KCl. ○, standard procedure (see text), no preequilibrium of apo-BLA with Mn^{+2}; ●, 1 h of preequilibration with Mn^{+2}. - - -, Mn^{+2} binding in the absence of Ca^{+2}. (From Bratcher, S. C. and Kronman, M. J., *J. Biol. Chem.*, 259, 10875, 1984. With permission.)

units. If Mn^{+2} binds to apo-BLA in the presence of Ca^{+2}, the binding cannot be competitive since the predicted value of pK_a should have been reduced by about 1.6 units under these conditions. These observations are strong evidence for concluding that the two cations ions bind at *different* sites on the BLA molecule.

Observations of binding of $^{54}Mn^{+2}$ in the presence of a fixed concentration of Ca^{+2} are more difficult to interpret since binding of the former ion can occur at a minimum of three sites (Table 7). It is apparent, however, that the binding of Mn^{+2} to apo-BLA is markedly suppressed when Ca^{+2} is bound to its high affinity site (compare ○ with - - - -, Figure 13). The dependence of $r_{Mn^{+2}}$ on (Mn^{+2}) for binding to Ca^{+2}-BLA is consistent with its binding at a single site with pK_a of 3 to 4. This is significantly smaller than the value of 4.6 obtained for the binding of Mn^{+2} to its high affinity site on the BLA molecule in the *absence* of Ca^{+2}. This decrease in pK_a implies a coupling of the binding affinities at the two sites similar to that found for the binding of two terbium ions to the apo-BLA molecule (Figure 9). This phenomenon is discussed later in considering the molecular character of the Ca^{+2}- and Mn^{+2}-binding sites.

The competition experiments described earlier were carried out by adding apo-BLA to solutions containing *both* Ca^{+2} and Mn^{+2}. However, *preequilibration* of Mn^{+2} with the protein *prior* to addition of Ca^{+2} dramatically alters the binding behavior[40] (compare ○ and ●, Figure 13). The binding of Mn^{+2} after preequilibration with protein increases more than twofold compared with that observed with *simultaneous* mixing at comparable cation concentrations. Furthermore, the pattern of binding of Ca^{+2} is altered as well (Figure 14). While apo-BLA binds only a single calcium ion in 0.1 *M* KCl with no Mn^{+2} present, preequilibration

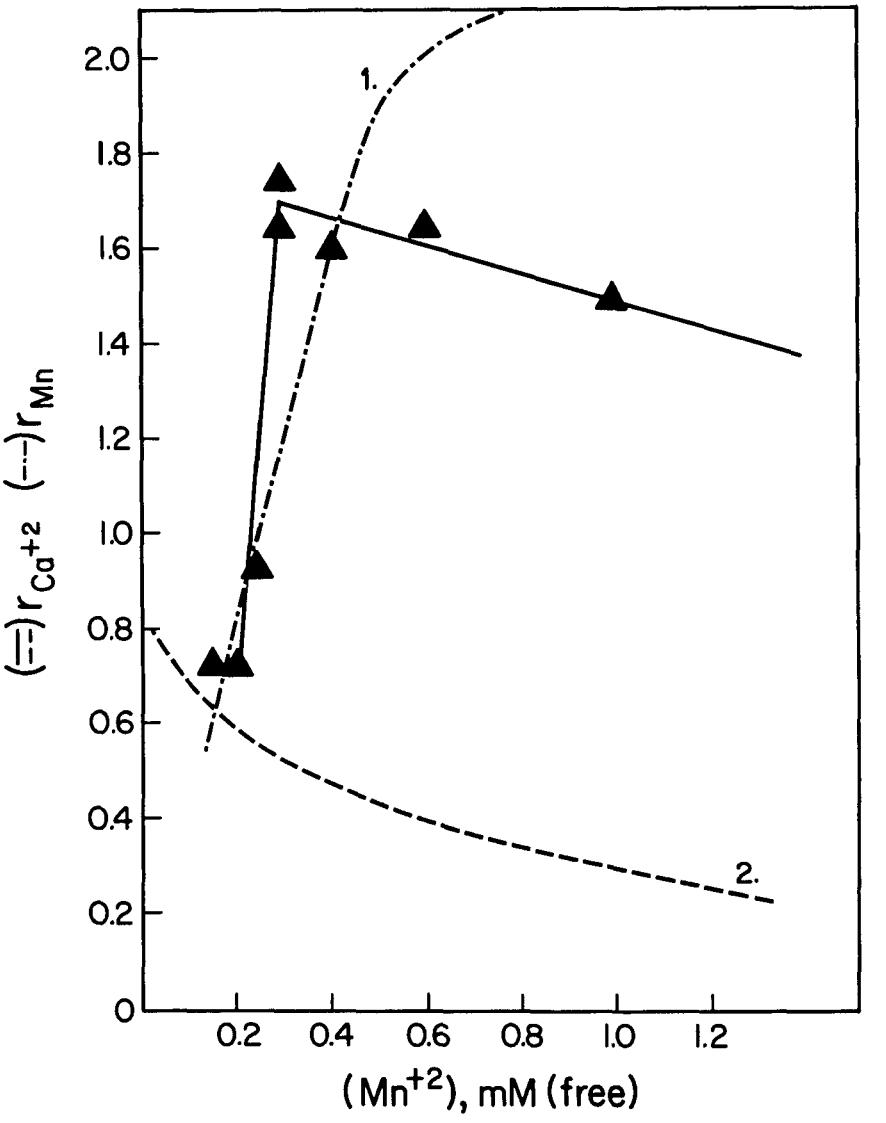


FIGURE 14. Binding of Ca^{+2} to apo-BLA as a function of (Mn^{+2}). Buffer, pH 6.9, 0.02 *M* Tris, 0.1 *M* KCl. (Ca^{+2}), 8.5 μ*M*. Binding of calcium ion determined after preequilibration of apo-BLA with Mn^{+2}. Curve 1, smoothed data for binding of Mn^{+2} after preequilibration. Curve 2, predicted binding of Ca^{+2}, assuming competative binding with Mn^{+2}. (From Bratcher, S. C. and Kronman, M. J., *J. Biol. Chem.*, 259, 10875, 1984. With permission.)

with the latter cation results in binding of Ca^{+2} at *two* sites. Making the assumption that one of the calciums binds at the high affinity site (pK_a, 6.4), K_a for the second one is estimated to be ca. 4×10^5, tenfold greater than the value determined in the absence of KCl for binding of calcium at the site of lower affinity.[36]

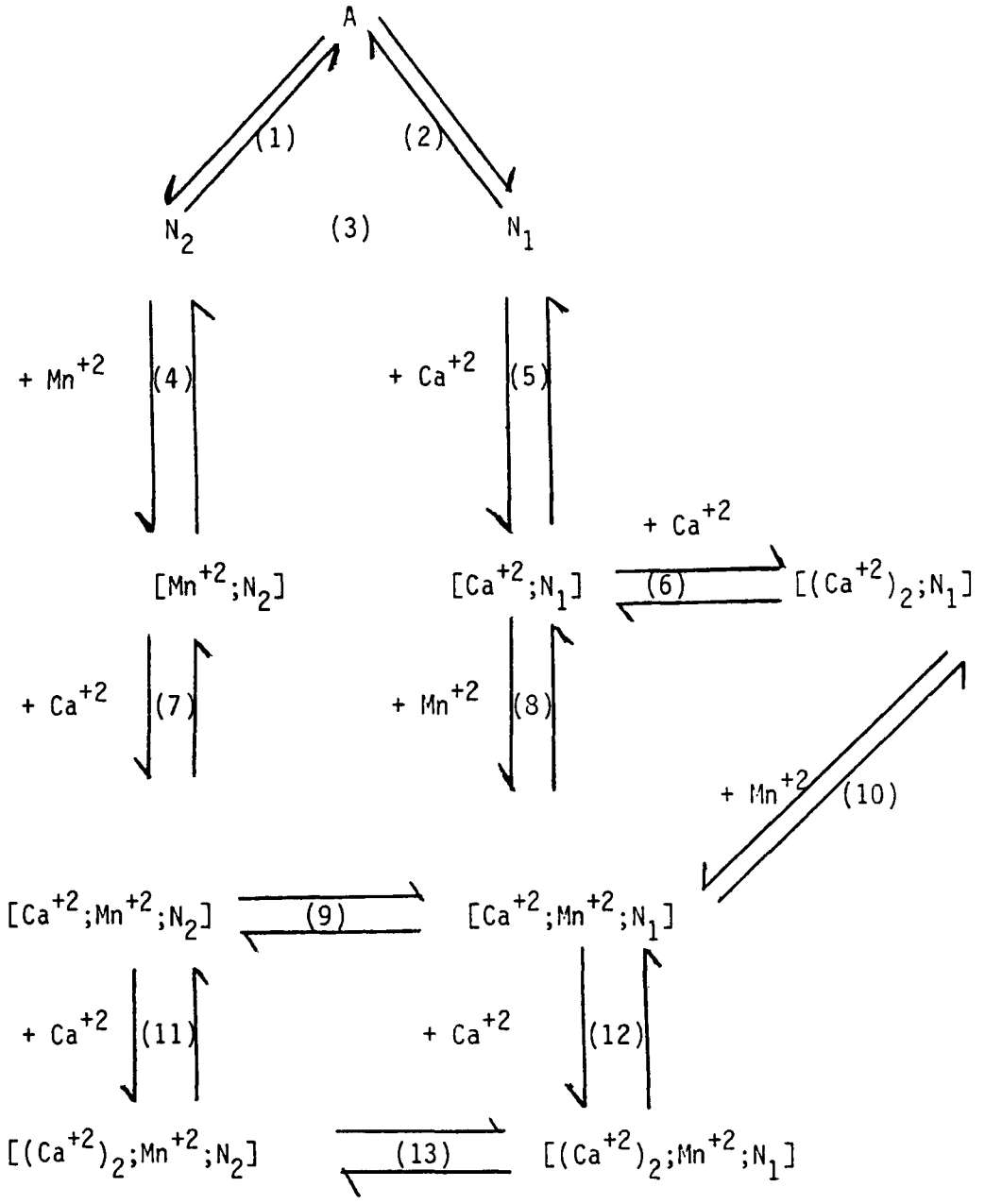
The NMR observations of Gerken[41] are consistent with simultaneous binding of Mn^{+2} and Ca^{+2} at their respective high affinity sites.[265] It is not clear, however, whether binding of the second calcium ion to Mn^{+2}-equilibrated protein (Figure 14) corresponds to that seen in the absence of Mn^{+2}. The fluorescence properties of the molecular species $[(Ca^{+2})_2;(Mn^{+2})_2$-BLA)] is indistinguishable from those of $(Mn^{+2}$-BLA) and $(Ca^{+2}$-BLA).[262]

It has been difficult to compare the observations of Bratcher and Kronman[40] with those

of Murakami et al.[105] since the latter data are presented as Scatchard plots with considerable scatter of the data, particularly in experiments where binding of Mn^{+2} to Ca^{+2}-BLA was determined. Their ESR measurements of Mn^{+2} binding were made at protein concentrations of ca. 0.4 m*M*, where extensive self-association of the Mn^{+2}-liganded protein would be expected. The effect of such self-association on their observations is not apparent. Both Bratcher and Kronman and Murakami et al. observed that prior binding of Ca^{+2} to BLA reduced the extent of binding of Mn^{+2}; this reduction in binding was interpreted by the latter authors, however, as the *absence* of binding of Mn^{+2} at its high affinity site. They conclude that Mn^{+2} and Ca^{+2} bind at the same high affinity site and that the inability of Mn^{+2} to displace Ca^{+2} is the consequence of the very large association constant for the latter cation (see Section II.B).

It is apparent from the observations of Bratcher and Kronman cited above that binding of Mn^{+2} to apo BLA and/or the concomitant change in conformation must be slow compared with that of Ca^{+2}. Indeed, careful measurements of tryptophan fluorescence indicate a difference in time dependence for binding of Mn^{+2} and Ca^{+2} (References 40 and 107); these spectral changes are virtually instantaneous for Ca^{+2}, while a monotonic change is observed with Mn^{+2}, requiring up to 3 min to attain a constant value.[40] Comparable time dependencies of CD-spectra have been reported in comparing the binding of Ca^{+2} and the monovalent cations Na^{+} and K^{+} (Reference 107) (Section II.E). The binding measurements discussed earlier suggest that (1) Mn^{+2} and Ca^{+2} bind to different sites on the α LA molecule and (2) the conformational states stabilized by binding of the two cations are different, in spite of their classification as N states. Further differences in the characteristics of the Ca^{+2}- and Mn^{+2}-mediated conformational states are discussed later.

While the binding of Mn^{+2} and Ca^{+2} to different sites with concomitant formation of different N-like states accounts for many of the observations made with these two cations, left unanswered is the question as to why the extent of binding of both Mn^{+2} and Ca^{+2} to apo-BLA depends on their *order of mixing* with the protein. Scheme IV describes a system of equilibria for binding of Ca^{+2} and Mn^{+2} to different high affinity sites with stabilization of two different conformational states, N_1 and N_2. The principle of microscopic reversibility requires that the positions of these conformational and binding equilibria depend only on the concentrations of the two cations and not on their order of mixing. The observation that the cation-binding pattern changes when the protein is preequilibrated with Mn^{+2} (Figure 13) demonstrates that binding and formation of the characteristic conformational state must have *kinetic* determinants. When the two cations are mixed simultaneously, conformation N_1 is formed which is not readily converted to N_2; similarly, preequilibration with Mn^{+2} leads to formation of N_2 which is not readily converted to N_1.



Scheme IV

A similar situation has been observed with concanavalin A which also binds Ca^{+2} and/or Mn^{+2} and can exist in two conformational states with different metal-ion-binding properties.[119,120] It has been proposed that these two states with similar ground state free energies are separated by an energy barrier which prevents their rapid interconversion. The two metal-ion-binding sites of this protein are known from the crystal structure to share common aspartates as coordinating groups.[121,122] The NMR study of Gerken[41] provides evidence for a Mn^{+2}-binding site on the BLA molecule adjacent to the Ca^{+2} site seen in the crystal structure of BLA. These two sites may also share common coordinating groups. The relationship between the calcium and the putative Mn^{+2} sites is discussed in Section II.F.

4. NMR Studies of Ca^{+2}- and Mn^{+2}-Liganded α LA

Gerken[41] used ^{13}C-amino group methylation and ^{13}C-NMR spectroscopy to probe amino group molecular environments and metal-ion interactions of BLA. The spectral properties of the α amino group of α LA were highly sensitive to removal of Ca^{+2} from its high affinity site and to binding Zn^{+2}, Mn^{+2}, and Ca^{+2} at a site distinct from the high affinity calcium-binding site. The ^{13}C-methyl groups on the α amino group have a characteristic resonance when calcium is bound to its high affinity site. When very small amounts of Mn^{+2} were added to Ca^{+2}-saturated BLA [final concentration of Mn^{+2}, 40 μ*M*; (Ca^{+2}-BLA,) 0.49 m*M*], the resonance line remained fixed in position but exhibited paramagnetic broadening indicative of binding of Mn^{+2} close to the ^{13}C-methyl groups on the α amino group. Since it is unlikely that Ca^{+2} was displaced from its high affinity site by 40 μ*M* Mn^{+2}, the later ion must bind at a site which is relatively close to the α LA amino group and distinct from the high affinity calcium site. Simultaneous binding of Mn^{+2} and Ca^{+2} has also been observed from measurements with isotopic metal ions (see earlier discussion). Gerken proposed in his original publication[41] that Ca^{+2} and Mn^{+2} bind at the same high affinity site. He indicates now, however, that his NMR observations are equally consistent with Ca^{+2} occupying its high affinity site distinct from a high affinity site for Mn^{+2} close to the α LA amino group.[265]

When additional calcium ion was added to Ca^{+2}-Mn^{+2}-BLA, the broadened α amino resonance narrowed, demonstrating displacement of Mn^{+2} as a second calcium ion binds at site II. Insufficient information is available, however, to ascertain if this corresponds to the second calcium ion which binds to BLA in the absence of KCl or one whose pK_a was so low as to preclude detection in measurements using $^{45}Ca^{+2}$ (References 36 and 40).

E. BINDING OF COBALT AND ZINC TO α LA

1. Binding of Co^{+2}

Murakami and Berliner[106] reported changes in tryptophan fluorescence on titration of apo- and Ca^{+2}-BLA with Co^{+2} and concluded that binding of this cation promoted formation of a conformational state whose fluorescence properties are intermediate between those found for N and A states. Binding of this ion has also been reported by Musci and Berliner.[123] These observations are in disagreement with those of Kronman et al.,[36] who showed that 1 m*M* Co^{+2} did not displace $^{45}Ca^{+2}$ from $^{45}Ca^{+2}$-BLA. Fluorescence measurements have repeatedly failed to detect changes when this cation was added to apo- or Ca^{+2}-BLA.[262] The reason for this discrepancy is not obvious. This difference should be resolvable using radioisotopes of Co^{+2} to detect and characterize possible binding.

2. Binding of Zn^{+2}

Kronman et al.[36] were the first to demonstrate that BLA binds Zn^{+2} (Figure 15). Addition of 1.36 m*M* Zn^{+2} to Ca^{+2}-BLA resulted in a time-dependent increase in tryptophan fluorescence, giving rise to a spectrum characteristic of an "A-like" state (Figure 15, compare spectra A and B of insert). Determination of the binding of $^{45}Ca^{+2}$ to apo-BLA in the presence and absence of 1 m*M* Zn^{+2} indicated displacement of the calcium from its high affinity site.[36] The observations suggested at the time that Zn^{+2} might bind to the Ca^{+2} site, with the differences in conformational states for the two forms of the protein resulting from differences in coordination properties of the two cations. Subsequent investigations demonstrated that the binding of Zn^{+2} and its effects on the molecular conformation of the α LA molecule was considerably more complex than the interpretation given here indicates.

The following observations provide evidence for binding of Zn^{+2} *at two different sites* with values of pK_a of 5.2 and 2.8, respectively.[42,92] Binding at the high affinity site gives rise to a small increase in the quantum yield of tryptophan fluorescence relative to the metal-free state; the characteristics of the spectrum classifies the conformation to be "A-like".

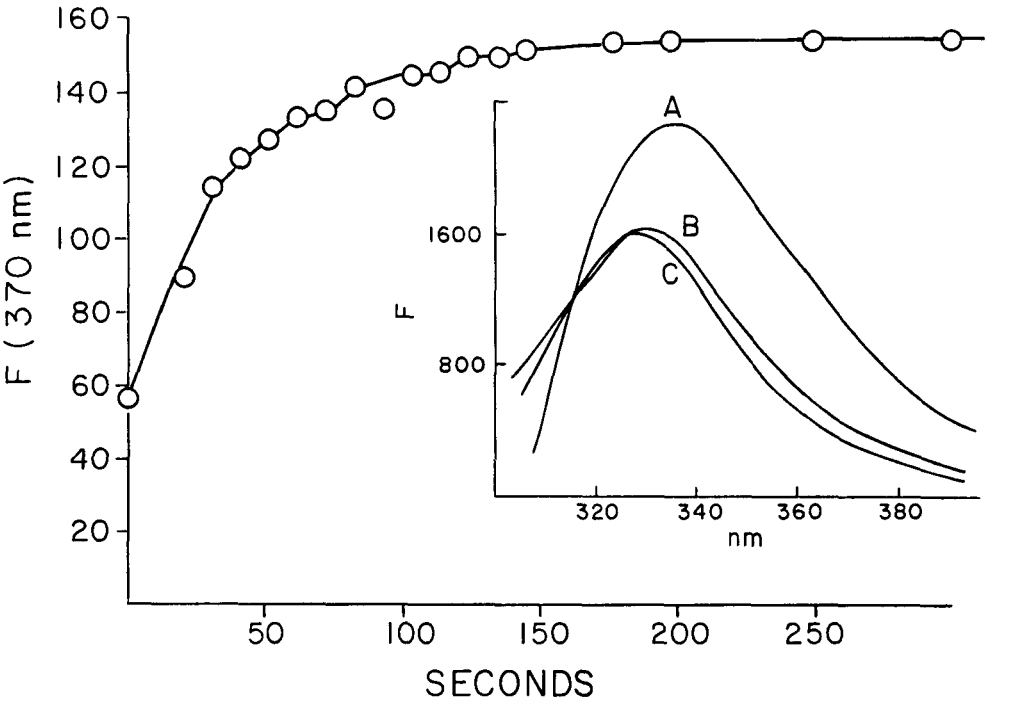


FIGURE 15. Time dependence of displacement of Ca^{+2} from native BLA by Zn^{+2}. BLA concentration, 1.46 μM; Zn^{+2} concentration, 1.36 mM. Inset, comparison of the tryptophan emission spectra of native bovine BLA in the absence of added metal-ion (curve B) and in the presence of 1.36 mM Zn^{+2} (curve A). Spectra were scanned after spectral changes were complete, 10 to 20 min. (From Kronman, M. J., Sinha, S., and Brew, K., *J. Biol. Chem.*, 256, 8582, 1981. With permission.)

Binding of Zn^{+2} at the site of lower affinity on the BLA *and* the apo-BLA molecule gives rise to time-dependent increases in tryptophan fluorescence, with constant values being attained in times of the order of minutes, depending on the concentration of zinc. The magnitude of the change in emission intensity also depended on the Zn^{+2} concentration, but unlike similar time-dependent changes observed with high concentrations of Tb^{+2} (Section II.C), those with Zn^{+2} were independent of pH. These data obtained with Zn^{+2} can be treated as a fluorescence titration curve; the Scatchard plots calculated from the smoothed data (Figure 16) yield an apparent pK_a of 2.7 (compare with the value 2.78 deduced from binding measurements made with $^{65}Zn^{+2}$).

The changes in the quantum yield, of tryptophan fluorescence of *apo-BLA* on addition of Zn^{+2}, are of particular interest (Figure 17). These data were obtained from the emission spectra after the fluorescence had attained a constant value. The dependence of Q on zinc concentration is biphasic for apo-BLA; a small increase in Q was observed as Zn^{+2} was bound to the high affinity site, with a further increase occurring as it is bound to the site of lower affinity. The maximum value of Q was almost *twice* that observed for the metal-free protein. These observations suggest that binding of zinc ion at the site of lower affinity stabilizes a molecular state different from either the metal-free state or that formed on binding of Zn^{+2} at its high affinity site. This unique state has been referred to as the "expanded A" state.[42] Addition of high concentrations of Zn^{+2} to either Ca^{+2}- or Mn^{+2}-BLA gives rise to comparable time dependencies of the tryptophan fluorescence with apparent displacement of either cation and conversion of the protein to the expanded A form.

Measurements of binding of $^{65}Zn^{+2}$ to apo-BLA yielded a value of pK_a of 5.05 ± 0.02 (Figure 18).[40] These data also support the conclusion that there is a weaker Zn^{+2}-binding

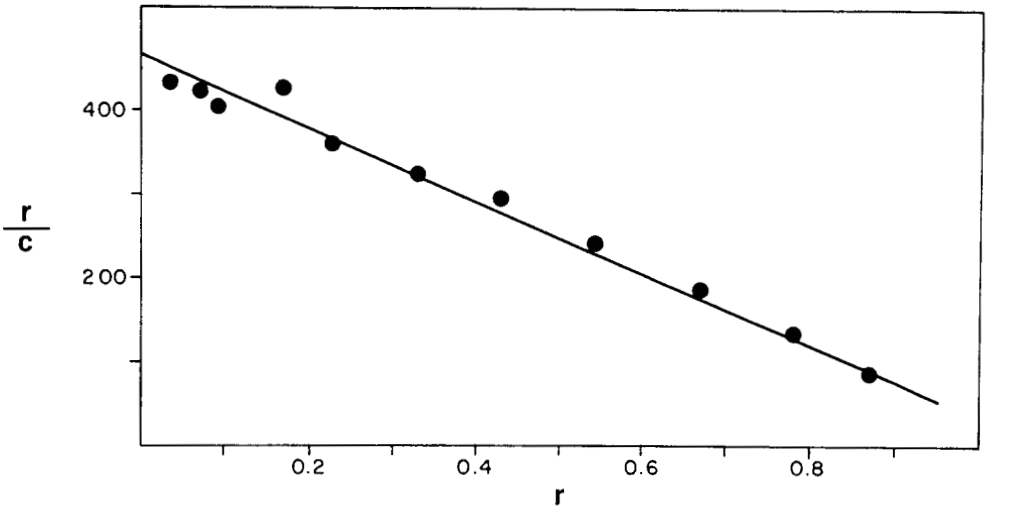


FIGURE 16. Scatchard plot of the binding of Zn^{+2} at the low affinity site of apo-BLA. Binding determined from equilibrium values of the fluorescence at 370 nm. Buffer, pH 7.4, 0.02 *M* Tris; protein concentration, ca. 1.3 μ*M*. (Data from Kronman, M. J. and Bratcher, S. C., *J. Biol. Chem.*, 259, 10887, 1984. With permission.)

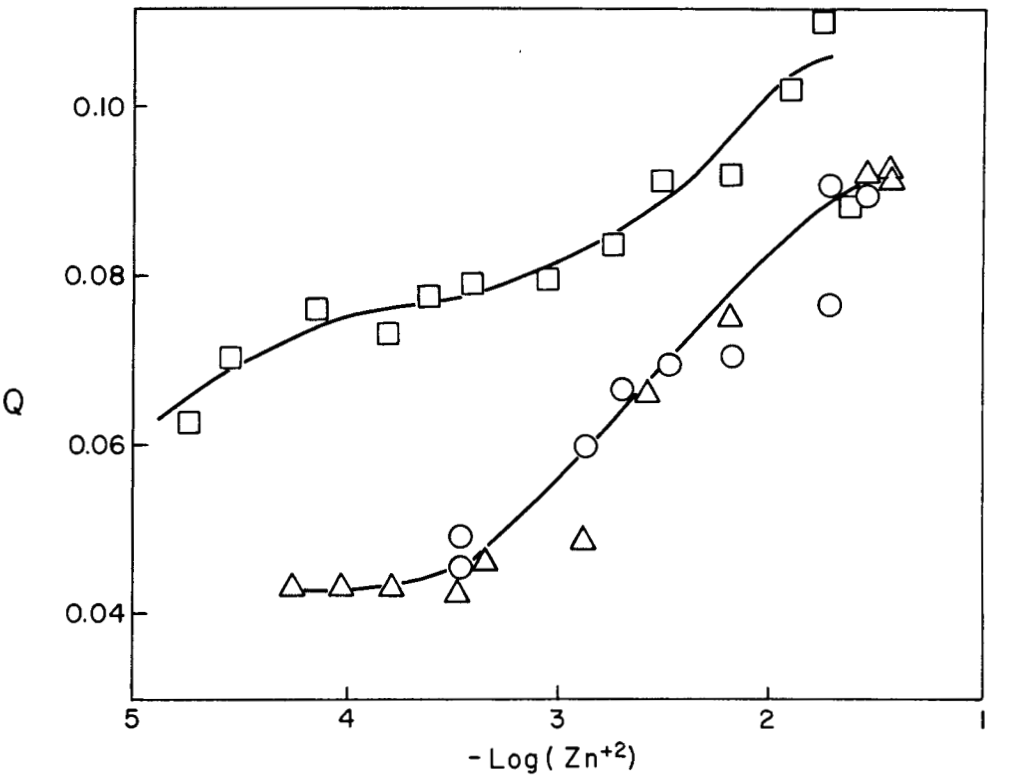


FIGURE 17. Dependence of tryptophan quantum yield of α LA on (Zn^{+2}). ○, Ca^{+2}-BLA; △, apo-BLA + 2 m*M* Mn^{+2}; □, apo-BLA. Buffer, pH 7.4, 0.02 *M* Tris. Protein concentration 1.2 to 1.5 μ*M*. (From Kronman, M. J. and Bratcher, S. C., *J. Biol. Chem.*, 259, 10887, 1984. With permission.)

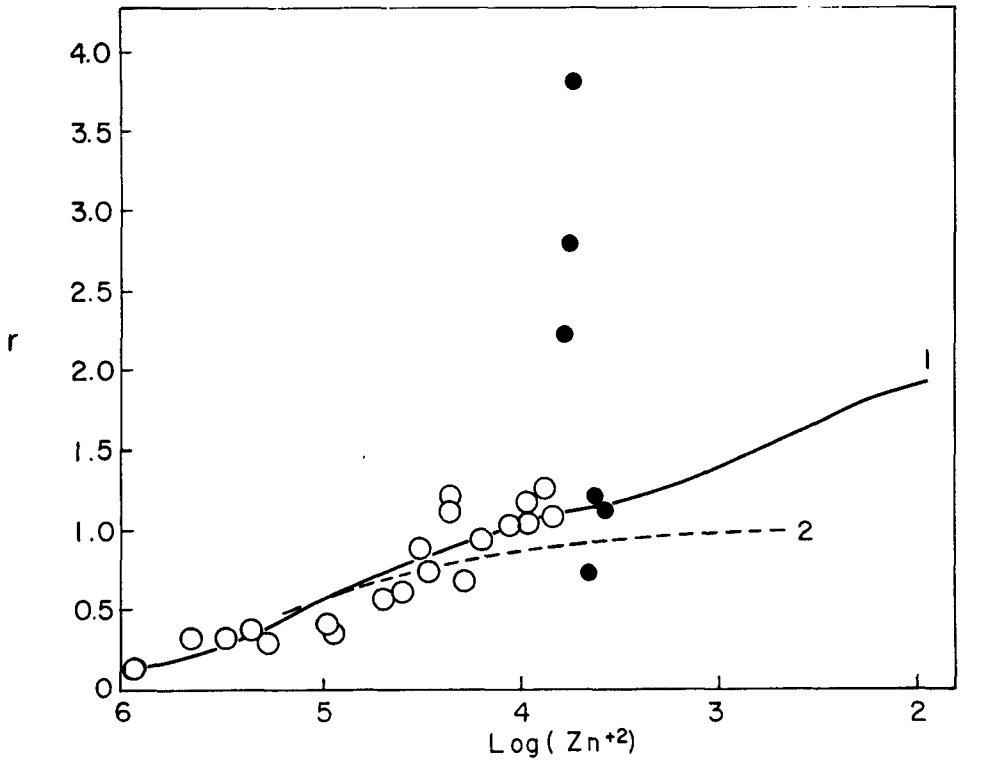


FIGURE 18. Zn^{+2}-apo-BLA-binding profile. Buffer, pH 7.3, 0.02 *M* Tris, ○ KCl. ●, data not included in regression analysis (see text). Curve 1, binding of Zn^{+2} computed from the parameters of a two-site nonlinear regression analysis of the data ($pK_{a,1}$, 5.05; $pK_{a,2}$, 2.78). Curve 2, binding of Zn^{+2} computed from the parameter of a one-site analysis of the data (pK_a, 5.15). (From Bratcher, S. C. and Kronman, M. J., *J. Biol. Chem.*, 259, 10875, 1984. With permission.)

site with a pK_a of 2.78 ± 0.01. It should be noted that at (Zn^{+2}) > ca. 0.2 m*M* precipitation of the protein occurs (Figure 18), suggesting that BLA in the "expanded A" state undergoes significant self-association. Self-association was also observed in fluorescence measurements, where observations of visible turbidity at protein and zinc iron concentrations above 5 μ*M* and 0.2 m*M*, respectively, necessitated use of much lower protein concentrations than were typically employed.[92] The effect of self-association on the binding properties of Zn^{+2} has not been evaluated, but it might be expected to be significant as demonstrated for self-association of Mn^{+2}-BLA (see Section II.D). The ESR measurements of Murakami and Berliner,[106] which show displacement by Zn^{+2} of Mn^{+2}-BLA, might be expected to be significantly influenced by self-association since they were carried out at with protein concentrations of 0.37 m*M*.

Observations of Zn^{+2} binding made by Murakami and Berliner[106] are quite different from those discussed previously. This study appeared to employ a rather narrow range of zinc ion concentrations, typically 0.2 to 2 m*M*, compared with 0.02 to 40 m*M* (fluorescence titration) and 0.001 to 0.2 m*M* (binding measurements) employed by Kronman and Bratcher[92] and Bratcher and Kronman,[40] respectively. This difference probably accounts for the conclusion reached by Murakami and Berliner that there is a single Zn^{+2}-binding site on BLA. Their observations are discussed further in considering competitive binding of Zn^{+2}, Ca^{+2}, and Mn^{+2} (Section II.E.4).

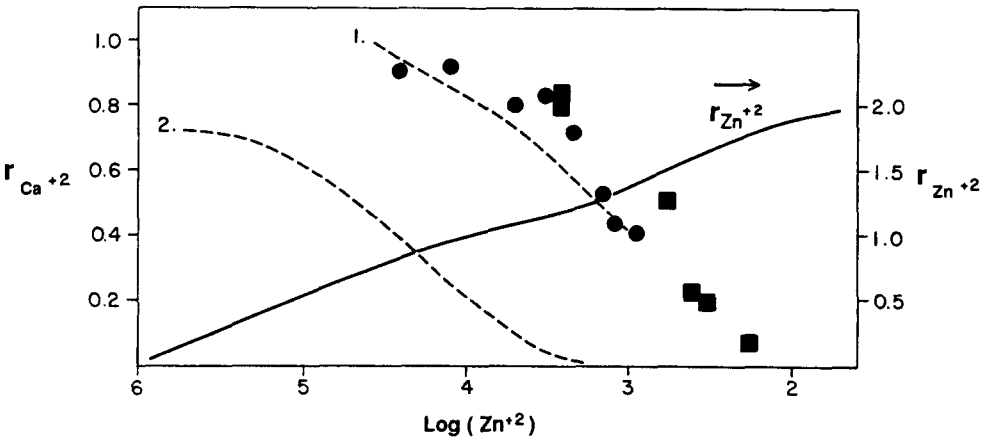


FIGURE 19. Effect of Zn^{+2} on the binding of Ca^{+2} to BLA. ●, Binding of $^{45}Ca^{+2}$ to apo-BLA in the presence of Zn^{+2}; (Ca^{+2}), 32 μ*M*; protein concentration 1.2 to 1.5 μ*M*. ■, Binding of calcium ion calculated from fluorescence titration of 1.3 μ*M* Ca^{+2}-BLA with zinc (see text). Binding of zinc calculated from pK_a values of 5.05 and 2.78. Curves 1 and 2 are the predicted binding of Ca^{+2} assuming its competition with Zn^{+2} for measurements with $^{45}Ca^{+2}$ and for fluorescence titration, respectively. The binding and fluorescence titration data were taken, respectively, from Bratcher and Kronman[40] and Kronman and Bratcher.[92]

3. Do Zn^{+2} and Ca^{+2}, Compete for Binding to BLA?

Determination of possible competition of binding of Zn^{+2} and Ca^{+2} is complicated by binding of the former cation at a site of lower affinity on the BLA molecule with a concomitant time-dependent transformation of its conformational state. This is evident from the observations made in Figure 19 of $^{45}Ca^{+2}$ binding in the presence of Zn^{+2} by the Hummel-Dryer method (○) and by fluorescence titration (□).[40,92] Calculation of Ca^{+2} from the fluorescence titration data assumes that the increase in fluorescence observed with increasing Zn^{+2} concentration (Figure 19) results from displacement of Ca^{+2}. Since binding of Zn^{+2} to the low affinity site of BLA also results in an *increase* in the quantum yield of tryptophan fluorescence, the values of r_{Ca+2} calculated from the fluorescence change may be somewhat *underestimated*. Curves 1 and 2 show the predicted binding of Ca^{+2}, assuming binding competition between the two ions. Also shown is a plot of r_{Zn+2} vs. $-\log (Zn^{+2})$ calculated from the association constants for the two sites.

Comparison of the experimental values of $r_{Ca+}{}^{2}$ (●,■ Figure 19) with the predicted values (curve 1), indicates that binding of Ca^{+2} persists until the low affinity site is saturated with Zn^{+2}, i.e., *simultaneous* binding of Ca^{+2} and Zn^{+2} and their respective high affinity sites can occur. Murakami and Berliner[106] concluded that binding of Ca^{+2} and Zn^{+2} is mutally exclusive; this conclusion does not seem tenable in view of the observations discussed here. In a later paper, Musci and Berliner[124] reported that Ca^{+2} was still bound to Zn^{+2}-BLA after prolonged dialysis of Ca^{+2}-BLA vs. 0.4 m*M* Zn^{+2}, further evidence against the mutally exclusive binding of the two cations proposed by Murakami and Berliner. These observations indicate that Ca^{+2} and Zn^{+2} bind simultaneously to BLA until the low affinity Zn^{+2} site is occupied, whereupon Ca^{+2} is displaced on formation of the "expanded A" state.

Although Murakami and Berliner[106] observe an apparent displacement of Ca^{+2} from BLA at high concentrations of Zn^{+2}, such displacement is totally inconsistent with the value of pK_a, 9.7, that they reported[105] for binding of Ca^{+2} at its high affinity site. If one assumes competition of binding of Ca^{+2} with zinc bound at *either* of its two sites, the magnitude of pK_a is too high to observe significant displacement of the former ion, e.g., K_{app} for binding of calcium in the presence of 2 m*M* zinc ion is calculated to be 1.4×10^7, corresponding

to a value of $r_{Ca^{+2}}$ of 0.99. This observation is further evidence that the magnitude of pK_a reported by Murakami et al.[105] for binding of Ca^{+2} at its high affinity site is in error.

The ^{13}C-NMR studies of ^{13}C-methylated BLA of Gerken[41] are useful in amplifying and extending the relationship of Ca^{+2} and Zn^{+2} binding. Not surprisingly, addition of Zn^{+2} to metal-free BLA had no effect on any of the resonances of the ^{13}C-methylated amino groups since Zn^{+2}-liganded BLA exists in A-like conformations regardless of the site occupied. Gerken concluded from experiments where Ca^{+2} and Zn^{+2} were added to Ca^{+2}-BLA that low concentrations of Zn^{+2} do not displace Ca^{+2} bound at the high affinity site. He observed that Zn^{+2} was in fast exchange with Ca^{+2} bound at the second site (see Section II.B for a discussion of binding of Ca^{+2} at a second site). When high concentrations of Zn^{+2} were added to Ca^{+2}-BLA, Gerken observed a shift in the ^{13}C-resonance of the methylated α-amino group to a position characteristic of the apo-state. Gerken concludes that such a shift might indicate the presence of second zinc-binding site, which at the time his paper was published had not been established.

4. Do Zn^{+2} and Mn^{+2} Compete for Binding to BLA?

Although both Bratcher and Kronman[40] and Murakami and Berliner[106] carried out measurements to determine if Zn^{+2} and Mn^{+2} bind competitively, these experiments are faulty and are inconclusive in answering the question of whether these two cations might bind at the same site. The ESR measurements of Murakami and Berliner show that about 90% of the Mn^{+2} bound to BLA is displaced by *total* concentrations of zinc approaching 1.3 m*M* [(Mn^{+2}), 0.4 m*M*; (apo-BLA), 0.37 m*M*]. However, since no determination of zinc binding was carried out, the stoichiometry of its binding on displacement of Mn^{+2} is uncertain. Interpretation of their observations is further complicated by the extensive self-association, if not precipitation, of the zinc and manganese liganded proteins at the concentrations required for resonance measurements. Does binding of zinc at its high affinity site displace Mn^{+2} from its high affinity site? Can binding of Mn^{+2} and Zn^{+2} occur simultaneously, as was seen with Ca^{+2} and Zn^{+2}? These questions remain unanswered. The displacement of Mn^{+2} by *high* concentrations of Zn^{+2} observed by Murakami and Berliner most likely results from formation of the "expanded A" state promoted by binding of the latter ion at its low affinity site, as was found with Ca^{+2}-BLA.

The experiments of Bratcher and Kronman[40] suffer from a similar defect. While the binding of $^{65}Zn^{+2}$ was determined as a function of Mn^{+2} concentration, binding of Mn^{+2} in the presence of Zn^{+2} was not measured. Furthermore, since the concentrations of Zn^{+2} in these experiments were chosen to saturate the *high* affinity binding site, without binding at the site of low affinity of this metal-ion, relatively high concentrations of Mn^{+2} were required to observe displacement of Zn^{+2}. The possibility that binding of Zn^{+2} at its high affinity site is influenced by binding of Mn^{+2} at sites of lower affinity thus cannot be excluded. The observed displacement was less than predicted, assuming that both ions compete for binding at the same site, e.g., at free Mn^{+2} and Zn^{+2} concentrations of 1 m*M* and 87 μ*M*, respectively, the experimental value of $r_{Zn^{+2}}$ was 0.4, while the predicted value was ca. 0.2. More detailed binding measurements need to be made using both $^{65}Zn^{+2}$ and $^{54}Mn^{+2}$ to resolve the questions posed earlier. Such experiments should also be directed toward determining if competitive binding of Zn^{+2} and Mn^{+2} to BLA might have kinetic determinants, as was the case with Ca^{+2} and Mn^{+2}.

Gerken[41] has proposed that Zn^{+2} and Mn^{+2} bind at the same site on the BLA molecule. If this were true, the paramagnetic broadening of the ^{13}C-resonance of the methylated α LA amino group which occurs on binding of Mn^{+2} to Ca^{+2}-BLA should narrow when low concentrations of Zn^{+2} are added. This experiment, however, does not appear to have been carried out.

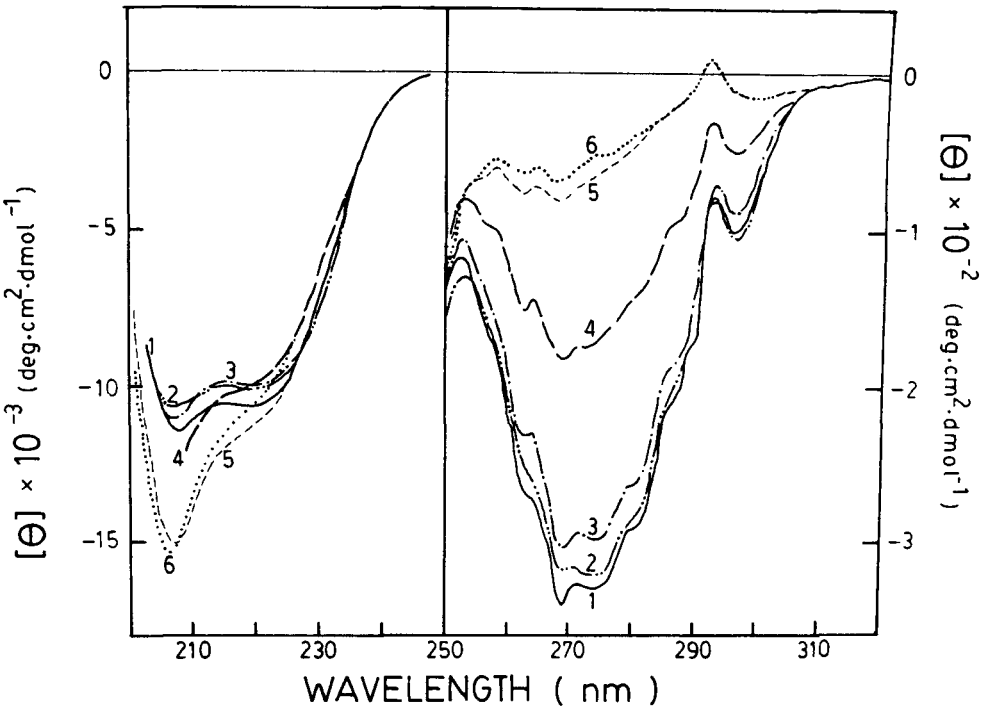


FIGURE 20. CD-spectra of apo-α LA at pH 8.0: curve 1, in 20 m*M* Tris in the presence of 200 m*M* NaCl at 25°C; curve 2, in 20 m*M* Tris in the presence of 1.29 m*M* $CaCl_2$, at 25°C (native holo-state); curve 3, in 20 m*M* Tris in the presence of 500 m*M* KCl at 25°C; curve 4, in 0.02 *M* Tris at 25°C; curve 5, in 20 m*M* Tris at 25°C; curve 6, in 20 m*M* Tris at 51°C. Protein concentration was 2.3 to 3.5 × 10^{-5} *M*. (From Hiraoka, Y. and Sugai, S., *Int. J. Pept. Protein Res.*, 26, 252, 1985. With permission.)

F. BINDING OF MONOVALENT CATIONS

Monovalent cations were shown by Kronman and Bratcher[92] and by Hanssens et al.[114] to stabilize the N conformational state of BLA, but their observations were incorrectly interpreted as ionic strength effects. The findings of Hiraoka and Sugai[107] that the effects of K^+ and Na^+ manifest themselves in different concentration ranges, however, rule out this interpretation and indicate rather that it is binding of these monovalent cations which stabilizes the N state of the protein. The effect of sodium ion on the thermal stability of apo-BLA[104,125] is likewise consistent with the binding of the cation. The binding of K^+ is of particular interest since this cation has been found to inhibit lactose synthase action (see Section V).

1. Binding of Na^+ and K^+ — Effect on the Conformational State of BLA

CD measurements have demonstrated that the conformational change occurring on binding of Na^+ and K^+ to apo-BLA is time dependent.[107] The following discussion is based on measurements where ellipticities had attained constant values. CD-spectra of K^+- and Na^+-liganded apo-BLA are essentially identical to those of the calcium form of the protein (compare curves 1 and 3 with curve 2 in Figure 20). The ellipticities of BLA and 270 nm on saturation with Na^+, K^+, and Ca^{+2} are -350 ± 1, -345 ± 2 (Reference 107), and -325 ± 43 (References 98, 99, and 262), respectively. Tryptophan fluorescence spectra of apo-BLA saturated with K^+ gave spectral parameters comparable to those observed for Ca^{+2}-BLA.[92] Thus it appears that binding of monovalent cations stabilizes a conformational state indistinguishable spectroscopically from those observed on binding of Ca^{+2} or Mn^{+2}.

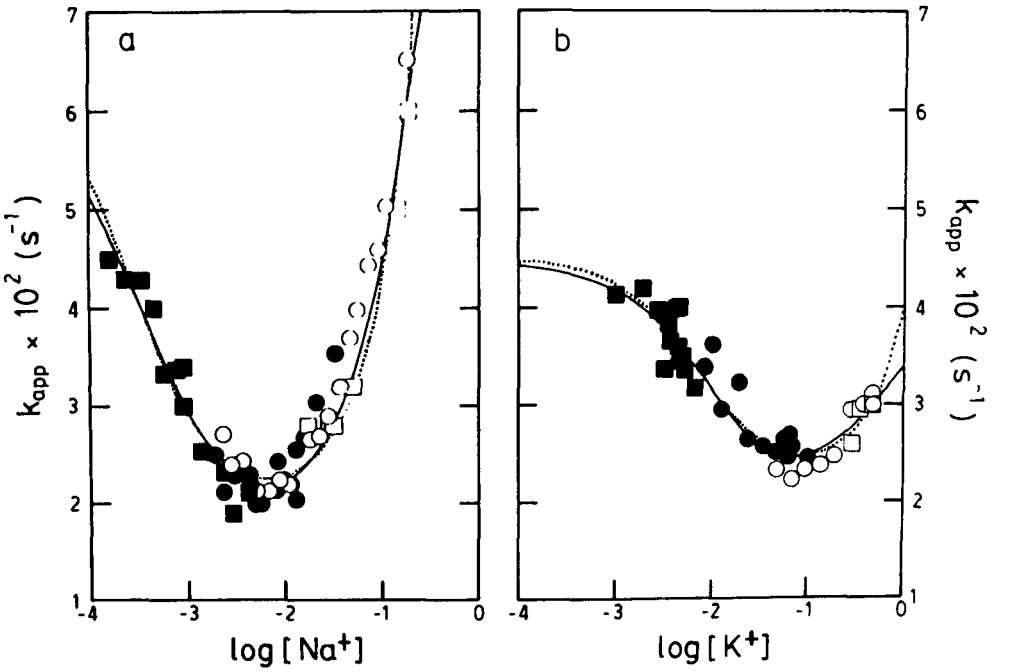
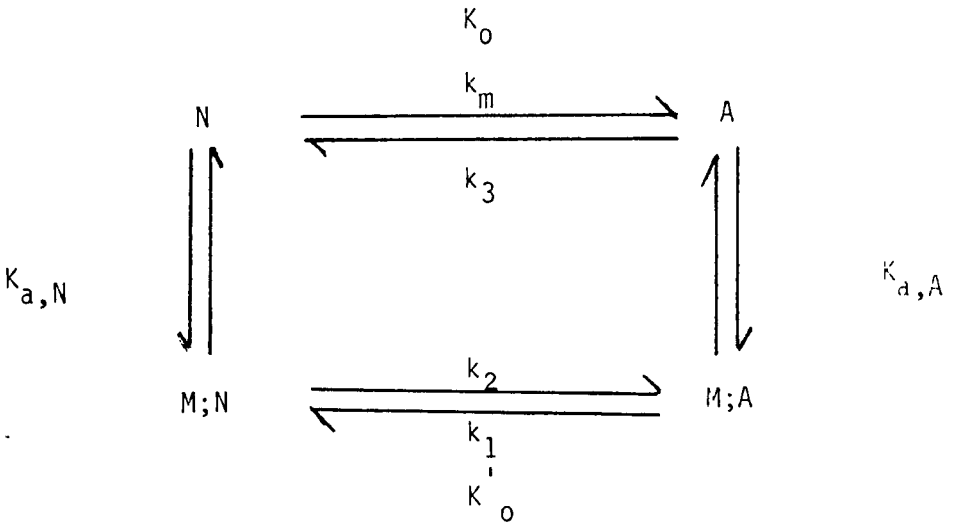


FIGURE 21. Dependence of the apparent rate constant of the conformational changes induced by Na^{+2} and K^+ on the final alkali-metal concentration at pH 8.0 and 25°C. (a) Na^+-induced unfolding (●,■) and refolding (○,□); initial concentrations of Na^+ are (●) 200 m*M*, (■) 9.5 m*M*, (○) 0 *M* and (□) 9.5 m*M*. (b) K^+-induced unfolding (●,■) and refolding (○,□); initial concentration of K^+ are (●) 500 m*M*, (○) 0 *M* and (□) 50 m*M*. Solid and dotted lines are theoretical curves obtained from the parameters listed in Table 10. (From Hiraoka, Y. and Sugai, S., *Int. J. Pept. Protein Res.*, 26, 252, 1985. With permission.)

CD measurements on titration of apo-BLA with Na^+ and K^+ yielded pK_a values of 2.1 and 0.2, respectively,[107] comparable to those obtained by other investigators (Table 8).

2. Kinetics of the Binding of Na^+ and K^+

The time dependence observed for formation of the conformational state on binding of Na^+ or K^+ is of particular interest since the process associated with the binding of calcium ion is very fast,[40,107,126] while that of Mn^{+2} is slow.[107] The CD study of Hiraoka and Sugai[107] is noteworthy in that the kinetics of the conformational change have been determined and the results analyzed in terms of equilibria between metal-ion-liganded conformational states. Since these measurements reflect the conformational change between the two states (A and M^+; A) and (N and M^+; N), the measured rate constant is an apparent one. The dependence of K_{app} on (Na^+) and (K^+) has a parabola-like form (Figure 21), which was accounted for using the following equilibrium scheme:[107]



Scheme V

The intrinsic equilibrium constants are described by:

$$K_o = (A)/(N) = k_4/k_3 \quad (8a)$$

$$K_o' = (M;A)/(M;N) = k_2/k_1 \quad (8b)$$

$$K_{a,N} = (M;N)/(N)(M) \quad (8c)$$

$$K_{a,A} = (M;A)/(A)(M) \quad (8d)$$

K_{app} is related to the intrinsic rate constants by the expression:

$$K_{app} = \frac{K_3[1 + K'']}{[1 + K_{a,N}](M)} + \frac{K_4[1 + K'']}{[1 + K_{a,A}](M)} \quad (9)$$

where

$$K'' = K_{a,A}k_1/k_3 = K_{a,N}\,k_2/k_4 \quad (10)$$

The parameters derived from the apparent rate constants (Figure 21) by nonlinear regression analysis are summarized in Table 10.

It would be useful to have similar kinetic data for the conformational change for apo-BLA as a function of calcium ion concentration, but no such data appear to have been obtained to date. A crude comparison, however, can be made with rate constants determined by Kuwajima et al.[126] using stopped flow-CD measurements for the low pH N-A equilibrium. These measurements yielded rate constants for the process, $[Ca^{+2}; N] \dashrightarrow A + Ca^{+2}$, (pH jump, 7.39 $\dashrightarrow$ 1.52) and for $Ca^{+2} + A \dashrightarrow [Ca^{+2}; N]$ (pH jump, 2.11 $\dashrightarrow$ 6.88) of ca. 8 and 1 s^{-1}, respectively. These value are about 100-fold larger than those observed for N $\dashrightarrow$ A (k_4, Scheme V) and A $\dashrightarrow$ N (k_3, Scheme V) (Table 10). The rate constant for dissociation of Ca^{+2} from Ca^{+2}; N must be comparatively large, e.g., the off-rates for dissociation of Ca^{+2} from calmodulin are >50 and 600 s^{-1} respectively,

TABLE 10
Kinetic Parameters of the Na^+- and K^+-Induced Conformational Changes of α LA

Ligand	$10^2 \times k\ (s^{-1})$	$10^4 \times k\ (s^{-1})$	$10^2 \times k\ (s^{-1})$	$10^2 \times k\ (s^{-1})$
Na^+	(15.6 ± 35.8)	(3.93 ± 0.44)	(1.70 ± 0.16)	(4.31 ± 0.76)
K^+	(4.12 ± 1.95)	(7.55 ± 1.07)	(1.93 ± 0.35)	(2.53 ± 0.29)

From Hiraoka, Y. and Sugai, S., *Int. J. Pept. Protein Res.*, 26, 252, 1985.

for the high and low affinity binding sites.[243] The differences in rate constants seen for formation of the Ca^{+2}-, K^+-, and Na^+-liganded forms of BLA must, therefore, reflect differences in the conformation of the N-like states promoted by binding of these cations.

3. Do Sodium and Potassium Compete for Binding with Calcium Ion?

It has been proposed that monovalent cations bind at the high affinity calcium site of α LA,[36,107,127] but until recently no demonstration of their competitive binding had been made. Hamano et al.[102] carried out a potentiometric titration of apo-BLA with calcium ion in the presence of sodium ion using an electrode sensitive to the former one. The relationship observed between the apparent binding constant and the concentration of sodium ion was that anticipated for competitive binding of the two ions, i.e., the associations constants for the individual cations obtained from these data were comparable to those obtained in their laboratory.

One would anticipate that potassium, like sodium, might bind competitively with calcium. However, the few data available do not substantiate this prediction. Calcium binding determined in the absence of KCl showed two sites (pK_a, 6.43 ± 0.03; 4.49 ± 0.03),[36] while only a single site was observed (pK_a, 6.40 ± 0.01)[40] in the presence of 0.1 *M* KCl. If Ca^+ and K^+ were to compete for binding at the high affinity site, the apparent pK_a for binding of calcium would be 5.92 in the presence of 0.1 *M* KCl compared with 6.43 obtained in its absence. A change of this magnitude would have been readily detected; we therefore concluded that potassium ion does not compete with calcium for binding to the high affinity site.

If K^+ were competitive with binding of Ca^{+2} at its site of lower affinity, pK_a would be reduced from 4.49 (the value observed in the absence of KCl) to 4.01 ($\Delta pK_a = 0.48$). A change as small as this would have been readily detected since the measurements with $^{45}Ca^{+2}$ were made over a range of cation concentrations from 0.1 to 500 μ*M*.[40] Binding of K^+ to BLA would have to decrease pK_a by more than 1.5 units for it to be undetectable. A large decrease in the binding affinity of K^+ for Ca^{+2}-BLA could occur if the monovalent cation were to bind to the second calcium site without displacement of the divalent cation from its high affinity site. The effect of K^+ observed for binding of Ca^{+2} at the second site could then be explained if pK_a for the equilibrium $K^+ + (Ca^{+2}; N) \rightleftharpoons (K^+; Ca^{+2}; N)$ were much larger than for $K^+ + A \rightleftharpoons (K^+; N)$. This mechanism implies that binding of calcium ion at its high affinity site alters the binding affinity for K^+ at the second site. This is similar to the "site-site interactions" observed with binding of Tb^{+3} (see Section II.B).

G. CATION-BINDING SITES ON α LACTALBUMIN

The binding of Ca^{+2}, Mn^{+2}, Tb^{+3}, Co^{+2}, Zn^{+2}, Na^+, and K^+ to BLA has been discussed, including the possible commonality of their binding sites and the conformational states that they promote. We now consider here the molecular character of the binding sites for these cations (Table 13), particularly Ca^{+2} and Mn^{+2}, and present a plausible unified model for their binding at multiple sites on the BLA molecule. The relationship between

binding of these ions, conformational state, and interconversion of molecular states is considered in Sections III and IV.

Hiraoka et al.[35] and Permyakov et al.[28] proposed sites for binding of Ca^{+2} (high affinity site) to α LA; inspection of the lysozyme-analogy model of BLA, however, showed them to be implausible.[40] Bratcher and Kronman, using this model, identified four potential binding sites for calcium ion, with the most plausible of these, site I, containing a large number of aspartate groups (asp 82-84, 87, and 88) which might serve as coordinating moieties. This region of the α LA molecule has proven to be the binding site for the calcium ion.

The recently published 1.7 Å resolution crystal structure of BBLA[33] has established the location and character of the high affinity Ca^{+2}-binding site, site I. Since Mn^{+2}, Na^{+}, K^{+}, and other cations promote formation of N-like states (spectral criteria), a number of investigators proposed or assumed that these cations bind at site I as well. The characteristics of the binding of Mn^{+2} to its high affinity site, including the absence of its competitive binding with Ca^{+2} (Section II.C), however, overwhelmingly favors separate sites for these two cations. The location of the Mn^{+2}-binding site, site II, has been deduced by Gerken[41] to be close to the α LA amino group of glu 1, adjacent to site I. The characteristics of sites I and II are considered later, as well as the possibility that other cations bind at these sites. (The designation of sites as "I-IV" bears no relationship to an earlier designation.[40])

1. The Three-Dimensional Structure of Site I, the High Affinity Ca^{+2} Site

Stereoscopic views of the Ca^{+2} site are given in Figure 22. The seven coordinating atoms are all oxygens from carboxylate ions and carbonyl groups of the protein, together with two from water molecules. The coordination observed in the crystal structure is a slightly distorted pentagonal bipyramid, with its apices formed by carbonyl and carboxylate oxygen atoms with the water molecules arranged approximately opposed, a pattern expected for a coordination number of 7. The Ca^{+2} - - - 0 distances range from 2.3 to 2.5 Å in "...accord with the observation that this site represents one of the tighest complexes observed."[33]

Berliner et al.[128] concluded from the $^{113}Cd^{+2}$ NMR spectrum of cadmium-BLA that the binding site for this cation has the same character as found in "EF-hand" calcium-binding sites including octahedral coordination to oxygen atoms. The crystal structure, however, demonstrates that the coordination of Ca^{+2} is *not* octahedral (coordination number, 6). Furthermore, as is discussed later, the conformation of the site is very different from that seen in "EF-hand" calcium-binding sites.

Murakami and Berliner[106] also concluded that Cd^{+2} binds at the high affinity Ca^{+2} site. The $^{113}Cd^{+2}$ NMR observations with (Cd^{+2}; apo-BLA),[128] however, may pertain instead to site II, the high affinity Mn^{+2} site (Section II.G.2). While Cd^{+2} binds to the calcium sites of calmodulin,[129,130] sites which also bind Mn^{+2} (Reference 131), it also binds at sites which may bind other cations as well. Liver alcohol dehydrogenase, for example, which normally binds zinc at its catalytic site, can bind Cd^{+2} (References 132 to 134), Mn^{+2} (Reference 132), and Co^{+2} (References 133 and 134). Likewise, Cd^{+2} can be substituted for the Zn^{+2} of alkaline phosphatase, which also binds Mn^{+2}, Co^{+2} and Cu^{+2} (References 129 and 135). It is also of interest to note that the low affinity metal-binding site of GT binds a wide range of different metal-ions, including Cd^{+2}, Ca^{+2}, Mn^{+2}, Zn^{+2}, and Co^{+2} (Section I). The possibility of binding Cd^{+2}, Zn^{+2}, and Co^{+2} at the high affinity Mn^{+2} site of BLA is considered in Scheme VI.

The coordinating residues in the crystal structure of the Ca^{+2} site (Figure 21) form part of what Stuart et al. refer to as the "calcium binding elbow" (Scheme VI).

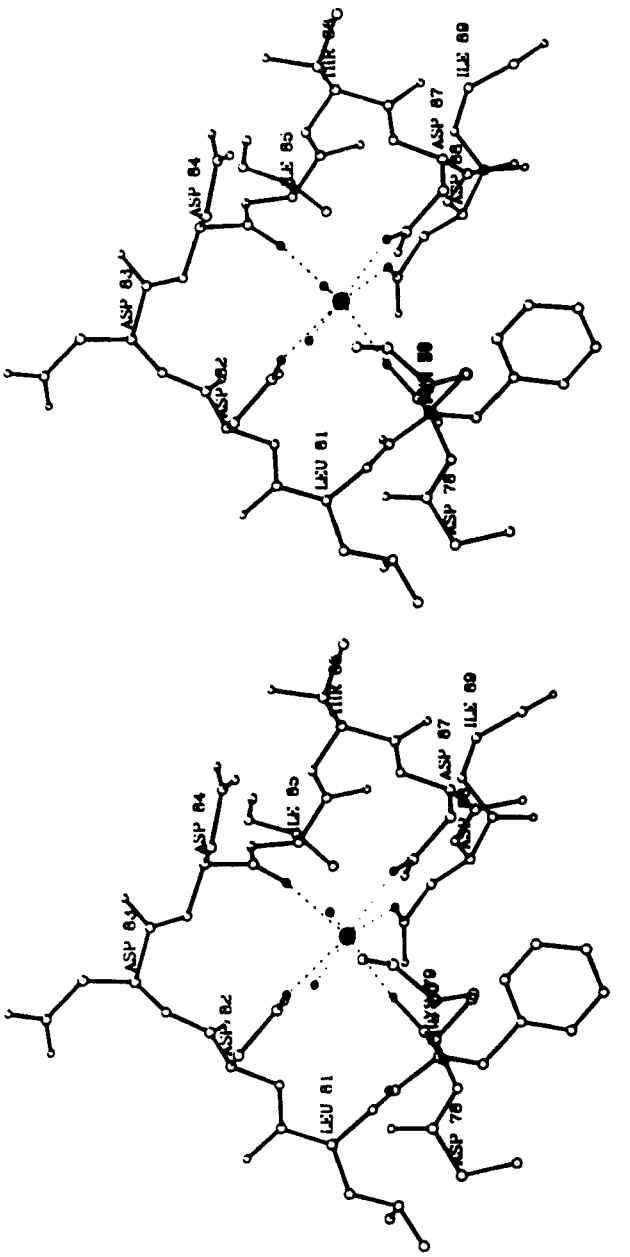
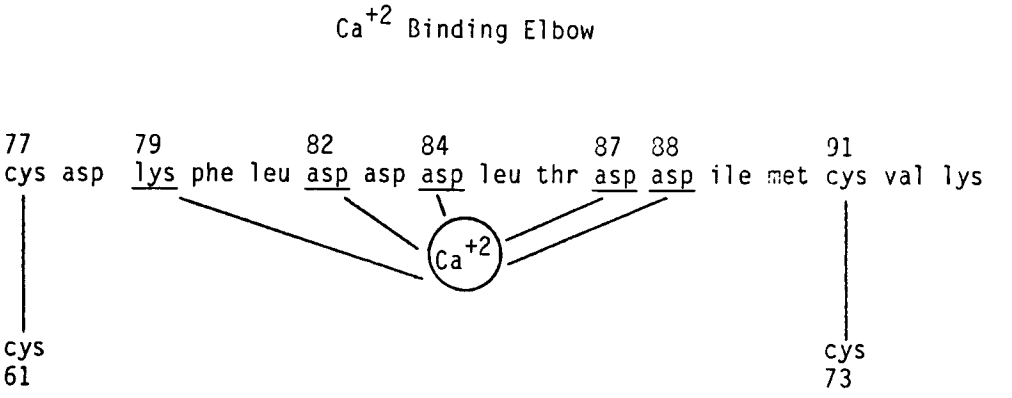


FIGURE 22. Stereoscopic views of the calcium-binding loop in α LA. Residues 78-89 plus the calcium ion (larger black dot) and its water ligands. Dashed lines join the calcium to its ligands. (From Stuart, D. I., Acharya, K. R., Walker, N. P. C., Smith, S. G., Lewis, M., and Phillips, D. C., *Nature*, 324, 84, 1986. With permission.)



Scheme VI

Sequence of BLA in Region of the Ca^{+2} Binding Site

This elbow forms a loop between two nearly orthogonal helical segments, probably residues 76-82 and 85-93 (Section III), with asp 82 acting as a spacer between the helices. This structure is reminiscent of the EF-hand characteristic of calcium-binding sites of troponin C, carp parvalbumin, intestinal calcium-binding protein, and calmodulin.[136,224,225] As Stuart et al.[33] point out, however, there are marked differences in the characteristics of the EF-hand and the calcium-binding elbow of α LA: (1) while there is only a single turn in the binding elbow, there are two in the EF-hand; (2) the EF-hand consists of 12 residues, while the elbow has only 10; (3) the elbow and EF-hand differ in the pattern of coordination within their structures. The calcium-binding elbow has five coordinating protein moieties at positions 1, 4, 6, 9, and 10 within the loop, while the EF-hand has typically six coordinating moieties at positions 1, 3, 5, 7, 9, and 12 of its loop.[136] The coordination pattern is likewise different from that found for the four calcium sites of thermolysin, where coordination numbers of 6 to 8 are observed.[137,138,227]

It is highly significant that the amino acid sequence of the calcium-binding elbow is very nearly constant in all of the α LAs which have been sequenced (Table 11), suggesting that the calcium-binding capability has been conserved. Fluorescence titration[105] of BLA, GLA, HLA, and GPLA and CD measurements of CLA[262] indicate that all of these proteins do indeed have a high affinity calcium-binding site. These observations suggest that binding of Ca^{+2} to α LA per se might have a functional role in the lactose synthase reaction, but as yet none have been demonstrated.

It is interesting that the conformations of the polypeptide backbone of hen's egg white lysozyme and BBLA are essentially identical in the region corresponding to the Ca^{+2}-binding site of the latter protein. The seeming inability of lysozyme to bind Ca^{+2} thus appears to be the consequence of the absence of coordinating asp residues in the amino acid sequence of lysozyme (Table 11). Nitta et al.[111] recently demonstrated that human lysozyme, lacking asp 82, 85, and 87, does not bind calcium ion, while the equine protein (ELYS), having these residues, binds this cation very tightly. It is of great interest, however, that addition of EDTA to Ca^{+2}-ELYS has almost no effect on its near-UV CD-spectrum, in contrast to α LA, where removal of Ca^{+2} profoundly alters this region of the spectrum. These observations suggest that, while calcium ion may occupy the same binding site in both proteins, its removal from ELYS does not give rise to a conformational change comparable to that seen with α LA (Sections III and IV). This conclusion, however, requires verification using other techniques.

TABLE 11
Amino Acid Sequences in the α LA Ca^{+2}-Binding "Elbow"; Comparison with Lysozymes

Residue	BLA	HLA	GLA	CLA	GPLA	RBLA	RLA	WLA	ELA	HLYS	CLYS	ELYS
79[a]	lys	lys	lys	lys	lys	asn	lys	lys	lys	ala	ala	*lys*
80	phe	phe	phe	phe	leu	phe	phe	phe	phe	leu	leu	leu
81	leu	leu	leu	leu	leu	leu	leu	leu	leu	leu	leu	leu
82[b]	asp	asp	asp	asp	asp	asp	asp	asp	asp	*gln*	*ser*	*asp*
83	asp	asp	asp	asp	asp	asp	asp	asp	asp	asp	ser	asp
84[a]	asp	asp	asp	asp	asp	met	asp	asp	asp	asn	asp	*asn*
85	leu	ile	leu	leu	leu	leu	leu	ile	leu	ile	ile	ile
86	thr	thr	thr	thr	thr	thr	ala	thr	thr	ala	thr	asp
87[b]	asp	asp	asp	asp	asp	asp	asp	asp	asp	asp	*ala*	*asp*
88[b]	asp	asp	asp	asp	asp	asp	asp	asp	asp	ala	*ser*	*asp*

Note: Coordinating residues are for baboon α LA.[33] Abbreviations and references: BLA, HLA, GLA, CLA, GPLA, RBLA, RLA, WLA, and ELA: bovine,[13] human,[9] goat,[12] camel,[14] guinea pig,[8] rabbit,[11] rat,[10] wallaby,[13] and equine[7] α LA, respectively. HYLS, CLYS, and ELYS: human,[220] chicken,[220] and equine[111] lysozyme, respectively. Boxed residues are invariant in all species of α LA. Italicized residues for ELYS correspond to those which coordinate to calcium ion in the α LAs.

[a] Residue of α LA which coordinate calcium ion through carbonyl oxygens.
[b] Residues of α LA which coordinate calcium ions through carboxylate oxygens.

TABLE 12
Distances of the N-Terminal Nitrogen Atom from Ca^{+2} Coordinating Residues of BLA

Residue	Atom	Distance (Å)
lys 79	Carbonyl 0	8.3
asp 82	Carboxyl 0	8.8
asp 84	Carbonyl 0	9.3
asp 87	Carboxyl 0	13.9
asp 88	Carboxyl 0	9.9

Note: Distances are calculated from coordinates of Warme et al.[30]

2. The Location of Site II, the Mn^{+2}-Binding Site

Although a high resolution crystal structure has defined the Ca^{+2}-binding site of α LA, the complete structure of the protein is known to a resolution of only 4.5 or 6 Å.[33] Therefore, the detailed tertiary structure has not been established in the region of the α LA molecule which includes the Mn^{+2}-binding site proposed by Gerken.[41] The features of the secondary structure predicted by Browne et al.[29] and Warme et al.[30] have been essentially confirmed in the crystal structure.[33,121] The following discussion, therefore, is based on interatomic distances determined from the model of BLA constructed according to Browne et al. or from coordinates derived from the energy minimization studies of Warme et al.

The location of a Mn^{+2}-binding site near the α amino group of BLA has been determined by ^{13}C NMR measurements[41] (see Section II.C for further description of these experiments). The distance of the metal-ion to the α-amino group was found to be 7.4 ± 0.3 Å. Comparison of this distance with those from the α-amino group to the coordinating atoms in site I (Table 12) indicates the unlikelihood of Mn^{+2} binding at the high affinity calcium site. Gerken originally assumed that this site was *not* the high affinity Mn^{+2}-binding site. Currently, however, he indicates that his observations are equally consistent with it being the high affinity Mn^{+2} site.[265]

The site for binding of Mn^{+2}, as proposed by Gerken, places the metal-ion 7.4 Å from the α-amino group (Figure 23). The potentially coordinating atoms are taken to be the oxygens of glu 1, 7, and 11 carboxyls, those of the peptide carbonyls of glu 2, thr 4, and asp 83, and of the hydroxyl of thr 4. If the Mn^{+2}-binding site on the α LA molecule were conserved (binding of Mn^{+2} has been demonstrated for BLA, GLA, and HLA),[106] we might anticipate conservation of glu 1, 7, and 11. Amino acid substitutions for these residues, however, are quite varied for the nine α LA whose sequence is known (see Table 11 for identification of these proteins). Coordination of the metal-ion to the peptide carbonyl oxygen should be essentially independent of amino acid substitutions.

The coordinating atoms proposed in this model are all oxygens, in agreement with the Cd^{+2} ^{113}NMR measurements of Berliner et al.,[128] if one assumes that Cd^{+2} binds to the Mn^{+2} rather than the Ca^{+2} site. Although Mn^{+2} commonly coordinates to nitrogen atoms in proteins, e.g., those found in the metal-ion-binding sites of alkaline phosphatase[135] and carbonic anhydrase,[244] it also binds to calmodulin[131] at sites whose coordinating atoms are oxygens[139] and to a transition state analog complex of creatine kinase[140] where all six ligands are oxygens.

Two features of the putative Mn^{+2}-binding site proposed by Gerken are of particular interest: (1) the carbonyl oxygen of asp 83 is proposed as a coordinating atom; (2) the peptide bond and side chain of leu 85 lay directly over the Mn^{+2} ion. Both of asp 83 and leu 85 are part of the Ca^{+2}-binding elbow observed in the crystal structure of α LA (Figure

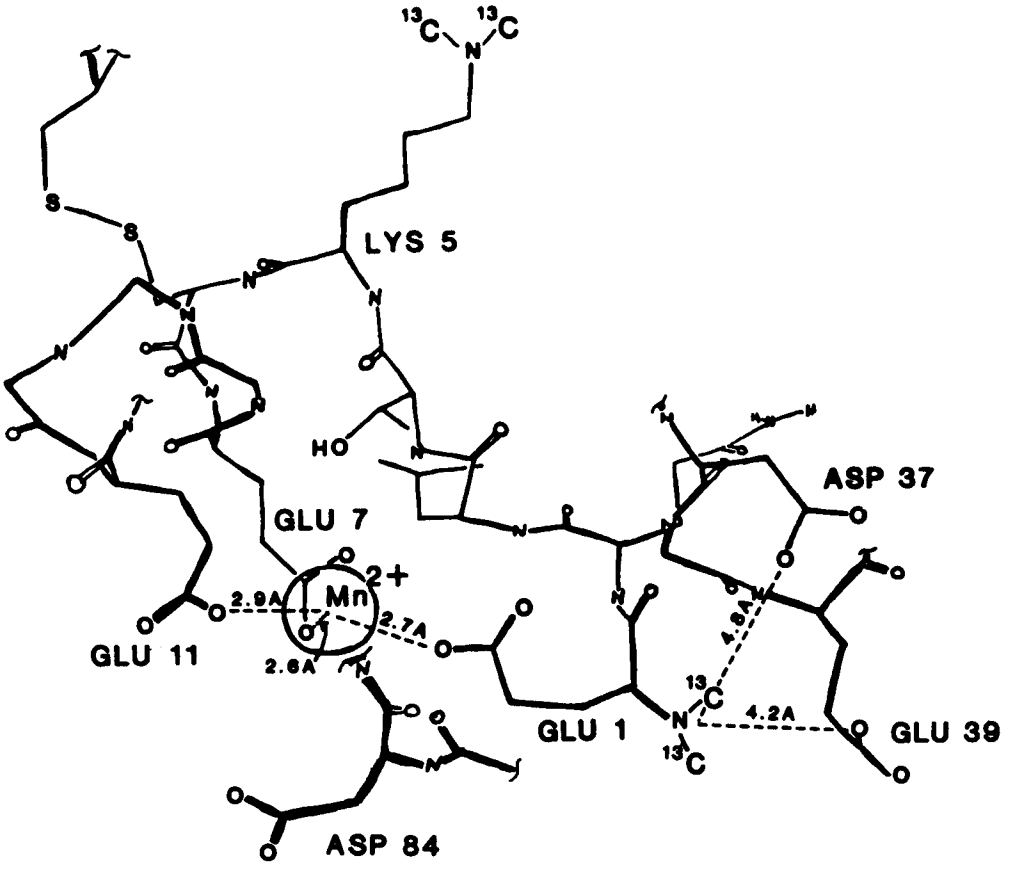


FIGURE 23. Proposed structure of the NH_2-terminal region of bovine α LA. The structure was obtained from the coordinates derived from the energy minimization study of Warme et al.[30] The NH_2-terminal ion pair interactions and a likely low affinity Ca^{+2}-, Mn^{+2}-, and perhaps Zn^{+2}-binding site are indicated. The peptide and side chain of leu-85 are directly above the metal-ion in this figure and are not clearly shown. (From Gerken, T. A., *Biochemistry*, 23, 4688, 1984. With permission.)

21). Thus, binding of Ca^{+2} or Mn^{+2} at their respective sites might induce similar "N-like" conformational states (conformers N_1 and N_2, Scheme IV). The spatial relationship of these two sites is discussed in Sections III and IV in considering the nature and interconversion of molecular conformers of α LA.

It should be noted that the features attributed to site II, the putative Mn^{+2}-binding site, other than the proximity of Mn^{+2} to the α LA amino group, are in large part speculative since they are based on the conformation of the Ca^{+2}-liganded protein. Since it appears that the molecular conformations of the Mn^{+2}- and Ca^{+2}-liganded proteins are not identical (see earlier discussion and Section III.F), the positions of coordinating groups in the Mn^{+2} protein remains uncertain until a crystal structure has been established for this form of α LA.

3. Do Sites I and II Bind Cations Other Than Ca^{+2} and Mn^{+2}?

The following section considers the binding of Tb^{+3} (three sites), Zn^{+2} (two sites), Ca^{+2} (low affinity site), and Na^+ and K^+ to sites I and II and at two others on the BLA molecule. Observation of the simultaneous binding of two Ca^{+2} and two Mn^{+2} ions to BLA when the protein is preequilibrated with Mn^{+2} prior to addition of Ca^{+2} (Figure 14) requires that there be a minimum of four cation-binding sites.

Since binding of Mn^{+2} at site II or Ca^{+2} at site I (Table 13) gives rise to formation of

TABLE 13
Metal-Ion-Binding Sites on the BLA Molecule

Sites	Metal-ion bound	Remarks
I	Ca^{+2}, Tb^{+3}, Eu^{+3}, Na^{+}, K^{+} (?)	Ca^{+2} site identified in the crystal structure;[34] binding of Tb^{+3} and Eu^{+3} at this site deduced from energy transfer measurements; binding of Na^{+} at this site deduced from its competative binding with Ca^{+2}
II	Mn^{+2}, Ca^{+2}, Tb^{+3}, Zn^{+2}, Co^{+2}, K^{+} (?)	Location of binding site for Mn^{+2}, Ca^{+2}, and Zn^{+2} deduced from ^{13}C-NMR measurements;[41] binding site for Co^{+2} deduced from energy transfer measurements[123]
III	Zn^{+2}, Tb^{+3}	Occupancy of this site promotes formation of the "expanded A" state (see text and Section IV.A)
IV, V, VI	Ca^{+2} (?), Mn^{+2}, K^{+}, (?)	Existence of sites postulated on the basis of the stoichiometry of binding of Mn^{+2} [40,105] and of the simultaneous binding of Ca^{+2} and Mn^{+2} [40]

Note: Sites I and II have specific locations in the α LA molecule (crystal structure and ^{13}C-NMR measurements). A *possible* location for Site III is discussed in Section IV. No specific locations are proposed for Sites IV to VI.

N-like states, the binding of terbium, which also promotes formation of N states (Section II.B), likely binds at these two sites as well. The Tb^{+3} and Ca^{+2} forms of the protein are probably very similar in molecular conformation, e.g., crystallographic studies of the lanthanide complexes of thermolysin indicate that replacement of calcium ion by terbium causes very little perturbation of the protein structure.[245] The coupling of binding affinities for two of the sites of BLA for Tb^{+3} is consistent with a model of adjacent interacting sites. There is a third site for Tb^{+3}, site III, which probably also can bind Zn^{+2} (low affinity site) (Section II.D), with binding of both ions promoting formation of the "expanded A" state (see Section IV).

Musci and Berliner[123] estimated intersite distances from fluorescence luminescence energy transfer measurements with Co^{+2}, Eu^{+2}, and Tb^{+2} and fluorescent dyes as donors or acceptors. The distance from fluorescein-labeled met 90 of BLA to the Co^{+2}-binding site was found to be ca. 17 Å. The average calculated distance from the coordinating residues of site I to the sulfur atom of met 90 is 11 ± 1 Å, while that from the α-amino group to met 90 is ca. 19 Å. It seems likely, therefore, that Co^{+2} binds to site II rather than site I. Energy transfer measurements yielded a distance of ca. 11 Å from an Eu^{+2} site to the Co^{+2} site, while the corresponding Tb^{+3} to Co^{+2} distance was ca. 10.5 Å. The average distance from the α-amino group to the coordinating residues in site I (Table 12) is of the order of 9 to 10 Å, comparable to the Eu^{+2} or Tb^{+3} to Co^{+2} distance determined by energy transfer measurements and consistent with the lanthanides and Co^{+2} occupying sites I and II, respectively.

Simultaneous binding of Ca^{+2} (site I) and of Zn^{+2} (low affinity site) has been demonstrated by determination of binding in solutions of apo-BLA, Ca^{+2}, and Zn^{+2} (Reference 40) and by ^{13}C-NMR measurements[41] (Section II.D). The latter observations also provide evidence for the proximity of the Zn^{+2}-binding site and the α LA amino group, i.e., Zn^{+2} binds at site II without displacing Ca^{+2} from site I. Binding of Zn^{+2} to Ca^{+2}-BLA was shown to perturb the 13C α-amino resonance, indicating the proximity of this group to metal-ion-binding site II. The position of the resonance, however, is characteristic of the *N* state, indicating that both Zn^{+2} and Ca^{+2} bind simultaneously. Only after addition of *high* concentrations of zinc ion does this resonance shift to a position characteristic of the A

conformation. The latter confirms the observations made of two distinct binding sites for this metal-ion.[40,92] The NMR data likewise show that a second calcium ion binds at site II of Zn^{+2}-Ca^{+2}-BLA with displacement of the zinc ion.

The imidazole nitrogens of histidine residues are commonly coordinating atoms for zinc ions.[141-143] The histidines of BLA, however, are too distant from site II to participate in binding of Zn^{+2} (model of Browne et al.). It is possible, however, that the binding site for Zn^{+2}, while in the same region as that for Mn^{+2} (Figure 22), is displaced somewhat so as to permit its coordination to the N terminal α-amino group. Crystal structures of Zn^{+2} complexes of peptides show coordinating of α-amino groups.[144] Furthermore, Christianson and Lipscomb[145] recently found that the Zn^{+2} of carboxylpeptidase A coordinates to the α-amino group of bound glycosyl-L-tyrosine.

The competitive binding data cited earlier suggest that sodium ion binds at site I, the high affinity calcium site. However, the time dependence of the binding of sodium and its absence on binding of calcium ion suggest that the local conformation of the binding site may not be the same with the two bound cations. As discussed previously, there is no clear-cut evidence for binding of potassium ion at the high affinity calcium site, although the binding of sodium to this site suggests that it should be true for potassium as well. Further work will be required to clarify the relationship between binding of calcium and monovalent cations.

Since binding of both Zn^{+2} and Tb^{+3} at low affinity sites gives rise to formation of the "expanded A" conformer, it is likely that the two cations bind at the same site, site III. A possible location for site III is considered in Section IV as part of a general discussion of the molecular events which take place during the N-A conformational change.

Finally, it is necessary to postulate the existence of perhaps three additional cation-binding sites, sites IV to VI, on the basis of the stoichiometry of binding of manganese and that found when calcium and manganese binding is determined after preequilibration with the latter cation. The latter experiments demonstrate the existence of four distinct binding sites, two each for Mn^{+2} and Ca^{+2}. The former measurements show there to be either three[40] or four[105] Mn^{+2} sites.

III. MOLECULAR CHARACTERISTICS OF N AND A STATES OF α LACTALBUMIN

A. INTRODUCTION

Transformation of native α LA (N state) to states with A-like character can be achieved in a number of ways, including removal of the metal ion by chelators,[103] exposure to low[91,115,146,147] and high pH,[115,147-149] and heating above 50°C at neutral pH.[97,146,150] The A conformation, furthermore, has also been detected and characterized as a molecular intermediate in the denaturation of α LA by guanidine-HCl.[151-153]

The following observations strongly suggest that none of the A-like states of α LA bind calcium ion:

1. Binding of $^{45}Ca^{+2}$ demonstrates a sharp decrease in affinity below pH 5.5 (Figure 8).
2. Analysis of the fluorescence titration of apo-BLA with calcium indicates that the association constant for binding of the cation at neutral pH to the A conformer per se is zero within experimental error.[40]
3. The effect of calcium ion on the thermal transition of BLA indicates that the A conformer does not bind the divalent cation.[154]
4. Binding of Zn^{+2} and Ca^{+2} at their respective high affinity sites occurs simultaneously with formation of the N state, but binding of a second Zn^{+2} forms the "expanded A" state with dissociation of calcium ion (Figure 19). No definitive test of the binding of Ca^{+2} the high pH A conformer appears to have been made, but it seems likely that the calcium ion is dissociated on formation of this state as well.

TABLE 14
Helical Content of the α LA Molecule

Crystal structure		Predicted helices, residues[c]						Measured helical content
		BLA			HLA			
HEWL[a]	BBLA[b]	I	II	III	I	II	III	
5—16	5—14	7—13	1—15	5—16	7—13	4—11	7—15	
24—34	22—34	37—43			23—29	25—32	25—32	26[e], 33[f]
80—85	76—82[d]			77—89			77—89	
88—96	85—93[d]			91—99			91—99	19[g], 22[h]
119—124		104—110	111—123	105—123	104—123	101—123	105—123	
33%	32%	17%	23%	41%	17%	31%	47%	

[a] Hen's egg white lysozyme.[33,34,88]
[b] Baboon α LA.[33,34]
[c] I and II refer to the prediction procedures of Chou and Fasman and Robson and Suzuki, respectively, as used by Nozaka et al.[190] for BLA and HLA. III is a prediction of Lim.[191]
[d] Stuart et al.[33] indicate that asp 82 in the calcium-binding "elbow" acts as a spacer between two helices, but neither they nor Smith et al.[34] identify the segments (see Scheme VI, Section III.F). We have indicated these as the residues corresponding to the two helical segments, 80-85 and 88-96, of HEWL.
[e] CD measurements, BLA.[99]
[f] Fourier transform infrared measurements, BLA.[221]
[g] CD measurements, BLA.[117,190]
[h] CD measurements, HLA.[32,190]

B. SECONDARY STRUCTURE IN N AND A STATES

1. Content of Helix and β Structure

The crystal structure of native BBLA[33,34] indicates that there are four segments of helix which are comparable to those of the homologous protein, hen's egg white lysozyme (Table 14). A helical segment homologous with residues 119-124 of lysozyme appears to be absent in α LA. The helices in BBLA make up ca. 32% of the molecule. The number of peptide hydrogens of BLA and hen's egg white lysozymes, which exchange slowly with deuterium, are 35 and 44, respectively,[155] consistent with the helical content of the two proteins. The prediction of helical regions of BLA and HLA using the methods of Chou and Fasman, Robson and Suzuki, and Lim are not very accurate and the amount of helix tends to be either too high or too low. The helical segments indicated for BBLA were deduced from the low resolution structures. A more definitive assignment of helix and β structure must await completion of a high resolution diffraction analysis.

The percent helix derived from far-UV CD measurements appears to be significantly lower than shown by the X-ray structure. Although the far-UV CD-spectra of the N and A states of BLA are not identical in the wavelength range generally associated with peptide bond transitions in helical and β structures, it is apparent that no gross unfolding of the protein molecule has occurred, i.e., the spectra for the low pH and high temperature A conformers are markedly different from that found for the denatured protein (compare curves 2 and 3 with curve 4 in Figure 24).

Robbins and Holmes,[99] in their analysis of CD-spectra of α LA conformers, obtained percentages of helix and β structure for N and A summarized in Table 15. These calculations show that formation of either the low or high pH conformer leads to a small apparent increase

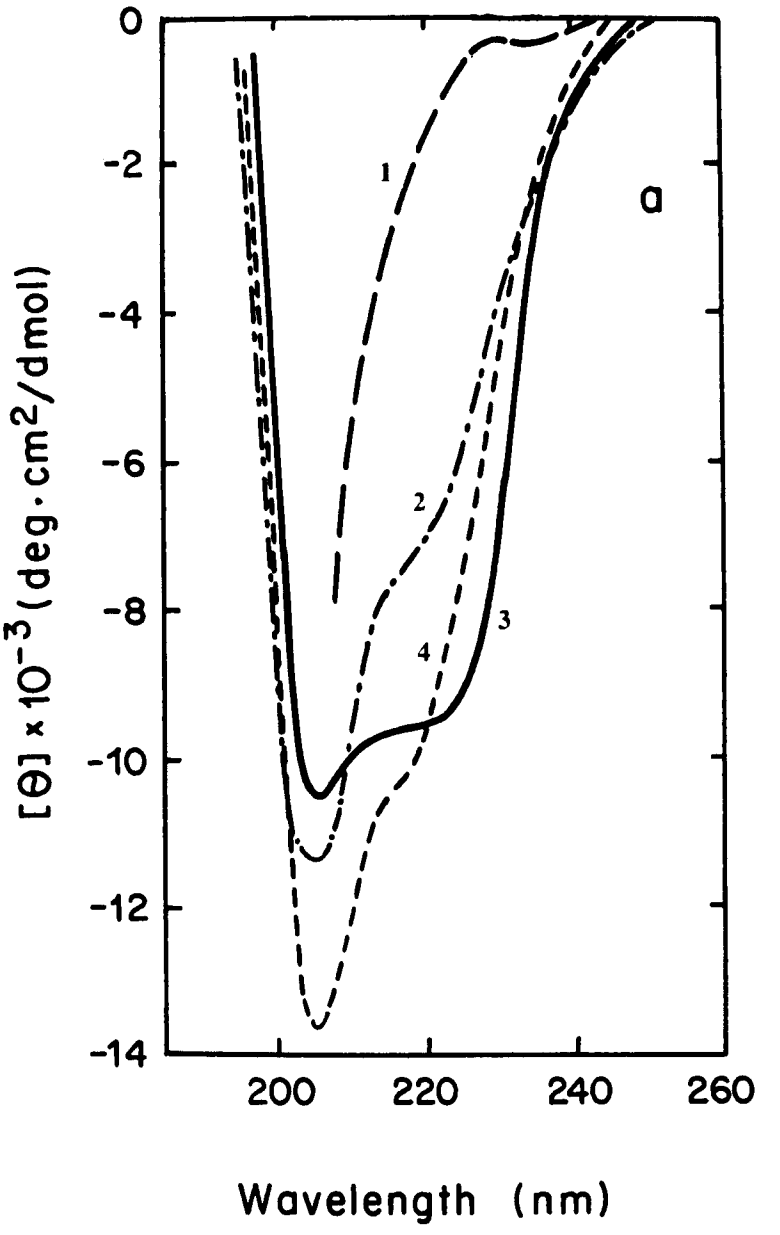


FIGURE 24. Far UV CD-spectra for BLA. ——, N state, pH 7, - - - -, A state, pH 2; ·-·-·-, A state, pH 7, 90°C. - - - -, denatured in 6 *M* guanidine-HCl. (From Dolgikh, D. A., Abaturov, L. V., Bolotina, I. A., Brazhnikov, V. E., Gilmanshin, R. I., Lebedev, Y. O., Semisotnov, G. V., Tiktopulo, E. I., and Ptitsyn, O. B., *Eur. Biophys. J.*, 13, 109, 1985. With permission.)

in helix content at the expense of β structure. While the N-A transformation undoubtedly involves some change, albeit small, in secondary structure, the following remarks suggest that the magnitude cannot be deduced in an unambiguous manner from CD-spectra.

The magnitudes of the changes in ellipticity and the shape of the spectra for the N and A states of BLA (curves 1 to 3 in Figure 24) are comparable to those measured for the N states of BLA, HLA, and CLA (Figure 25). These proteins have different numbers of tryptophan residues and give somewhat different near-UV CD-spectra.[262] The percent helix

TABLE 15
Apparent Changes in Secondary Structure of BLA Occurring During the N-A Transition[a]

State	Percent of residues		
	Helix	β structure	Unordered
N[b]	26	14	60
A[c]	36	4	60
A[d]	36	4	60

[a] Percentage of structural elements calculated from the far-UV CD-spectra of BLA[99] by the method of Greenfield and Fasman.
[b] pH 7—9.
[c] pH 2—3.
[d] pH 11.3.

calculated from the ellipticity at 208 nm are ca. 35, 30, and 22% for the N states of BLA, HLA, and CLA, respectively. It seems highly improbable that the secondary structures of these three proteins could be as different as the calculations suggest. A more likely explanation is that the ellipticities corresponding to the aromatic side chains such as tryptophans are superimposed in the far-UV region on those characteristic of secondary structure. Similar conclusions have been reached for other proteins.[31,32,156-159] Attempts to use relatively small changes in ellipticity in the far-UV to assess alterations in secondary structure are, therefore, likely to be thwarted by the uncertainty in the magnitude of the contributions of the aromatic residues in the N and A conformational states of α LA.

As might be expected, the absence of major changes in secondary structure suggests that the difference in molecular volumes of N and A states should be minimal (Section III.B.2). It will be apparent from the following discussion that the N-A conversion reflects primarily changes in tertiary structure and that removal of calcium ion brings about *global* changes in the BLA molecule.

2. Molecular Dimensions of the α LA Molecule in the A State

A variety of physical measurements indicate that the molecular size and/or shape of α LA in the N state undergoes only a small change on transformation to the A state (Table 16). The Einstein-Stokes radius of the molecule increases 10 to 15%, with a corresponding change in molecular volume of 30 to 35%. The intrinsic viscosities for α LA in N and A states are nearly identical, comparable to those typically found for native globular proteins.[26,97] Denaturation of BLA, in contrast, leads to significantly different values of intrinsic viscosity, corresponding to a 40% increase in Einstein-Stokes radii and a nearly threefold increase in molecular volume.[193]

Pfeil[160] used gel filtration to compare the Stokes radii of BLA in various conformational states and found that the radius of the protein molecule increased by about 40% on denaturation with guanidine-HCl, while formation of the low pH A conformer gave rise to an increase of only 10%. These observations are comparable to those obtained by viscosity measurements. Of particular interest is his finding that the molecular size of the high temperature conformer is essentially identical to that of the complete denatured protein. Sommers and Kronman[146] demonstrated that the transfer of excitation energy among tryptophans is markedly different in the low pH and high temperature A conformers, indicating that these two conformers are not identical (Section III.C.5). The observation of Pfeil of a difference in the apparent molecular sizes of these conformers confirms this earlier conclusion.

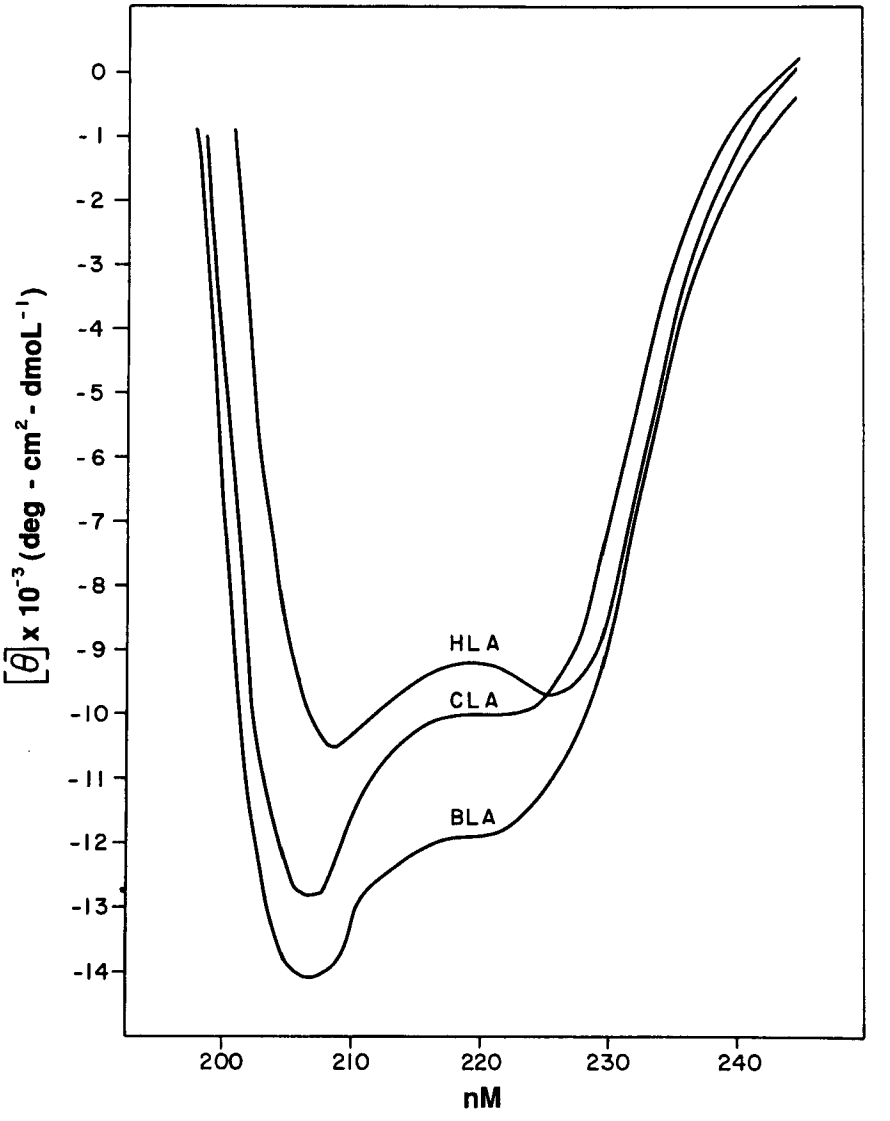


FIGURE 25. Far-UV CD-spectra of native human, camel, and bovine α LA. Buffer, pH 6.9, 0.05 *M* Tris, 0.1 *M* KCl. These spectra were measured with a computer-interfaced Aviv-Cary CD spectrometer. Each of the curves presented is an average of two independently measured spectra obtained with freshly prepared solutions. Each of the pair of independently measured spectra was the sum of five repetitive scans at 0.2-nm intervals.[262]

A low degree of swelling of the BLA molecule on formation of the A state has also been demonstrated by Damaschun et al.[161] in their high-angle, X-ray diffuse-scattering study of the packing of side chains in the conformers of BLA. The scattering curves for both N and A forms of the protein exhibited broad maxima at Bragg distances of 0.4 to 0.6 nm, which the authors assign to Van der Waals interaction distances. The maximum observed for the A state of the protein, however, is shifted relative to that of the N state, indicating that side chains in the A state, on the average, are somewhat less tightly packed than in the N state.

TABLE 16
Molecular Dimensions of the α LA Molecule in N and A States

State	Dimension (Å)	Technique	Ref.
N	17[a,b]	Sedimentation velocity	20
A	21[a,b,c]	Sedimentation velocity	20
N	14.5[d]	X-ray scattering	192
N	16.7[d]	X-ray scattering	222
N	15.5[d]	X-ray scattering	97
A	15.7[c,d]	X-ray scattering	97
N	17.7[a]	Quasielastic light scattering	193
A	18.8[a,e]	Quasielastic light scattering	193
A	19.9[a,c]	Quasielastic light scattering	193
A	19.6[a,e,f]	Quasielastic light scattering	193

[a] Radius, Einstein-Stokes sphere.
[b] Original data corrected to a MW of 14,200.
[c] Low pH conformer.
[d] Radius of gyration.
[e] Metal-free protein.
[f] High temperature conformer.

3. Accessibility of the Peptide Bonds of the N and A Conformers

Hydrogen-deuterium exchange of the N-H groups of the polypeptide chain of BLA reveals significant differences in N and A states. While the *rapid* exchange (up to 50 s) of such groups for deuterium observed is suppressed in the N state, the overall rate of exchange is higher in the A state under comparable conditions (compare + and △ in Figure 26), indicating that the dynamic accessibility of partially "buried" N-H groups is higher in the A state.

An increase in accessibility of peptide bonds in the A state has also been observed in experiments where BLA was treated with subtilisen.[263] While the calcium form of the protein is essentially resistant to proteolysis, the rate of cleavage for the apo-form is relatively rapid. The rate of proteolysis of Mn^{+2}-BLA was measurable, but significantly slower than that found for the apo-protein, while the rate of cleavage for Na^{+}-BLA was only marginally different from that of apo-protein. These observations are comparable to those made on trace labeling of BLA with acetic anhydride (Section III.F), which indicated that the molecular conformations of K^{+}- and Mn^{+2}-BLA were significantly different from that of the calcium form of the protein.

C. MOLECULAR ENVIRONMENTS OF TRYPTOPHYL RESIDUES

Kuwajima et al.[154] observed differences in tryptophan proton-NMR resonances for Ca^{+2}- and Ca^{+2}-free BLA and HLA. These measurements, unfortunately, were carried out in solutions containing relatively high concentrations of sodium ion. Since Na^{+} is bound to α LA under these conditions, the differences that they observed probably correspond to both molecular species, Ca^{+2}- and Na^{+}-BLA.

Fluorescence,[27,28,36,40,92,97,105,108,146] absorption,[81,91,126,147,154] and near-UV CD and ORD[38,96,99,104] measurements indicate that the molecular environments of tryptophan residues are altered in the N-A conformational change. In the following discussion, we consider how such spectral changes may relate to alteration of the character of the α LA molecule.

1. "Exposure" of Tryptophan Residues in N and A States

The character of the spectral changes observed with BLA suggested initially that formation of the low pH A state might be the result of unfolding of the protein molecule in

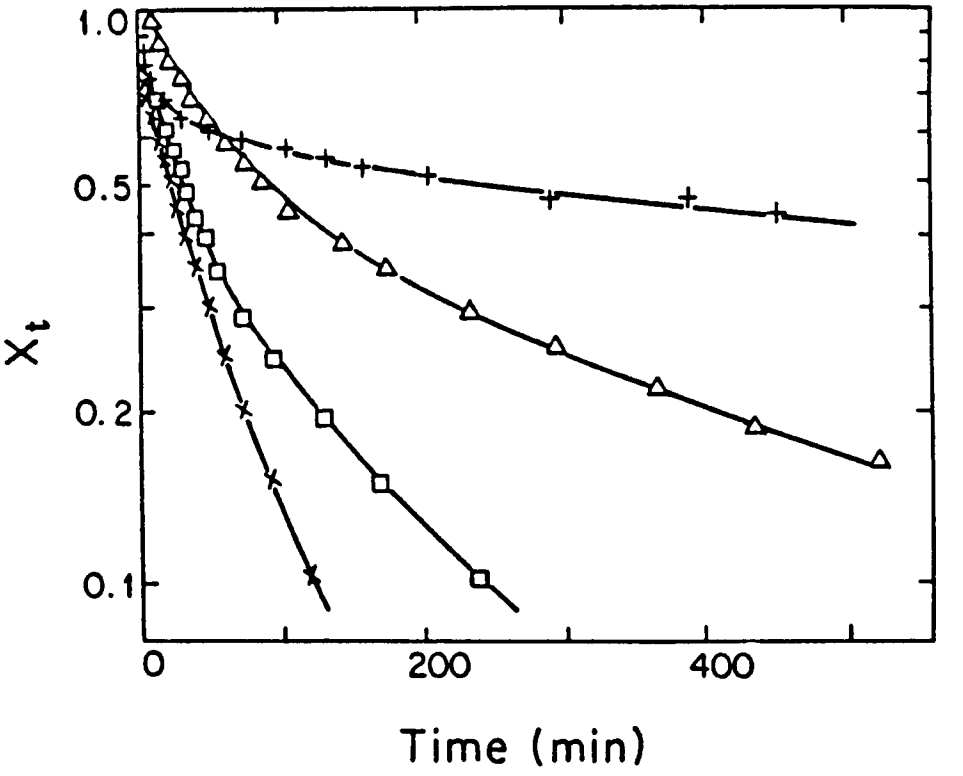


FIGURE 26. Time dependence of the unexchanged fraction of NH groups in the course of the H-D exchange for the native form of BLA at pH 4.0 and 10°C (+ + +), as well for the acid form of BLA at pH 1.9 and 10°C (△) and 20°C (□). For comparison, the change in rate for oxidized ribonuclease at pH 1.9 and 10°C is also presented (× × ×). Protein concentrations were 15 to 25 mg/ml. (From Dolgikh, D. A., Abaturov, L. V., Bolotina, I. A., Brazhnikov, V. E., Gilmanshin, R. I., Lebedev, Y. O., Semisotnov, G. V., Tiktopulo, E. I., and Ptitsyn, O. B., *Eur. Biophys. J.*, 13, 109, 1985. With permission.)

order to bring tryptophan residues in the hydrophobic interior of the molecule into contact with the aqueous medium.[94] Solvent perturbation measurements, however, are not consistent with this conclusion. The average number of groups "exposed" for BLA[94] and GLA[95] were found to be about *two* in *both* N and A states with respect to a number of different perturbing solvents (BLA and GLA contain tryptophans residues 26, 60, 104, and 118; Figure 1). The possibility that binding of perturbants such as glycerol, ethylene glycol, or sucrose might shift the equilibrium between N and A states was excluded by the constancy of spectral- and optical rotatory-dispersion properties of the two conformers in the presence and absence of perturbants.

The use of NMNC as a conformational probe gave somewhat different results for BLA than had been observed by solvent perturbation. This procedure showed that only a single tryptophan residue formed a change transfer complex with the probe in the N state, while *two* tryptophans were complexed in the A state.[82]

The differences in the observations made by solvent perturbation and with the charge transfer probe, NMNC, most likely reflect differences in what these two techniques "see". In the latter procedure, a 1:1 complex is formed between the indole ring of a tryptophan residue and the molecular probe, with complex formation requiring ring-to-ring opposition of donor and acceptor moieties.[162,163,230] Formation of such a complex might entail a local conformational change of the protein to permit ring contact. The solvent perturbation procedure, in contrast, does not ordinarily involve complex formation of chromophores with

perturbant molecules. The magnitude of the spectral perturbation, which forms the basis for determining the extent of "exposure", depends on the bulk solvent properties of the medium and does not require direct contact of chromophore and perturbant.[164]

The fact that solvent perturbation gives an *average* of two "exposed" groups in the N state, with only a single tryptophan forming a charge-transfer complex, suggests that one tryptophan may be completely exposed or that its indole ring has sufficient mobility to form the complex. The observation of two "exposed" tryptophans in the A state by solvent perturbation and two residues forming charge complexes with NMNC suggests a greater freedom of motion of side chains in this state.

2. Near-UV CD Properties of N and A States of BLA

The near-UV CD-spectrum of α LA exhibits marked changes on transforming native BLA to the A state (compare curves 1 to 4 in Figure 27). The features of the near-UV CD-spectrum of Ca^{+2}-, K^{+}-, and Na^{+}-liganded BLA are characteristic of tryptophan residues,[259] although the possibility of there being underlying tyrosyl spectra cannot be excluded. Tryptophans 26, 60, and 104 form a cluster in the BLA molecule[29,30] and are close to his 107 and tyr 103 (Table 17). Trp 118 is somewhat removed from the other tryptophans, closer to phe 31 and his 32. Dipole-dipole coupling of the ^1L_a and ^1L_b electronic transitions from these tryptophans with those of other aromatic rings of tyrosine, histidine, and phenylalanine, as well as with transitions of the peptide bond, appears to be the major mechanism for generating near-UV CD bands.[259] Coupling is also possible for the $^1B_{a,b}$ transition leading to ellipticity contributions in the far-UV. Such coupling depends on the distances between the dipoles of the chromophores, as well as on their mutual orientation, with significant contributions to the ellipticity when the distances between residues are less than 10 Å.[259]

As the intraresidue distances of Table 17 indicate, all four tryptophans of BLA are sufficiently close to aromatic rings to contribute to their near-UV CD-spectra in the N state. The marked decrease in ellipticities of the near-UV CD-spectrum in the A state (curves 2 and 3 in Figure 27) suggests that structural fluctuations in a more fluid-like BLA molecule virtually abolish dipole-dipole coupling of tryptophyl with other structural moieties.

It is of interest to note that the near-UV CD-spectra of BLA, HLA, and CLA are very similar in form in the N state and these characteristic spectra are essentially abolished in the A state.[262] The ellipticities in the N state, however, are 20 to 30% lower for HLA and 20 to 30% higher for CLA compared with BLA. These changes primarily reflect the replacement of trp 26 of BLA by leu and of leu 123 by trp for CLA and indicate that the molecular environment of residues 26 and 123 is altered in the N-A transition.

3. Molecular Motion of Tryptophan Residues in the A State

Dolgikh et al.[26,97] proposed that the side chains in the A state of BLA undergo *slow* fluctuations in spatial position and that the abolition of the near-UV CD-spectrum of the protein in the A state (Figure 27) results from time averaging of the spectra for the rotational conformers of the tryptophan residues. Changes in the NMR spectra in the region between -0.7 and $+0.8$ ppm, probably indicative of shifts in the resonances of aliphatic side chains by the ring currents of the aromatic groups, indicate that the characteristic spatial relationships of aliphatic and aromatic side chains of the N state are abolished in the A state.[97] However, these observations are also consistent with *rapid* fluctuations in spatial positions of tryptophans, contrary to the conclusion reached by Dolgikh et al. that they are slow.

The latter conclusion was based on measurements of the polarization of the steady-state fluorescence of tryptophan residues, showing that the rotational diffusion constant of BLA undergoes only a small change on transformation of BLA to the A state (16 and 19 ns for N and A states, respectively). These rotational constants, however, are of the appropriate order of magnitude for rotational diffusion of the *entire* BLA molecule[165] and are consistent

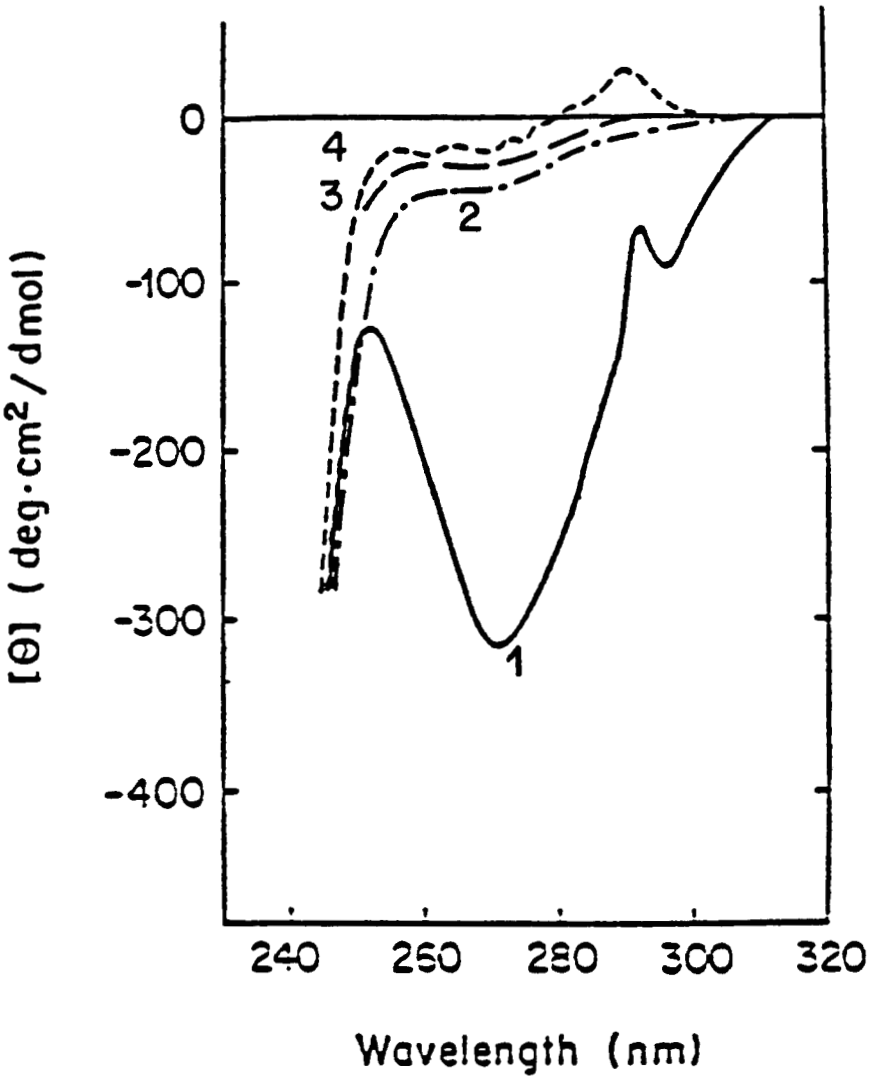


FIGURE 27. Near-UV CD-spectra for BLA in the N and A states. Curve 1, N state, pH 7 Tris, 0.05 *M* KCl; curve 2, thermally induced A state, pH 7 Tris, 0.05 *M* KCl, 90°C; curve 3, protein denatured in 6 *M* guanidine-HCl; curve 4, A state, pH 1.7, HCl, 0.05 *M* KCl. (From Dolgikh, D. A., Abaturov, L. V., Bolotina, I. A., Brazhnikov, V. E., Gilmanshin, R. I., Lebedev, Y. O., Semisotnov, G. V., Tiktopulo, E. I., and Ptitsyn, O. B., *Eur. Biophys. J.*, 13, 109, 1985. With permission.)

with a low degree of molecular swelling on formation of the A state. However, they tell little about internal rotation of the tryptophan residues.

Measurements of anisotropy decay[166] indicate that such internal rotations typically occur in times <1 ns. The values of P_o, the limiting polarization of tryptophan fluorescence, was found to be of the order of 0.1 for both conformers and comparable in magnitude with that observed with completely denatured protein, where considerable freedom of rotation might be anticipated.[97] However, freedom of motion of tryptophans in the N state would not appear to be consistent with the observation of a near-UV CD-spectrum for this conformer. The low value of P_o observed for *both* of these states, however, may be the result of energy transfer among the tryptophans, which occurs for both conformers. Until anisotropy decay measurements[151] are made for N and A states of α LA, no unequivocal answer can be given to the question of whether the structural fluctuations of tryptophans are rapid or slow.

TABLE 17
Spatial Relationship of Tryptophyl and Other Aromatic Residues of BLA

Tryptophan	Adjacent rings	Distance (Å)
26	trp 60	9
	his 107	8
60	trp 26	9
	trp 104	6
	tyr 103	4
104	trp 60	6
	tyr 103	8
	his 107	9
118	phe 31	6
	his 32	8

Note: Distances are calculated from the coordinates of Warme et al.[30]

TABLE 18
Comparison of the Difference Extinction Coefficients for N and A States for Four α LAs[a]

α LA	$-(\Delta\epsilon_{293})$
Bovine	2300[b]
Goat	2500[c]
Human	2200[b]
Guinea pig	1400[b]

[a] Values for the low pH N-A conformational change.
[b] Data of Takase et al.[81]
[c] Data of Sommers.[194]

4. Which of the Four Tryptophans of BLA Are Involved in the N-A Conformational Change?

Although significant changes in the tryptophan absorption and fluorescence spectra of α LA are observed during the N-A conformational change, there has been only limited success in establishing unequivocally which of the tryptophans were affected. Some information can be deduced by comparing the magnitudes of the difference in extinction coefficients obtained for the N-A transformation for BLA, GLA, HLA, and GPLA.[81] Comparison of the quantum yields of tryptophan fluorescence for these α LAs will be less reliable since emissions from individual residues are less likely to be additive (Section III.C.5). The tryptophans of BLA and GLA are at sequence positions 26, 60, 104, and 118, while those of HLA and GPLA are at 60, 104, and 118, and 26, 104, and 118, respectively. The $\Delta_{\epsilon 292}$ for GPLA is ca. 40% lower than that observed for BLA and GLA, indicating that the molecular environment of trp 60 is significantly altered during the conformational change (Table 18). Using the same criterion, trp 26 is only marginally affected by the N-A transformation. Thus, trp 104 and/or 118 must give rise to nearly 60% of the spectral change.

Chemical modification studies indicate that the N-A conformational change is global in character, affecting most of the molecule (Section III.C.6). It is likely, therefore, that the molecular environment of trp 118 is also altered as a result of this change. The secondary structure of the C-terminal end of the α LA is at present undefined in the crystal structure. Warme et al.[30] found in their energy minimization study of the BLA molecule that the C-terminal tail region could exist in several conformations of nearly equivalent energy. In two of these, T3 and T4, trp 118 is shielded from solvent, while in another, T2, this residue is exposed to solvent. It is significant, therefore, that Smith et al.[34] report in their 4.5 to 6 Å resolution crystal structure of BBLA that the electron densities of residues 115 to the end are weak and less continuous than in hen's egg white lysozyme. It seems possible that this region of the molecule may indeed exist in a number of energetically degenerate conformations as predicted by Warme et al. The possibility of conformational motility of the tail region of the molecule is discussed in Section III.C.5.

5. Energy Transfer Among Tryptophans

The proximity of tryptophans 26, 60, and 108 (Table 17) might be expected to lead to energy transfer among them on their excitation. Such transfer has been deduced by Sommers and Kronman[146] and Sommers et al.[232] from a comparison of the quantum yields of tryptophan fluorescence for BLA, GLA, and GPLA. Trp 60 of BLA and GLA is replaced by phenyl-

TABLE 19
Quantum Yields of Tryptophan Fluorescence of N and A States of α LA

Protein	Quantum yields		
	N State[a]	A State (low pH)[b]	A State (high temp.)[c]
BLA	0.030	0.041	0.016
GLA	0.029	0.039	0.019
HLA	0.031	0.030	0.018
GPLA	0.070	0.086	0.022

[a] pH 6.0, 0.15 *M* KCl, 25°C.
[b] pH 2.5, 0.15 *M* KCl, 25°C.
[c] pH 6.0, 0.15 *M* KCl, 77°C.

Data from Sommers, P. B. and Kronman, M. J., *Biophys. Chem.*, 11, 217, 1980.

alanine in GPLA. The quantum yield for GPLA was found to be more than twofold greater than that observed for BLA or GLA. If all of the tryptophans were to emit independently, the measured quantum yields should be number averages of those for the individual residues and

$$Q_{60} = 4Q_{LA} - 3Q_{GPLA} \tag{10}$$

Q_{60} for the N state is calculated to be -0.09, a physically meaningless result, indicating that the tryptophans do *not* emit independently.

Sommers and Kronman[118] proposed that as a result of energy transfer trp 26 and 108 funnel their excitation energy to trp 60; the proximity of the latter residue to S-S bridges 61-77 and 73-91 results in quenching of their fluorescence. This "transfer-quenching" mechanism explains the very low yield of BLA, GLA, and HLA compared with other tryptophan-containing proteins.[167] Such energy transfer persists in the low pH A conformer, i.e., $Q_{GPLA} \gg Q_{BLA}$ or Q_{GLA}. It would appear that, in spite of the more fluid-like character of the A state, trp 26, 104, 60, and the S-S bridges retain the spatial relationships requisite for energy transfer and quenching.

In contrast, energy transfer appears to be minimal in the high temperature A state,[146] the apparent quantum yield for trp 60 approaching a value >0 at this temperature (Table 19 and Equation 10). The difference in molecular environment of tryptophans in the two A states is also evident from differences in the emission maxima for the two forms of the protein.[146] These observations indicate that, while the low pH and high temperature conformers are very similar, significant differences exist in their side chain environments. The observation of a molecular volume for the high temperature A conformer, larger than that of the low pH form of the protein,[160] suggests that the BLA molecule in the former state may be more "fluid-like" than in the latter one. Increased mobility of tryptophans in the high temperature A state, or their increased spatial separation from disulfide bridges, would account for the vanishing of the energy transfer/quenching process.

The metal-free and low pH A states differ also from the "expanded A state" formed on the binding of Tb^{+3} or Zn^{+2} to a site of low affinity on either native or apo-BLA. The tryptophan-fluorescence properties of the "expanded A" states are markedly different from those of either the metal-free or low pH forms of the protein.[92]

6. Chemical Reactivity of Tryptophyl Residues

Although chemical reactivity studies in principle should be able to provide information about the extent of "exposure" of tryptophan residues, the published observations are too ambiguous to be useful. Barman[168,169] and Barman and Perry[170] found, respectively, that trp 26, 104, and 118 of native BLA and trp 60, 104, and 118 of native HLA react with 2-hydroxy-5-nitrobenzyl bromide. Bell et al.,[83] in contrast, found that only trp 26 and 118 react with this reagent. Bell et al. also reported that trp 26 and 118 reacted specifically with N-bromosuccinimide. Kronman and Brew[171] have shown, however, that this reagent *does not* react specifically with the tryptophans of either BLA or HLA, with reaction of histidines and tyrosines occurring to a significant extent, comparable to what had been observed earlier on reaction of this reagent with hen's egg white lysozyme.[172]

7. Photo-CIDNIP Measurements

Laser-photo CIDNIP measurements[173,228] indicate that the only "exposed" tryptophan of BLA, GLA, and HLA is trp 104, a somewhat different conclusion from that reached by solvent perturbation measurements, which indicate partial exposure of more than one residue. This difference likely reflects what the two techniques "see". Solvent perturbation provides information about the long-term dynamic accessibility of tryptophans to the medium, while the photo-CIDNIP procedure tends to emphasize accessibility occurring on a time scale of nano- to microseconds.[173] It is rather puzzling that these measurements also indicate that trp 118 of RBLA is also "exposed", but the authors offer no explanation for this difference found for the four α LAs studied.

D. MOLECULAR ENVIRONMENT OF TYROSYL RESIDUES

All four tyrosine residues of native BLA — 18, 36, 50, and 103 — titrate normally at alkaline pH with a value of pK_{diss} of 10.3 (Reference 115). A detailed analysis of the titration data using the Linderstrom-Lang equation, however, revealed an underlying pH-dependent conformational change. Subsequent studies[95,147] demonstrated a N-A transconformational change occurring above pH 9 which results in "normal" titration of the tyrosyl residues. Kuwajima et al.[231] subsequently carried out a stopped-flow spectrophotometric titration of the tyrosyl residues of BLA by which it was possible to estimate their individual dissociation constants in the *N state*. The titration curve for the tyrosyl residues was described by the following parameters: one group, pK_{diss}, 10.5; two groups, pK_{diss}, 11.8 and one group, pK_{diss}, 12.7 for the N state; and pK_{diss}, 10.3 for all four groups in the A state. The range of possible variation of these parameters yielding good fits of the titration curve is not given. The magnitudes of these dissociation constants suggest that one of the four tyrosyl residues should be much more reactive than the others, assuming that the reactive species is the tyrosinate ion.

Chemical studies indicate, however, that from one to four tyrosyl residues are reactive, depending on the reagent employed, its concentration, and the pH of the reaction.[95,147,174,226] Gorbunoff[174] found that all four tyrosyl residues of BLA react at pH 10, while at pH 9.3 only three of them react. Since these pH values are close to, or within, the N-A transition region, reaction probably occurred in both states of the BLA molecule. GLA, whose tyrosyl residues are homologous with those of BLA, gave a biphasic reaction profile on treatment with *N*-acetyl imidazole of pH 7.5, indicating acetylation at one and then two residues,[95] while reaction of BLA at pH 7.5 acetylated two to three tyrosines.[147] These data are of limited utility, however, since the derivatives are too labile to permit identification of the reactive residues and because their interpretation is complicated by a low degree of reaction of amino groups.

Modification of tyrosyl groups with tetranitromethane (TNM) has proven to be more useful in probing the molecular environment of tyrosyl groups of α LA. Denton and Ebner[78]

used this reagent under conditions where side reactions must have occurred since the nitrated BLA products were highly polymerized. No difference in the reactivity of the four tyrosyl residues was observed. Prieels et al.[79] nitrated HLA as a function of a TNM:protein molar ratio under conditions different from those employed by Denton and Ebner and polymer formation was minimal (the tyrosyl groups of HLA are homologous with those of BLA). Tyr 36 and 50 were found to be unreactive over a range of reagent-to-protein molar ratios of 32:1 to 128:1; tyr 103 was found to be more reactive than tyr 18 under comparable conditions.

The relationship of the tyrosyl dissociation constants of BLA observed by Kuwajima et al.[231] with BLA and the specific TNM-reactive and -unreactive residues of HLA is not at all obvious. Kuwajima et al. made the following identifications based primarily on the relative magnitudes of the dissociation constants for tyrosyl residues of hen's egg white lysozyme homologous with those of BLA and their chemical reactivity and crystal structure: tyr 103, 10.5; tyr 18, 11.8; tyr 36, 11.8; tyr 50, 12.7. One would expect that the reactivity of TNM with tyrosyl residues should increase with a decrease in their pK_{diss} and the assignment of $pK_{diss} = 12.7$ to tyr 50 and $pK_{diss} = 10.5$ to tyr 103, thus would seem reasonable. However, inspection of a BLA model[29] indicates that tyr 18 is more likely to be accessible to the bulky TNM molecule than tyr 103, which is located in a cleft-like region close to trp 60 and 104. Tyr 36, which is *unreactive* with TNM, appears to be at least as accessible to TNM as tyr 103. The only assignment which seems unequivocal in terms of the BLA model is $pK_{diss} = 12.5$ for tyr 50. The accessibility of the two ortho positions on its ring to the large TNM molecule would appear to be low, and the phenolic OH is probably hydrogen bonded to vicinal acceptor groups as it is in the homologous tyrosyl residue in hen's egg white lysozyme.[29]

Photo-CIDNIP measurements indicate that three of the four tyrosyl groups of native BLA, GLA, HLA, and GPLA are "exposed".[173]

E. MOLECULAR ENVIRONMENTS OF HISTIDYL RESIDUES

Although information about the molecular environments of histidine residues of α LA in the A state is comparatively sparse, more is known about their characteristics in the N state from studies of chemical reactivity and of proton NMR.

The relative rates of carboxymethylation of the three histidines of native BLA (residues 32, 68, and 107) with iodoacetate are in the order: His 68 > his 32 with his 107 being unreactive.[175] The relative rates of ethoxyformylation of native BLA with diethylpyrocarbonate are in the order: his 68 > his 32 with his 107 being unreactive.[25] The reagent 2-hydroxy-5-nitrobenzyl bromide typically reacts with tryptophan and thiol groups. Barman,[89,168] however, has observed that his 32 of BLA and HLA reacts to a low degree with this reagent near neutral pH, but not at all at pH 2.7 (Reference 168). It appears that this unique reaction can occur only in the N state of α LA.

Bradbury and Norton[176] identified the H-2 and H-4 proton-NMR resonances corresponding to the three histidines of BLA and determined their individual proton-dissociation constants. The assignments of pK_{diss} are his 32, 6.51; his 68, 6.49; his 107, 5.78. The average of these values, 6.26, is in reasonable agreement with the average value of 6.14 obtained by analysis of the pH-titration curve.[115] Bradbury and Norton conclude from the relatively low value of pK_{diss} for his 107 that this residue is partially "buried", consistent with its low chemical reactivity.

Takesada et al.[177] obtained proton-NMR results comparable with those of Bradbury and Norton and also measured the rates of hydrogen-deuterium exchange for the three histidines. The rate constants obtained at a pH of 8.5 and 35°C were 8×10^{-5}, 2.6×10^{-4}, and 8×10^{-5} min^{-1} for his 32, 68, and 107, respectively, while at 62°C the rate constants were essentially identical and equal to ca. 1×10^{-2} min^{-1}. Comparison of these rate constants

with those obtained for hydrogen-deuterium exchange of acetylhistidine-*N*-methyl amide leads to the conclusion that all three histidines were "exposed" to the medium at 62°C, while at 35°C his 32 and 107 were somewhat shielded from the solvent. These results suggest that the N-A conformational change occurring at elevated temperature alters the molecular environment of his 32 and 107.

It is of interest to note that analysis of the pH dependence of the N-A conformational change observed as the initial phase of the denaturation of BLA by guanidine-HCl demonstrates that the molecular environment of a group with a pK_{diss} of 5.8 in the N state becomes altered to give a value of 6.3 in the A state.[153] Comparison with the dissociation constants obtained by Bradbury and Norton suggests that this group may be his 107.

The low rate of hydrogen-deuterium exchange observed by Takesada et al. for his 32 suggest shielding of this residue by the medium, a conclusion that seems at odds with the magnitude of pK_{diss} obtained by Bradbury and Norton for this residue. Furthermore, his 68, whose dissociation constant is supposedly comparable with that of his 32, has an exchange rate markedly greater than that observed for this residue. It is apparent that a number of different environmental effects are operative in determining characteristic exchange rates and dissociation constants (see Bradbury and Norton for further discussion).

The lysozyme-analogy model of BLA indicates that (1) his 68 has comparatively few interactions with other structural moieties of the protein and is in contact with the medium; (2) his 107 appears to be rather shielded from the medium, being surrounded by glu 25 and 113, asn 102, and ser 112. His 32 lies in the cleft region of the BLA molecule, somewhat shielded from the medium by the vicinal groups, phe 31, ala 109, and leu 110. The exact spatial relationships between his 32 and other protein moieties will eventually be defined when a high resolution crystal structure of α LA is available. This is of particular importance since this residue appears to be highly significant for lactose synthase action (see Section I).

F. MOLECULAR ENVIRONMENT OF AMINO GROUPS

1. Chemical Reactivity of Amino Groups

Differences in the molecular conformation of apo- and metal-liganded forms of BLA in widely separated regions of the protein molecule have been evaluated from the chemical reactivity of the twelve ε- and single α-amino groups. Kronman and Brew[171] compared the relative rates of acetylation by acetic anhydride of the amino groups of Ca^{+2}-, Mn^{+2}-, and K^{+}-liganded BLA by a variation of the trace-labeling technique.[86,178,179] The data (Figures 27 to 29) are expressed as protection factors, PF, a value of PF > 1 indicating the rate of acetylation to be *less* than that found in the metal-free state.

Although binding of Ca^{+2}, Mn^{+2}, and K^{+} to apo-BLA promotes formation of N states (Sections II.C,D,F), the inherent reactivities of the amino groups of the three cation-liganded molecular species are quite different (Figures 28 to 30). Binding of Ca^{+2} at its high affinity site of apo-BLA, site I, results in a decrease in the average reactivity of the amino groups (PF = ca. 1.4 to 1.5), with binding at the second site, probably site II, resulting in a further decrease in reactivity (PF = ca. 2) (Figure 27).

In contrast, binding of Mn^{+2} to apo-BLA at site II, its high affinity site, conveys no protection whatsoever against acetylation (PF = 1, Figure 9). The *maximum* level of protection, corresponding to a value of PF of ca. 1.1 to 1.2, is observed only after binding of Mn^{+2} to *three* sites. It is worth noting that this maximum level of protection is significantly lower than that observed with Ca^{+2} (compare Figures 28 and 29). Quite unexpectedly, the binding of K^{+} was found to convey no protection whatsoever against acylation of amino groups, PF remaining at unity over the entire potassium concentration range (Figure 30).

These observations indicate that, although binding of Ca^{+2}, Mn^{+2}, and K^{+} promote formation of N-like states, such states are not identical. Their "classification" as N states

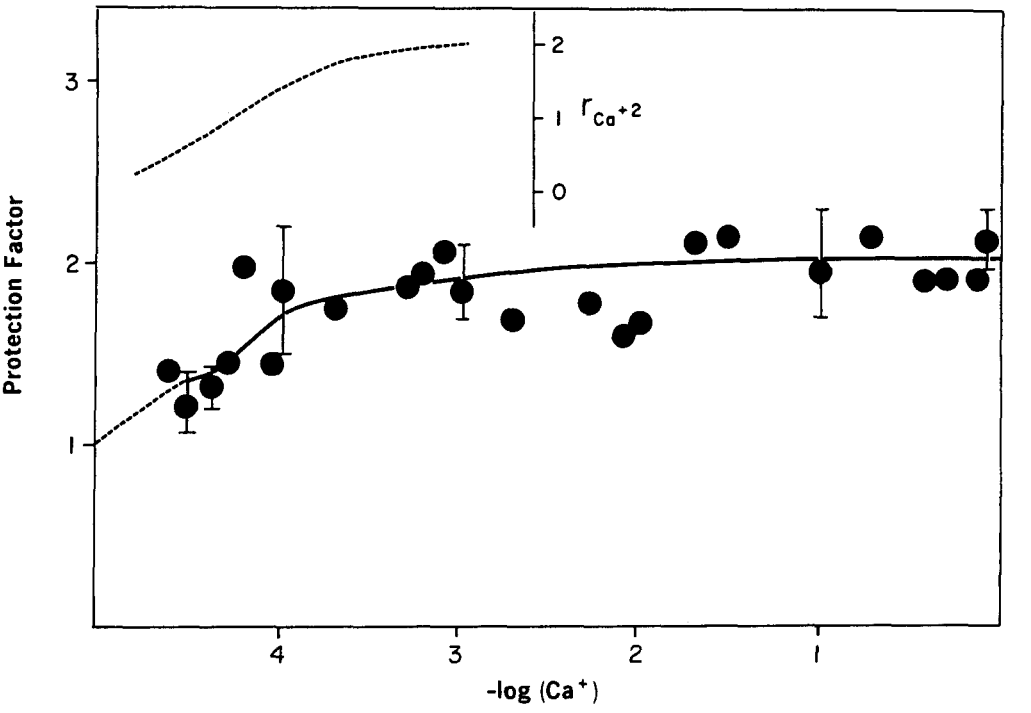


FIGURE 28. Ca^{+2} dependence of reaction of apo-BLA with acetic anhydride. (apo-BLA), 51.2 μ*M*; (acetic anhydride), 48 μ*M*. Buffer, pH 7.5, 0.02 *M* Tris. Error bars reflect the standard error of the mean for replicate determinations.[266]

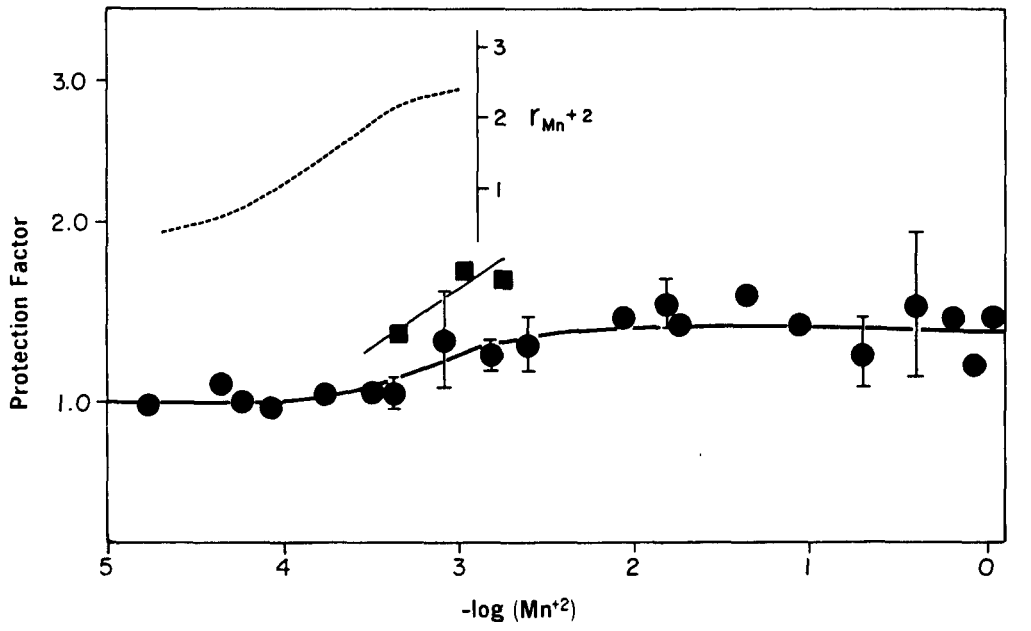


FIGURE 29. Mn^{+2} dependence of reaction of apo-BLA with acetic anhydride. Conditions are as in Figure 28. Mn^{+2}-binding data from Reference 40; ●, Mn^{+2} in the absence of Ca^{+2}; ■, Mn^{+2} in the presence of 0.66 m*M* Ca^{+2}.[266]

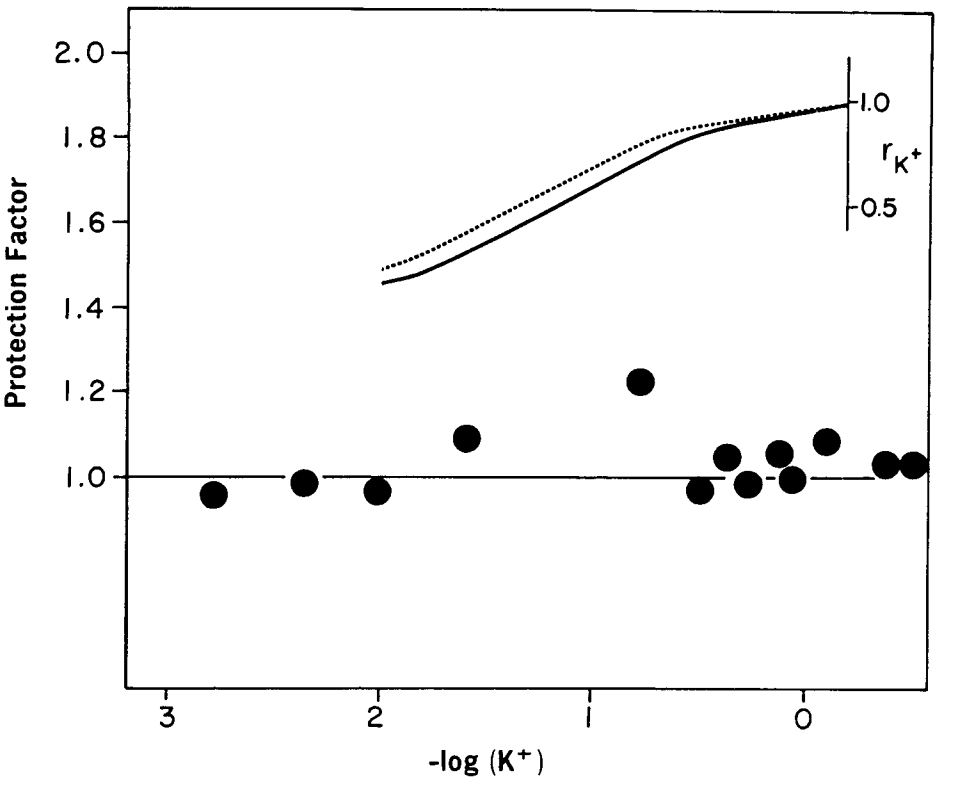


FIGURE 30. K^+ dependence of reaction of apo-BLA with acetic anhydride. Conditions are as in Figure 28. Binding of K^+: ——, calculations from circular dichroism measurements; - - -, calculations from UV-fluorescence measurements.[266]

means only that the molecular environments of tryptophan residues in these states are comparable, at least by spectroscopic criteria, but those of the amino groups, however, are markedly different.

The protection factors obtained in the experiments described earlier are averages for all thirteen amino groups of BLA. Experiments were also carried where protection factors were determined for acetylation of *individual amino groups* for Ca^{+2}- and Mn^{+2}-liganded BLA (Table 20). The most striking feature of these data is the comparative uniformity of the values of PF observed with individual peptides for both the Ca^{+2}- and Mn^{+2}-liganded proteins. This observation demonstrates that binding of Ca^{+2} or Mn^{+2} brings about significant changes in the reactivity of essentially *all* of the amino groups of BLA and that the conformational change occurring on removal of metal ions is *global* in character.

2. ^{13}C-NMR Measurements of ^{13}C-Dimethylated Amino Group of BLA

Gerken[41] concluded from observations made with ^{13}C-NMR measurements of the ^{13}C-dimethylated amino groups of BLA that removal of calcium elicits a global conformational change. He resolved 13 individual resonances for Ca^{+2}-BLA corresponding to the α-amino (two states) and 11 of the 12 ε-amino groups (Table 4) and found that removal of calcium with EGTA merged all of the resonances to give a broad peak at 42.75 ppm. Analysis of the pH dependence (N state) of the chemical shift yielded the dissociation constants for these amino groups. The observations made for the α-amino group are particularly striking. The value of pK_{diss} for this group in the N state of the protein is significantly higher than typically observed [resonance 13 (native), Table 21]. Addition of EGTA decreased pK_{diss}

TABLE 20
Protection Factors for Acetylation of Amino Groups of Ca^{+2}- and Mn^{+2}-Liganded Apo-BLA[266]

Peptide retention time (min)	Protection factor		Tentative assignment of amino groups
	Ca^{+2}	Mn^{+2}	
5.0	1.92	1.28	α amino
6.1	2.04	1.33	
10.4	2.15	1.25	lys 58
12.4	2.07	1.31	lys 16
22.5	2.30	1.36	lys 93, 94
28.2	2.17	1.14	lys 108
30.8	2.53	1.45	lys 114
38.7	2.20	1.28	lys 5 + 122
45.6	1.85	1.08	lys 79
48.7	3.00	1.36	lys 98
52.5	1.80	1.14	lys 62
Average:	2.19 ± 0.23	1.27 + 0.08	
Intact protein:	1.98	1.16	

Note: The procedure for acetylation of the protein with [^{3}H]- and [^{14}C]-acetic anhydride was described earlier.[86,178] Derivatives were prepared in the presence of saturating concentrations of Ca^{+2} and Mn^{+2}. The derivatized protein was reduced, carboxymethylated with iodoacetate, and fragmented with thermolysin. The radioactive peptides whose properties are described were isolated by reverse-phase HPLC. Identification of peptides was made by amino acid analysis.

by almost one unit, reflecting a change in the molecular environment of this group in the calcium-free state. The significance of this change in the molecular environment of the α-amino group is considered in Section IV as part of a general discussion of the molecular events occurring during the N to A transformation.

Three of the ϵ-amino groups of Ca^{+2}-BLA have values of pK_{diss} well below what is considered "normal" for such groups (resonances 1, 2, and 5, Table 21). Richardson and Brew[86] found in the course of a trace-labeling study of the interaction of native BLA with GT (Section I) that three of the α-amino groups corresponding to lys 5, 16, and 98 have strikingly higher reactivates with acetic anhydride than the others. Gerken used the relative reactivities of these residues to identify resonances 1, 2, and 5 with lys 5, 16, and 98. While it seems likely that lys 5, 16, and 98 correspond *as a group* to the amino groups with low values of pK_{diss}, it is questionable that the relative magnitudes of the reactivities observed by Richardson and Brew are sufficiently precise to warrent identification of specific resonances and dissociation constants with specific residues. A further complication in comparing the observations made with Ca^{+2}-BLA in the NMR and trace-labeling experiments is the fact that the former studies were carried out in the presence of potassium ion which probably binds to site II of α LA, while the latter were carried out in the presence of high concentrations of Mn^{+2} without displacement of calcium ion from site I. The differences in conformational states for α LA on binding of Ca^{+2}, Ca^{+2} + Mn^{+2}, and K^+ were discussed earlier.

G. HYDROPHOBIC SITES IN THE N AND A STATES

It has been commonly assumed that the region of contact of α LA and GT in the lactose synthase complex consists of hydrophobic "patches" on the molecular surface of each of the two protein components (Section I). Hydrophobic chromatography of GT demonstrates

TABLE 21
^{13}C-NMR pH Titration Properties of the Dimethylated Amino Groups of ^{13}C-methylated BLA[a]

Resonance	Chemical shift[b]	pK_a
1	43.14	9.40
2	43.06	9.58
3	43.07	10.05
4	[c]	[c]
5	42.91	9.15
6	43.07	10.00
7	43.14	10.00
8	43.07	10.12
9	43.03	10.36
10	43.08	10.40
11	43.17	10.52
12	43.25	10.88
13 (native)[d]	42.25	8.33
13 (apo)[d,e]	41.98	7.45

[a] Data from Reference 41; experiments carried out using 0.5 to 1.3 m*M* BLA in 0.05 or 0.10 *M* KCl.
[b] Chemical shifts given as ppm relative to tetramethylsilane. Since the positions of the resonances are pH dependent, the value given is the low pH limit.
[c] Not determined.
[d] Resonance assigned to α-amino group of α LA in the N state.
[e] Data obtained from protein in the presence of EGTA and 0.10 *M* KCl; resonance assigned to α-amino group in A state.

that such a site exists on the enzyme molecule.[80] Since the molecular conformation of the α LA moiety in the lactose synthase complex is not known, it is important to consider the evidence for such hydrophobic regions at the molecular surface in the various conformational states of the protein.

There are a number of investigations from which the existence of hydrophobic regions on the α LA molecule can be deduced:

1. Proton nuclear Overhouser effects for native BLA lead Koga and Berliner[180] to conclude that trp 60, ile 95, tyr 103, and trp 104 form a "hydrophobic box".
2. Barman[168] has shown that the dye 1-anilino naphthalene-8-sulfonate (ANS), which has a propensity for binding to hydrophobic regions, protects his 32 of native BLA against reaction with 2-hydroxy-5-nitrobenzylbromide. Phe 31, ala 109, leu 110, and perhaps trp 104 are all sufficiently close to his 32 to constitute the ANS-binding site.
3. The binding of apo-BLA to phenyl Sepharose and its elution by solutions of Ca^{+2} suggest that a site of perhaps greater hydrophobicity is found on the BLA molecule in the A state.[127] Desmet et al.[39] have shown that sodium ion is less effective than calcium in eluting apo-BLA from such columns, indicating that the surface of the Na^{+}-BLA molecule may have a "more hydrophobic character" than that of Ca^{+2}-BLA.

4. Amidination of lysine side chains of BLA, while having no effect on the conformation of the protein at pH 6, increased the extent of self-association, showing that hydrophobic interactions play a role in determining the state of aggregation of this protein.[253]
5. The propensity of native BLA to self-associate at neutral pH with extensive aggregation of the low pH form of the protein[20,21] suggests that differences exist in the hydrophobicity of the BLA molecule in the two states.
6. The increased tendency of N state-BLA to self-associate when Ca^{+2} is replaced by Mn^{+2} is evidence for differences in the hydrophobicity of these two N-like states.[40]
7. Binding of Zn^{+2} at its high affinity site gives rise to an A-like state with markedly lower solubility than that of the metal-free protein.

1. Binding of ANS and TNS to α LA

It has been common to probe hydrophobic sites of protein molecules with fluorescent dyes such as ANS, 2-*p*-toluidinyl naphthalene-6-sulfonate (TNS), and more recently with 4,4′-bis[1-(phenylamino)-8-naphthalene sulfonate] (bis-ANS). Early studies by Versee and Barel[152] and by Barel et al.[149] demonstrated that native BLA bound ANS and TNS, respectively. Rawitch and Hwan,[181] in a subsequent study of the binding of ANS to native BLA, concluded that such binding occurs only for associated or denatured protein. Mulqueen and Kronman[182] showed unequivocally, however, that association per se is not a requisite for binding of ANS or TNS by demonstrating the protein was monomeric under conditions where significant binding was observed. Binding of ANS and TNS, however, was weak (K_a ca. 10^3). A further complication was the observation of more than one binding site for the two fluorescent dyes, i.e., fluorescence decay measurements revealed two distinct lifetimes. While no *quantitative* conclusions concerning the binding of ANS to apo- and Ca^{+2}-BLA could be made, it appeared that ANS fluorescence was greater when the dye was bound to the apo-form of the protein. It is of interest that the pH dependence of ANS fluorescence in the presence of native BLA was almost exactly that observed for the N-A transition as measured by tryptophan fluorescence,[262] indicating that the affinity of binding of the dye and/or the emission properties of the dye liganded to the hydrophobic site(s) in the N and A states are different.

2. Binding of bis-ANS to α LA

The tighter binding of bis-ANS compared with ANS and TNS has made it possible to accurately determine association constants for its binding to apo- and metal-ion-liganded BLA (Table 22). Neither Musci and Berliner[124] nor Desmet et al.[39] consider the possibility of more than one dye-binding site. Binding of bis-ANS to apo-BLA was found by both groups of investigators to be stronger than to the calcium form of the protein, while the affinities of the dye to the Zn^{+2}- and Ca^{+2}-Zn^{+2}-liganded BLA were comparable. The sodium- and manganese-liganded protein bound the dye with affinities intermediate between that for the calcium and metal-free protein. This difference in dye-binding affinities for the three metal-ion-liganded forms of the protein is consistent with differences in their molecular character as revealed by kinetic labeling (Figures 27 to 29).

Musci and Berliner[124] proposed that the intermediate value of K_a observed for binding of bis-ANS to Mn^{+2}-BLA is the result of a conformational change which occurs on binding of Mn^{+2} at a *low affinity* site, as well as at sites of higher affinity. The implication of this explanation is that binding of Mn^{+2} *solely* at its high affinity site should give an association constant for bis-ANS comparable to that observed for Ca^{+2}-BLA. However, no experimental evidence is presented to support this view. It should be noted, however, that there is experimental evidence to support the view that the conformational state of BLA with a *single* bound Mn^{+2} is somewhat different from that with *multiple* binding of Mn^{+2} ions (see Figure 28). Furthermore, Lindahl and Vogel[127] have observed that while 1 m*M* Mn^{+2} elutes apo-BLA from a column of phenyl-Sepharose 10 m*M* Mn^{+2} *does not*.

TABLE 22
Binding of bis-ANS to Metal-Free and Metal-Ion-Liganded BLA

Molecular species	$10^{-5} \times K_a$	
	Musci and Berliner[124]	Desmet et al.[39]
apo-BLA	1.58 ± 0.10	0.66 ± 0.05
Ca^{+2}-BLA	0.158 ± 0.014[a]	0.17 ± 0.02[b]
Mn^{+2}-BLA	0.501 ± 0.114[c]	—
Zn^{+2}-BLA	2.00 ± 0.75[d]	—
Ca^{+2}-Zn-BLA	2.00 ± 0.50[e]	—
Na^{+}-BLA	—	0.27 ± 0.02[b,f]

[a] $(Ca^{+2})/(BLA)$, 1.
[b] $(Ca^{+2})/(BLA)$, 1.
[c] (Mn^{+2}), 1.6 m*M*.
[d] (Zn^{+2}), 0.4 m*M*.
[e] $(Ca^{+2})/(BLA)$, 1; (Zn^{+2}), 0.4 m*M*.
[f] (Na^{+}) (saturation).

The binding of bis-ANS to Ca^{+2}-BLA has been reported to be stronger in the presence of NaCl, suggesting that binding of the dye may be ionic strength dependent.[263] This observation needs to be tested with ammonium salts since this cation does not appear to bind to apo-BLA.[171] A question that neither Musci and Berliner nor Desmet et al. address is the possibility that binding affinities of the metal-ions may change on binding of bis-ANS. Experiments are required to test this possibility.

3. Reaction of α LA with [^{125}I]-TID

Van Ceuenebroeck et al.[24] have used the photoreactive probe 3-(trifluoromethyl)-3-(m-[^{125}I]-TID iodophenyl) diazarine, ([^{125}I]-TID), to study the hydrophobic site presumably corresponding to the bis-ANS-binding site. Incorporation of [^{125}I]-TID is tenfold greater for apo-BLA than that found for the calcium form of the protein. Binding of sodium ion was likewise found to reduce incorporation of [^{125}I]-TID. The covalent linkage of the protein and the radioactive probe after photoactivation will facilitate identification of those protein moieties which make up the hydrophobic site in different conformational states of the protein; such work is now in progress.[263]

It is significant that the extent of incorporation of [^{125}I]-TID into Ca^{+2}-BLA *increases* with an increase in protein concentration in the reaction mixture (Figure 31), suggesting that self-association of the protein may give rise to an increase in the binding affinity of the reagent prior to photoactivation. A second possibility is that formation of oligomers of BLA leads to a conformational change, giving rise to an additional hydrophobic site(s). This phenomenon needs to be investigated further since it raises a general question as to interpretation of observations carried out at millimolar concentrations of protein, e.g., those studies using NMR and ESR measurements.

4. Location of Hydrophobic Site(s)

Musci and Berliner[123] used fluorescence energy transfer measurements with bis-ANS bound to BLA to establish distances between the hydrophobic site and other moieties of the protein molecule. The distance they reported between fluorescein-labeled met 90 and the bis-ANS-binding site was 33 ± 3 Å. This value is physically meaningless since it is 10 to 15 Å *greater* than the distance from met 90 to the *most distant point* in the BLA molecule (distances were determined from the model of Browne et al.[29]).

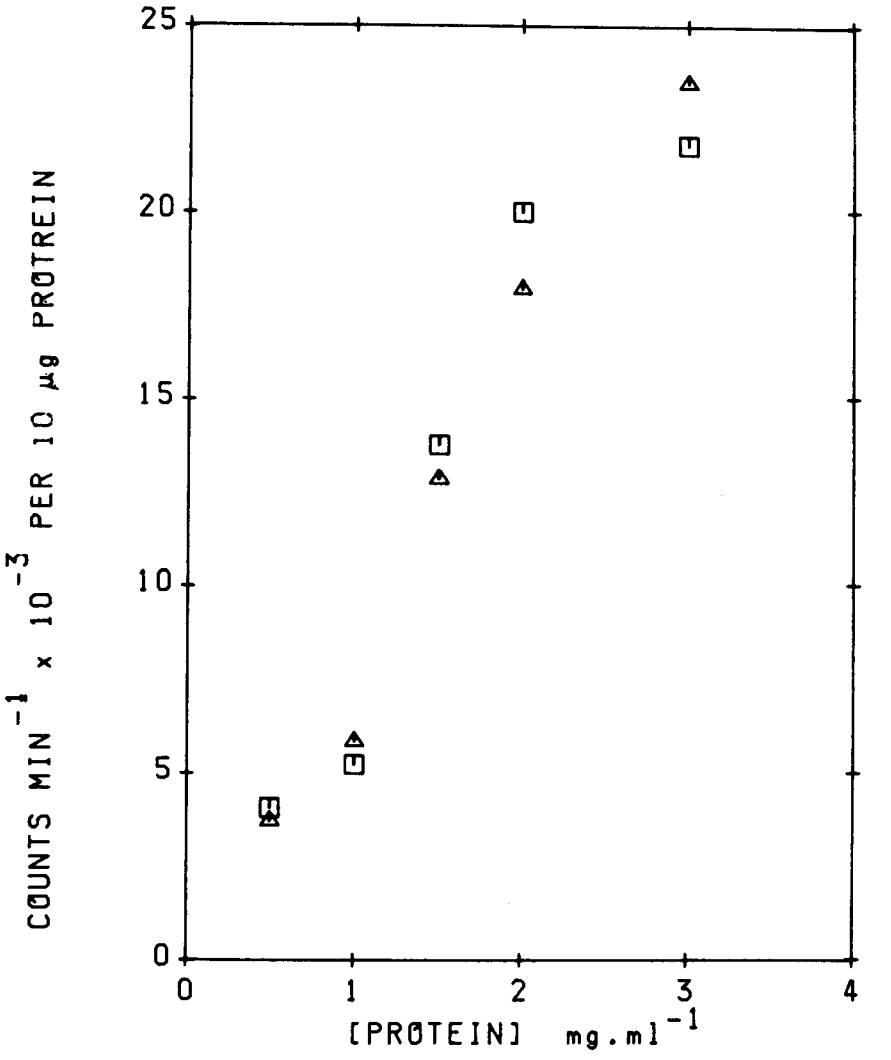


FIGURE 31. Protein concentration dependence of the incorporation of [^{125}I]-TID into Ca^{+2}-BLA. (From Van Ceunebroeck, C. J. C., Krebs, J., Hanssens, I., and Van Cauwelaert, F., *Biochem. Biophys. Res. Commun.*, 138, 604, 1986. With permission.)

A likely binding site for bis-ANS is the "hydrophobic box" (trp 60, ile 95, tyr 103, trp 104) deduced by Koga and Berliner.[180] The average distance of these residues from met 90 is ca. 15 Å, more than 50% lower than the distance obtained by Musci and Berliner.[123] The latter authors also report that the distance from the Co^{+2}-binding site to the bis-ANS site is 13.6 ± 1, somewhat lower than the distance of ca. 20 Å from either site I or II (Section II.F) to the residues of the hydrophobic box. A second possible binding site for bis-ANS may include the residues phe 31, ala 109, leu 110, and trp 104. The average distance of these residues is ca. 18 Å from met 90, considerably lower than the values measured by energy transfer.

H. MOLECULAR ENVIRONMENT OF GLUTAMYL AND ASPARTYL RESIDUES IN N AND A STATES

The pH titration curve of BLA indicates that the average value of pK_{diss} for the 17 glutamyl plus aspartyl residues is 4.25 (Reference 121). However, analysis of the data by

the Linderstrom-Land method indicates that the titration of carboxyl groups is paralleled by the N-A conformational change, reflected as a change in the electrostatic parameter *w* during the titration.

The pH dependencies of the binding of Ca^{+2} to apo-BLA (Figure 8) and of the N-A conformational change also yield information about differences in the dissociation of carboxyl groups in the two conformational states. The pH dependence of the low pH N-A transition for GLA could be accounted for by the presence of three aspartate and/or glutamate residues with pK_{diss} values of ca. 2 and 4 to 4.4 in N and A states, respectively.[148]

A similar analysis for the N-A transition observed as the initial phase of the denaturation of BLA by guanidine-HCl showed that there were three aspartate and/or glutamate residues with pK_{diss} of 3.3 and one with a value of 3.8 in the N state. The value for all four residues in the A state was 4.4 (References 116, 117, and 153).

The three carboxylate groups with low dissociation constants almost certainly correspond to the three aspartates, residues 82, 87, and 88 of the calcium-binding "elbow" (Scheme VI).[34] The electrostatic interaction of the coordinated calcium and the carboxylate ions would decrease their values of pK_{diss} below what is expected for a "normal" group. Asp 84, which coordinates Ca^{+2} through its carbonyl oxygen, may be the fourth carboxyl group whose pK_{diss} is 3.8 in the N state. Removal of the Ca^{+2} ion would leave the negative charges of asp 82, 84, 87, and 88 unneutralized and close together; we would thus expect their pK_{diss}'s to be *higher* than normal. Since the dissociation constants of these asp residues of the Ca^{+2}-binding elbow appear to have "normal" values in the A state, it seems likely that the conformational change results in their increased spatial separation.

IV. INTERCONVERSION OF N AND A STATES

The properties of the various N- and A-like states were contrasted previously, focusing primarily on the changes in the molecular environments of specific amino acid residues and other moieties in the α LA molecule. We present here a tentative account of how transformations from one state to another might be effected, considering the driving forces for these processes and how those *global* structural changes occurring on formation of A states might be propagated outward from the cation-binding sites to distant regions of the α LA molecule.

A large body of information indicates that dissociation of the calcium ion from its high affinity site of native α LA is sufficient to bring about the N-A conformational change. The conclusion of Dolgikh et al.[97] that electrostatic repulsion occurring at low pH, *in addition* to removal of calcium, is a requisite for formation of the A state is invalid. Their observations, which showed only a *decreased stability* of the N state near neutral pH, were made in the presence of EDTA and 0.05 *M* KCl, where stabilization of the N state by binding of monovalent cations and the Ca^{+2}-EDTA complex has been observed (Section II).

A. MOLECULAR ASPECTS

1. Introduction

Although binding of the divalent calcium and manganous ions and monovalent cations gives rise to N-like states, their character, particularly as reflected as differences in the molecular environment of amino groups, is quite different (Section III.F). The magnitudes of the spectral changes occurring on binding of these cations are sufficiently alike to warrant the conclusion that the conformational changes may involve comparable alterations in the molecular environments of the same tryptophan residues (Section III.C). Binding of Mn^{+2} or K^{+}, however, promotes formation of molecular states where the amino groups show a markedly increased reactivity with acetic anhydride compared with that of the conformer promoted by binding of Ca^{+2} (Figure 28 to 30).

These observations indicate that there are *separable* kinds of structural changes in the

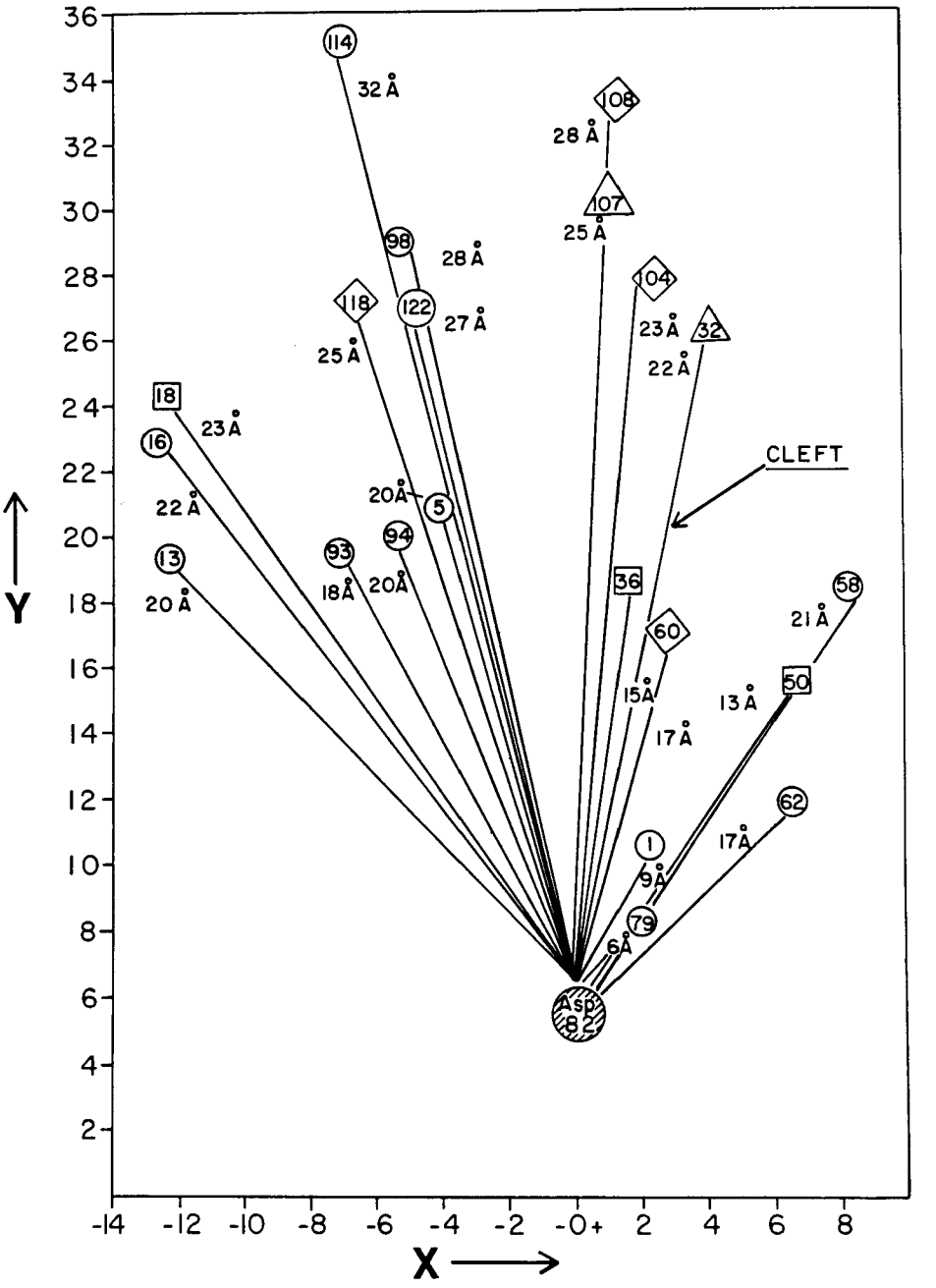


FIGURE 32. X-Y coordinate map of the amino acid residues of BLA whose molecular environments are altered in the N-A conformational change. Distances from asp 82 in the calcium-binding site were calculated using the coordinates derived from conformational energy computations.[30]

α LA molecule associated with N-A transformations: (1) one which alters the molecular environment of tryptophan residues and (2) another which alters the reactivity of the ϵ- and α-amino groups. Alteration of the molecular environments of tyrosyl and histidyl residues occurs as well (Section III.D and E), although nothing is known about the possible differences for these residues in the *individual* N- and A-like states. Figure 32 is an X-Y coordinate map of the amino acid residues in the α LA molecule affected by transformation of the

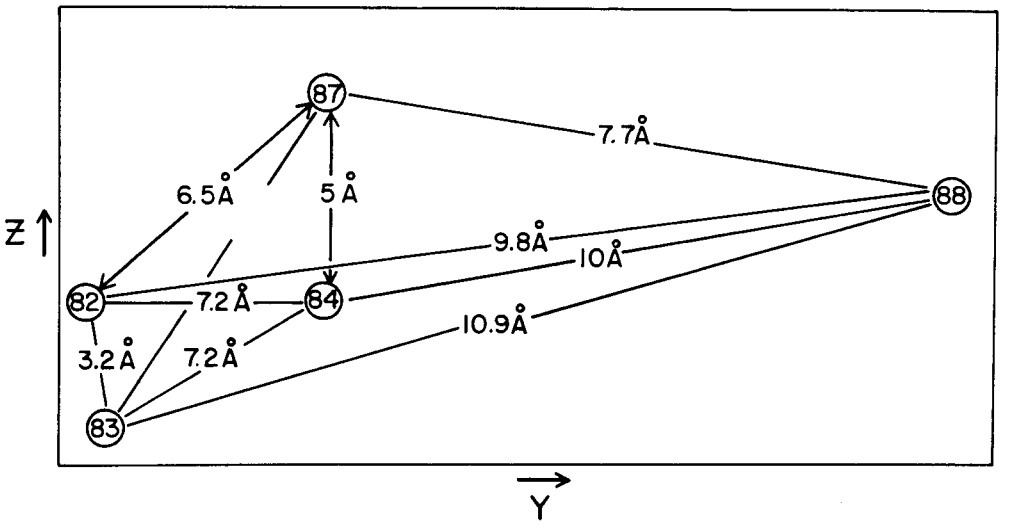


FIGURE 33. Z-Y map showing the distances between carboxylate ions in the calcium-binding site of α LA. Coordinates used in calculation of the distances were those of Warme et al.[30]

protein to the A state. The location and the distances of these residues from the calcium ion-binding site indicate that the N-A conformational change is global in scope and not localized in the region of the calcium-binding site.

These molecular changes may be viewed as resulting from alteration of the "fluidity" of the molecule, i.e., changes in the freedom of motion of side chains, with little or no change in the secondary structure. Dolgikh et al.[26,97] referred to this conformation of the α LA molecule as a "molten globule state". If the reactivity of amino groups is taken as a measure of the fluidity of a given conformational state, then the order of *increasing* "fluidity" is $(Ca^{+2})_2$-BLA < (Ca^{+2}) α-BLA < $(Mn^{+2})_3$-BLA < (Mn^{+2})-BLA = (K^+)-BLA = apo-BLA. It should not be assumed, however, that "melting-out" of the tertiary structure leads to a completely disordered side chain conformation since the maintenance of the secondary structure in the A state will considerably reduce per se their conformational freedom.[183] It is striking that (Mn^{+2})-BLA, having an N-like conformation, is as "fluid" as that of the metal-free A-state of the protein. It may also be true that binding of cations at sites I and II per se, e.g., Ca^{+2} and Mn^{+2}, gives rise to different molecular fluidities, but evidence is insufficient at present to warrant such a conclusion.

A fundamental difference in the N states promoted by binding at sites I and II may be that binding of a cation at site I leads to partial neutralization of the high-charge density in the region of aspartate ions 83, 83, 84, 87, and 88, while binding at site II does not. These carboxylate ions are separated in the N state by distances of the order of 3 to 11 Å (Figure 32), sufficiently close for marked electrostatic repulsion to occur. Coordination of calcium ion to carboxylate groups (Figure 33) would reduce this charge repulsion. The molecular event which triggers formation of the A state when the calcium ion is dissociated is likely to be the repulsion of aspartate ions and their concomitant spatial separation. These charges, however, would remain unneutralized on binding of Mn^{+2} or another cation at site II. The significance of such a high charge density at site I on binding of cations at site II is not known; it may account, however, for the apparent difference in the fluidity of the α LA molecule with different bound cations.

2. Dissociation of the α-Amino-Carboxylate Salt Bridge

Studies of the three-dimensional structure of the BLA molecule using an energy minimization procedure led Warme et al.[30] to propose that its protonated N-terminal amino

group forms a salt bridge with the carboxylates of asp 37 and glu 39 (Figure 23). Gerken[41] proposed that the abnormally high pK_{diss} observed for the α-amino group of Ca^{+2}-BLA reflects this interaction (see Section III.F for a more detailed discussion of these ^{13}C-NMR observations). Further evidence for the high dissociation constant of the α-amino group in the N state of α LA is drawn from the pH dependence of the equilibrium between N and A conformers on guanidine-HCl denaturation of BLA.[184] This pH dependence was quantitatively described assuming the dissociation constant of the α-amino group increased tenfold on formation of the A state. Gerken's observation that the α-amino group has a low reactivity toward methylation with formaldehyde and $NaCNBH_3$ (Reference 41) is further evidence for formation of ion pairs involving the α-amino group. The reduction of pK_{diss} for the α-amino group observed on removal of the calcium ion suggests that transformation to an A state of the protein molecule breaks the α-amino-carboxylate salt bridge. Dissolution of the bridge is likely to be the result of alteration of the spatial relationship of asp 37, glu 39, and the α-amino group on movement of the polypeptide chain segment including these residues.

Dissolution of the ion-pair bridge by dissociation of a proton from the α-amino group is not sufficient to transform α LA from N to A state. If this were true, an N-A conformational change should be observed in the pH range 7 to 9, where in fact no such change is observed until well above 9. While the contribution of the salt bridge to the energy of stabilization of the N state of the protein may be significant, its magnitude must therefore be small compared with the energy of binding of Ca^{+2} (Section IV.B). The significance of the salt bridge is subsequently considered in terms of the pH dependence of the formation of the "expanded A" state induced by binding of terbium ion and propagation of the N-A conformational change throughout the entire molecule.

3. The "Expanded A" State

Binding of zinc or terbium ions to sites of low affinity on the BLA molecule gives rise to a time-dependent transformation to the "expanded A" state, whose properties are quite distinct from those of the metal-free or the zinc-liganded protein[92] (see Sections II.C.4 and II.D for discussion of binding of Zn^{+2} and formation of the "expanded A state"). The molecular transformation promoted by zinc is independent of pH in the range 7 to 8, where terbium binding induces formation of the "expanded A" state (Figure 34), suggesting that the α-amino group may be involved in both processes. Although there is no definitive evidence that Zn^{+2} and Tb^{+3} bind to the same site and bring about the identical conformational change, this assumption seems reasonable.

If the binding sites and the conformational changes were identical for Tb^{+3} and Zn^{+2}, why is the process independent of pH for Zn^{+2}? The transformation promoted by zinc and terbium can be represented as (see Table 23 for designation of conformers):

$$Zn^{+2};A_1 + Zn^{+2} \rightleftharpoons Zn^{+2};A_2$$

$$(Tb^{+3})_2;N_5 + Tb^{+3} \rightleftharpoons Tb^{+3};A_2 \qquad (11)$$

Since binding of terbium transforms the α LA molecule from an N to an A state, with a concomitant decrease in pK_{diss} for the amino group from 8.3 to 7.4, a pH dependence is anticipated in the pH region above 7 where this group titrates. On the other hand, since the transformation promoted by binding of Zn^{+2} is from one A-like state to another, with no change in pK_{diss}, no pH dependence should be observed.

Addition of sub-millimolar concentrations of Tb^{+3} to apo- or Ca^{+2}-BLA above pH 8 does not promote formation of the "expanded A" state,[92] thus supporting the conclusion that dissolution of the amino-asp 37-glu 39 salt bridge per se does not alter the conformational

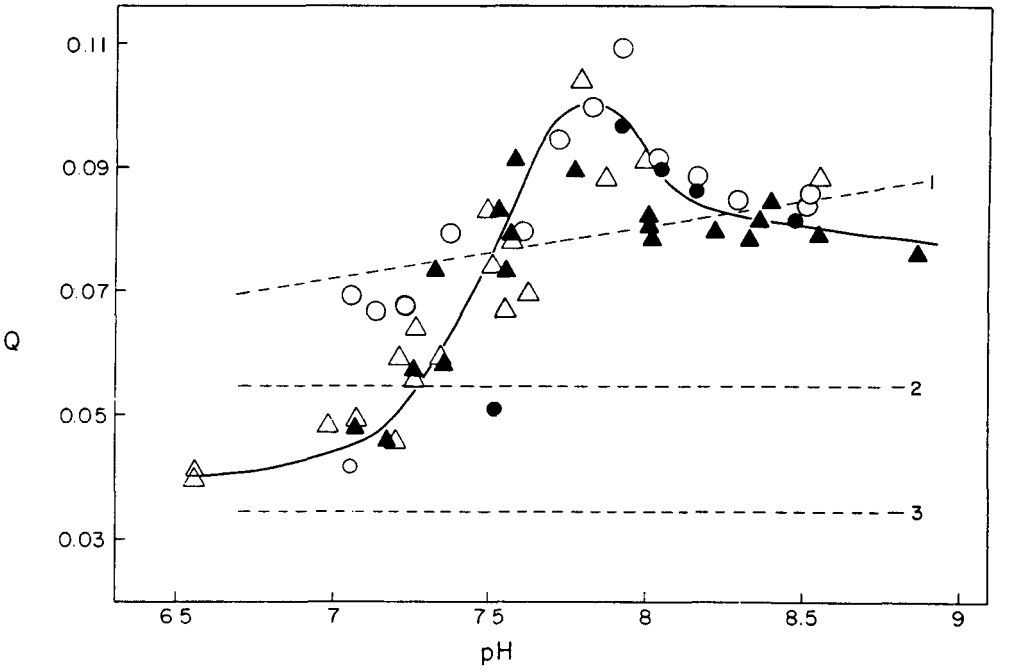


FIGURE 34. pH dependence of tryptophan quantum yields for BLA and apo-BLA in the presence of Tb^{+3}. Protein concentration, 4.5 μ*M*; Tb^{+3} concentration, 2.3 m*M*; ●, BLA, 0.02 *M* Tris; ○, apo-BLA, 0.02 *M* Tris; △, BLA, 0.02 *M* Hepes; ▲, apo-BLA, 0.02 *M* Hepes. Curves 1 to 3, pH dependence of quantum yields in the *absence* of Tb^{+3}: curve 1, apo-BLA, 0.02 *M* Tris; curve 2, apo-BLA, 0.02 *M* Hepes; curve 3, BLA, 0.02 *M* Tris. The differences in quantum yield reflect binding of buffer species. (From Kronman, M. J. and Bratcher, S. C., *J. Biol. Chem.*, 259, 10887, 1984. With permission.)

state of the protein. It appears that binding of Zn^{+2} or Tb^{+3} destabilizes the BLA molecule by binding initially at a site of low affinity with a subsequent relatively slow transformation to the "expanded A" state, whereupon their binding affinities increase markedly. The latter conclusion can be deduced from the observations that (1) the Zn^{+2}-protein requires addition of millimolar concentrations of Ca^{+2} to regain the N conformation and (2) the Tb^{+3}-induced conformational change cannot be reversed either by addition of high concentrations of Ca^{+2} or by procedures which might be expected to remove terbium ion.[92]

4. Where Are the Zn^{+2} and Tb^{+2} Sites Whose Occupancy Triggers Formation of the "Expanded A" State?

In principle, the binding site for zinc or terbium might be located anywhere on the α LA molecule. The involvement of the α amino group in the Tb^{+2}-promoted process does not require that it be part of the binding site for these cations. However, since the conformational state of α LA can be modulated by cation binding at two adjacent interacting sites, sites I and II, it is reasonable to assume that the conformational transition to the "expanded A" state might be promoted by binding at a third site located in the *same* region of the α LA molecule as the other two.

The coordinating atoms in this putative site (site III, Table 13) are the nitrogen of the α amino group, the carboxylate oxygens of asp 37 and 39, the OH oxygen of thr 38, and the carbonyl oxygen of glu 1 (model of Browne et al.). These are sufficiently close in the N state of the α LA molecule to comprise a cation-binding site ca. 7 Å from asp 82, the hinge in the calcium-binding elbow (Table 11), and ca. 7 Å from those residues which Gerken has proposed comprise site II, the putative Mn^{+2}-binding site (Figure 23).

TABLE 23
Apparent Conformational States of α LA

State	Cations bound in state[a]
N_1	Ca^{+2} or Tb^{+3}, site I[b]
N_2	Mn^{+2} at site II[c,d]
N_3	Mn^{+2} at three sites[c,d]
N_4	Na^+ or K^+ at site I or II[c,d]
N_5	Tb^{+3} at sites I and II[e]
N_6	Ca^{+2} at sites I and II[f]
N_7	Ca^{+2} and Zn^{+2} at sites I and II, respectively[g]
N_8	Ca^{+2} at site I and Mn^{+2} at site II (simultaneous equilibration of both cations with protein)[h]
N_9	Mn^{+2} and Ca^{+2} bound, each at two sites (preequilibration of protein with Mn^{+2})[h]
A_0	The metal-free state; the low pH conformer[j]
A_1	Zn^{+2} at site II (high affinity site)[j,k]
A_2	Binding of Zn^{+2} or Tb^{+3} at site III, "expanded A" state[j,l]
A_3	Formed at neutral pH at elevated temperature[j]

[a] See Section II.G for the rationale of assignment of binding sites for cations.
[b] Section II.C.
[c] Time dependence of formation of conformer on binding of cation (Section II.D).
[d] States N_1 to N_4 distinguished on the basis of amino group reactivity (Section III.F).
[e] State N_1 distinguished from N_5 on basis of "coupling" of binding affinities at sites I and II (Section II.C).
[f] May be the same as state N_5. States N_1 and N_6 distinguished on the basis of amino group reactivity (Section III.F).
[g] May be the same as states N_5 or N_6. Differences in coordination properties of Zn^{+2} suggest that the "local" conformation may be different from that with other cations bound at site II.
[h] Based on Mn^{+2}-Ca^{+2} "competition" experiments (Section II.D).
[j] States A_0 to A_3 distinguished on the basis of spectroscopic criteria (Section II.E).
[k] Section II.E.
[l] Section II.C and E, time dependence of formation of conformer on binding of cation.

The assumption that all three cation-binding sites are clustered in the same region of the α LA molecule is a very attractive one since it could account for the multiplicity of similar, but not identical, N- and A-like states, promoted by binding of different cations. This three-site model provides an elegant means of rationalizing the complex relationship between cation binding and the conformational state of α LA. Its validity, however, remains untested.

5. Molecular Changes Involving the C-Terminal Tail Region

Energy minimization calculations of Warme et al.[30] suggest that the C-terminal tail region of the α LA molecule exists in a number of energetically equivalent conformations. The ill-defined electron density maps obtained for this region of the molecule in the low resolution crystal structure[34] are consistent with the conformational mobility of the C-terminal segment. In two of the C-tail conformational states (T3 and T4) discussed by Warme et al., lys 5 forms a salt bridge with the C-terminal carboxyl of leu 123, while in a third state, T2, this ion pair is absent. A further significant difference is the presence of a hydrophobic box surrounding trp 118 in states T3 and T4, which is absent in state T2, the latter residue being in contact with the medium in this conformer. The low pH A-conformer of α LA, unlike the native protein, is irreversibly bound to phosphatidylserine/phosphatidylethanolamine vesicles.[249] It is striking that the segment of the α LA chain which is inaccessible to the milieu on binding to vesicles includes both the region of the calcium-binding site and a large portion of the C-terminal tail. This observation suggests that removal of the calcium ion shifts the equilibrium between T2, T3, and T4 conformers.

Sommers and Kronman[146] proposed that the properties of the tryptophans of BLA might be best accounted for if the C-terminal conformation were T3 or T4. The abnormally low pK_{diss} reported for lys 5 (Table 21) is also consistent with formation of an ion pair between lys 5 and leu 123. The removal of leu 123 by treatment of BLA with carboxypeptidase A, however, had no effect on either the dissociation constants or the chemical shifts for any of the amino groups,[41] apparently ruling out a lys 5-leu 123 ion pair. This still leaves unexplained, however, the origin of the molecular perturbation leading to the abnormally low pK_{diss} for lys 5. A definitive description of the tail conformation of the native α LA molecule must await determination of its high resolution structure.

The degeneracy of the C-terminal tail of the α LA molecule, if real, may provide a partial explanation of the multiplicity of conformational states of the protein mediated by binding of different cations. Changes in the liganded state of the protein might select one or another of the energetically comparable conformational isomers.

6. Molecular Events in the N-A Conformational Change

The studies discussed in Section III.B indicate that formation of the A state leads to virtually no change in the organization of the secondary structure of the α LA molecule. At the same time, however, marked alteration of the tertiary structure occurs, referred to earlier as changes in the "fluidity" of the conformer. Similar conformational changes have been observed for cytochome C[185] and ribonuclease A.[73,186,254] This type of molecular transformation is markedly different from the more common type of alteration seen in denaturation of proteins, where disruption of helical and β-structural regions of the molecule occur, as well as marked changes in the tertiary structure.

The N-A structural changes observed with α LA may be comparable to those observed with insulin, citrate synthase, and hemoglobin, which Chothia and co-workers[187-189] attribute to mutual rigid-body displacements of closely packed helical segments without internal disruption of these structural elements. These movements, of the order of 1.5 Å, result from small alterations in the conformation of the side chains forming the interface between the helices. It is of interest that this type of structural change, like that observed with α LA, is promoted by binding of ligands to the three proteins.

The α LA molecule consists of two lobes separated by a cleft-like region (Figure 4)[29,30,34] (the terms "left" and "right" hand lobes given in the following discussion are for the observer facing the cleft region). Alterations in the conformation of the α LA molecule appear to have their origin in molecular events occurring in the region of cation-binding sites I to III. The following is a plausable sequence of molecular events on removal of calcium ion from site I:

1. An increase in the local charge density in the binding site on dissociation of the cation followed by:
2. Mutual repulsion of the negatively charged carboxylate ions of asp 82, 83, 84, 87, and 88 in the calcium-binding "elbow" which:
3. Increases separation of these residues occuring as:
4. The relative positions of helical segments 76-82 and 85-93 hinged through asp 82 (Scheme V) change.

The movement of the two helical segments is constrained by disulfide bridges 61-77 and 73-91. Concomitant movements appear to occur also in a segment of polypeptide chain including asp 37 and glu 39 spatially close to site I, but "downstream" in the sequence. Movement of this peptide chain segment breaks the ion pair between the latter residues and the α-amino group.

How are these molecular changes, which are localized on the left-hand lobe of the α

LA molecule, communicated to the *right*-hand one? The rigid body movements seen in insulin and citrate synthase are transmitted from helical segment to helical segment.[187-189] Two other helical segments of α LA, 5-14 and 22-34 (Table 14), however, are located in the right-hand lobe of the molecule too distant from segments 76-82 and 85-93 for displacement to occur via direct contact. Transmission of the conformational change from its site of origin on the left-hand lobe to the right-hand one must, therefore, occur by a different route, e.g., by either or both of the following:

1. Small adjustments in the polypeptide backbone propagated through a segment of nonordered peptide chain (residues 32-37) running across the bottom of the cleft and bridging the two lobes of the molecule. Movement of this segment appears to be responsible also for breaking the amino salt bridge. It is also significant that this segment includes asp 37, one of the coordinating residues in the putative low affinity site (site III) for binding zinc and terbium.
2. Changes in the interactions of side chains which span the cleft region between the two lobes. These include tyr 36, phe 56, and trp 60 from the left-hand lobe and phe 31, his 32, thr 33, tyr 103, and trp 104 from the right-hand one. The molecular environment of these two residues, trp 60 and possibly that of trp 104, are altered in the N-A conformational change (Section III.C).

7. The Multiplicity of Conformational States

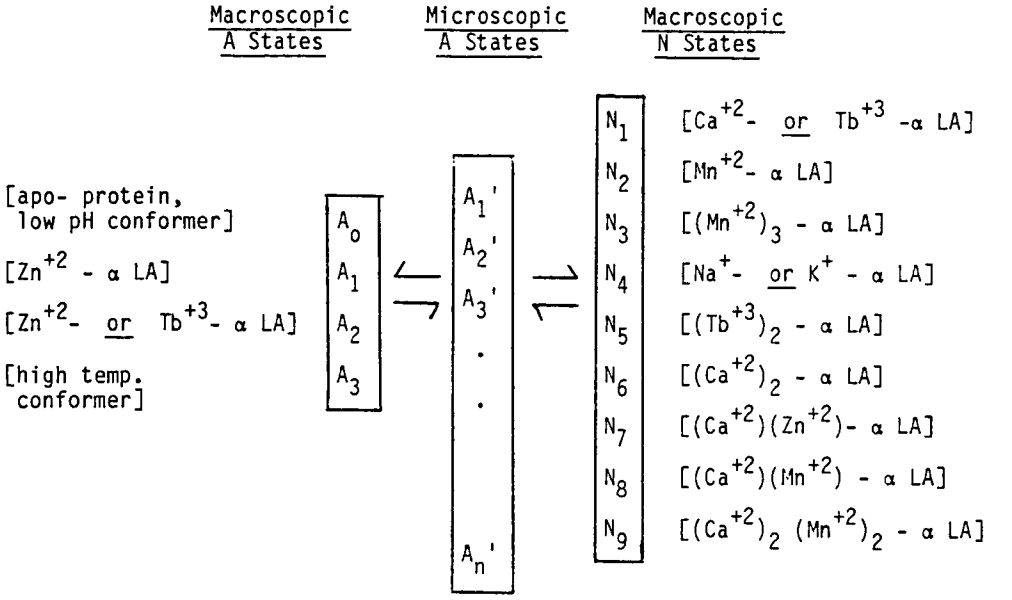
The characteristics of the binding processes and the properties of the protein in different liganded states of α LA strongly suggest that there may be a *number* of distinguishable N and A conformers. These are summarized in Table 23. In some instances major differences in such conformations may be manifest, e.g., states N_1 and N_2 differ greatly in molecular fluidity (amino group reactivity). States A_0 and A_3 have different molecular volumes[160] and greater fluidity. In other instances, the conformational differences may be more localized, e.g, states N_1 and N_5 are considered to be distinct conformations since the cation-binding affinities at two adjacent sites, I and II, are coupled.

The multiplicity of conformational states found for α LA, a comparatively small protein molecule, is rather surprising. We believe that such multiplicity is a reflection of equilibria between a large number of conformational states of nearly equivalent energy. Their observation under a range of conditions, including binding of different metal ions, reflects shifts in such equilibria, as well as stabilization of discrete forms due to energy barriers which prevent equilibration between different conformers. The latter was proposed to explain the dependence of the binding of Mn^{+2} and Ca^{+2} on the order of mixing of protein and cations (Section II.D.3).

Discussions of conformational states of protein have tended to be polarized between the static view of the protein molecule (crystal structural model) and a stochastic model, where the protein exists, in the words of Weber,[205] as a "kicking and screaming molecule" exhibiting time-dependent fluctuations in conformation. The reconciliation of these seemingly conflicting ways of looking at the character of globular proteins has been discussed by Cooper[206] and others. Ikeguchi et al.,[151,260] in their studies of denaturation of BLA by guanidine-HCL, proposed that there are multiple pathways of folding of denatured protein to the A conformer since the structure of the latter is highly sensitive to the folding conditions. The macroscopic A state can be described as an equilibrium distribution of microscopic states with the average properties of the microscopic states making up those of the macroscopic state. The fluidity of the A state implies facile equilibration of the microscopic distribution and the possibility of multiple pathways for folding from the denatured state.

A similar situation obtains for the folding of the A conformer to the N conformer, where multiple pathways are also observed involving different ligands (Table 23). We propose that

the various N- and A-like states result from *shifts* in the distribution of microscopic states for α LA on either side of that for the metal-free protein (Scheme VII).



Scheme VII

The extremes of the distributions represent molecular species with very low and very high fluidity, with all species having essentially the same secondary structure. In some cases, microscopic states may be selected having energies that are barriers to equilibration with other states. This appears to be true to conformers promoted by binding of Mn^{+2} which do not appear to be in equilibrium with those promoted by binding of Ca^{+2}. In other cases, however, where no energy barriers exist, the microscopic states are in equilibrium and shifts in the distribution of microscopic states can occur on binding of different ligands.

This indicates that α LA is a protein with an unusual degree of "conformational adaptability", its state depending markedly on the interactions it undergoes with small molecules and ions. The global nature of the conformational changes which α LA undergoes on binding of ligands, as well as the fluid character of some of these conformers, may have fundamental significance for formation of the lactose synthase complex and subsequent molecule events in the biosynthetic process. While the binding site for cations appears to be spatially distant from the region of the α LA molecule in contact with GT in the complex (Figure 4),[86,87] changes in their state of ligation may alter the conformation of the α LA moiety in the contact region (Section I).

B. THERMODYNAMIC ASPECTS

The denaturation of α LA has been extensively studied.[116,117,125,150,201,202,207-216,229] These investigations have focused largely on the overall transformation of the native protein to the denatured or D form of the protein and its relationship to the unfolding of the lysozyme molecule. These observations have been discussed most recently by Kuwajima et al.[212] and by Ikeguchi et al.[151] Our concerns here are primarily with the thermodynamics of the initial step in the denaturation, the $N \rightleftharpoons A$ equilibrium.

TABLE 24
Enthalpy and Entropy of Binding of Metal-Ions to Apo-BLA; Comparison with Binding of Metal-Ions to Other Proteins and Chelators[a]

ΔH (kJ mol^{-1})	ΔS (kJ^{-1}mol^{-1})	Species	Ref.
−110	−235	Ca^{+2}-BLA	113
−145	−327	Ca^{+2}-BLA	39
−118	−273	Ca^{+2}-BLA	100
−75	−142	Sr^{+2}-BLA	100
−143	−432	Na^{+}-BLA	39
−250 to −500[b]	—	Ca^{+2}-thermolysin	195
	—		
−37.2	+55.2	Ca^{+2}-parvalbumin	198, 199
−32.2	0[c] to −28[d]	Ca^{+2}-troponic C	198, 199
−16.4	+15	Ca^{+2}-porcine pancreatic phospholipase A (1)	196
+27	+130	Ca^{+2}-EDTA	197
+34	+96	Ca^{+2}-EGTA	197
+50	+130	Sr^{+2}-EDTA	197
+46	+63	Sr^{+2}-EGTA	197

[a] Calorimetric measurements, except where noted.
[b] Van't Hoff analysis of spectra data. The range of values reflects uncertainty in the number of bound calcium ions which stabilize the protein.
[c] Calcium-specific sites.
[d] Calcium-magnesium sites.

1. Binding of Metal-Ions

Binding of Ca^{+2} to apo-BLA is strongly exothermic, values reported for ΔH ranging from −110 to −145 kJ-mol^{-1} (Table 24). The lowest value of −110 kJ-mol^{-1} obtained by Van Ceunebroeck et al.[113] was measured with an apo-BLA preparation containing substantial amounts of sodium ion which proved to account for their apparent dependence of enthalpy on protein concentration.[39] The value of −145 kJ-mol^{-1} subsequently reported by Desmet et al.[39] was determined indirectly, assuming competition of binding of calcium and sodium ions; no dependence of enthalpy change on protein concentration was observed. However, independent determinations were not made of ΔH for sodium and for calcium ion binding. Their analysis yielded a value of K_a of 195 mol^{-1} for binding of calcium ion, somewhat higher than previously determined values (Table 8) and a value of ΔH for Na^{+} binding not significantly different from that obtained with calcium.

The value of the enthalpy of binding of Ca^{+2} to apo-BLA reported by Schaer et al.[100] is somewhat lower than that found by Desmet et al.[39] These authors *demonstrated* calorimetrically that Ca^{+2} and Sr^{+2} bind competitively. It is rather striking that the enthalpy of binding of Sr^{+2} to apo-BLA was almost 40% lower than the value obtained on binding of calcium ion, particularly in view of the observation of Desmet et al. that binding of sodium and calcium, two very dissimilar cations, gives rise to virtually identical enthalpy changes. Since comparatively little is known about the Sr^{+2}-liganded state of BLA, the significance of the thermodynamic differences found for binding of calcium, sodium, and strontium is not apparent.

The magnitude of the ΔH observed on binding of calcium to apo-BLA is unusually large compared with values obtained on binding of this cation to parvalbumin, troponin C, and phospholipase A2 (Table 24). Binding of calcium ion to thermolysin, in contrast, gives

rise to an even larger change in enthalpy corresponding to a change in molecular conformation which protects the enzyme against autolysis.[195] The enthalpy change observed on binding of calcium ion to α LA must be due primarily to the global conformational change which this protein undergoes. However, there may be contributions to the enthalpy change from other processes as well. If we assume that self-association of the protein and proton dissociation on binding of metal-ion have no effect on the enthalpy, as demonstrated by Desmet et al.[39] the enthalpy change can be described by:

$$\Delta H_{total} = \Delta H_{conf.} + \Delta H_{bind} \quad (12)$$

where $\Delta H_{conf.}$ and ΔH_{bind} are the enthalpy changes corresponding, respectively, to the change in conformational state from A to N and to the ion binding itself. There is no unambiguous way, however, of partitioning ΔH_{total} into $\Delta H_{conf.}$ and ΔH_{bind}. Desmet et al.[39] have estimated the binding enthalpy to be comparable to the values observed on formation of Ca^{+2}-chelator complexes (Table 24). Implicit in this estimate, however, is the assumption that the hydration of the coordinating aspartate groups in α LA is comparable to that of the chelators. As discussed by Eftink and Biltonen,[217] the energy of binding of calcium is primarily electrostatic in nature, but is offset by the energy required to disrupt the hydration of the coordinating groups. In the case of chelation of metal-ions, the enthalpy changes are small and endothermic (Table 24). The presence of *five* aspartate ions in the calcium-binding elbow, however, must give rise to a high degree of electrostriction of water molecules, and this will require considerably larger energies for disruption than that required on formation of chelator complexes of calcium ion.

While the magnitude of the conformational enthalpy may be uncertain, it is sufficiently large to be consistent with the global conformational change observed with α LA. The large *negative* entropy change observed on binding of calcium ion reflects in large part the decrease in "fluidity" of the α LA molecule on formation of the N state. In contrast, a small *positive* entropy change is observed on binding of this cation to parvalbumin, troponin C, and phospholipase A2, probably reflecting the net effect of disruption of the hydration of carboxylate ions in the binding site and a relatively modest conformational entropy change. The magnitudes of the enthalpy changes for binding of calcium to parvalbumin, troponin C, and phospholipase A2 likewise suggest more limited alterations in structure than that observed with α LA. The conformational change for troponin C promoted by binding of calcium ion to the Ca^{+2}-Mn^{+2} sites gives rise to formation of two short segments of helix[213] and a "tightening" of the structure, most likely in the region of the metal-ion-binding-site.[255] Although binding of Ca^{+2} to α LA does not appear to change the secondary structures, its effect on the conformation of the protein molecule must be considerably greater than that found with troponin C.

2. The N-A Conformational Changes Induced Thermally and by Guanidine-HCl

The values of the enthalpy changes reported for the thermally induced N-A conformational change and that observed at low pH and at moderate concentrations of guanidine-HCl span a range of about 100 kJ-mol^{-1} (Table 25). It is difficult, therefore, to compare values for formation of different A-like states. Comparison of the data of Tables 24 and 25 indicates that the thermodynamic parameters for the various N-A transformations are of comparable magnitude. The small differences found in character of the A conformer using other methods are not evident from the thermodynamic data.

As indicated earlier, the magnitudes of ΔH and ΔS are unusually large for dissociation of a metal-ion from a protein molecule and therefore probably reflect primarily a "global" conformational change. The magnitude of ΔC_p, ca. 4 to 6 kJ-deg^{-1}-mol^{-1}, is consistent with breaking of a number of stabilizing interactions of hydrophobic side chains during this

TABLE 25
Thermodynamic Parameters for Conformational Changes of Bovine α LA at 25°C

Protein	ΔH (kJ mol^{-1} K^{-1})	ΔS (kJ mol^{-1} K^{-1})	ΔC$_p$ (kJ mol^{-1} K^{-1})	Method[a]	Ref.
Ca^{+2}-BLA	130 ± 17	—	4.0 ± 0.8	C	200
Ca^{+2}-BLA	106	—		C	201
Na^{+}-BLA	175 ± 15	—	5.2 ± 0.6	C	202
Na^{+}-BLA	187 ± 18	—	4.6	VH	125, 154
Low pH and/or Guanidine-HCl-Induced N - - - A Transition					
Ca^{+2}-BLA	122 ± 19	—	—	C	200
	87	232	4.1	VH	153
Guanidine-HCl-Induced A - - - D Transition[b]					
Ca^{+2}-BLA	45	116	1.8	VH	153
Guanidine-HCl-Induced N - - - D Transition[b]					
Ca^{+2}-BLA	120	311	5.9	VH	153

[a] C, calorimetry; VH, Van't Hoff analysis of spectral data.
[b] D is the completely denatured state.

TABLE 26
Comparison of the Thermodynamic Parameters for the N-A, N-D, and A-D Transitions of α LA at 25°C

Transition	ΔH (kJ mol^{-1})	ΔH (J mol^{-1} deg^{-1})	ΔC$_p$ (J mol^{-1} deg^{-1})
N-A	87	232	4100
A-D	45	116	1760
N-D	120	311	5860

Note: Data taken from Reference 153 obtained from Van't Hoff analysis of spectral and CD data obtained in the presence of guanidine-HCl and extrapolated to zero denaturant concentration. D is the completely denatured state.

process.[195] The significance of these thermodynamic parameters can be best appreciated in contrasting their magnitude for the transitions: N - - - → D, N - - - → A, and A - - - → D. Kuwajima's data[153] indicate that about 75% of the enthalpy and entropy changes for denaturation of BLA (N - - - → D) occurs in the N - - - → A step (Table 26). Similarly, the magnitude of ΔC_p for the A - - - → D step is much smaller than that found for the N - - - → A step, indicating that a number of hydrophobic interactions disrupted in the latter process are significantly greater than that for denaturation of the A conformer. It is also significant that Sommers and Kronman[146] and Pfeil et al.[215] have found no indication of a cooperative thermally induced transition from A to D states. These observations indicate that the A conformer of α LA may be regarded, in a sense, as "a denatured protein with secondary structure."

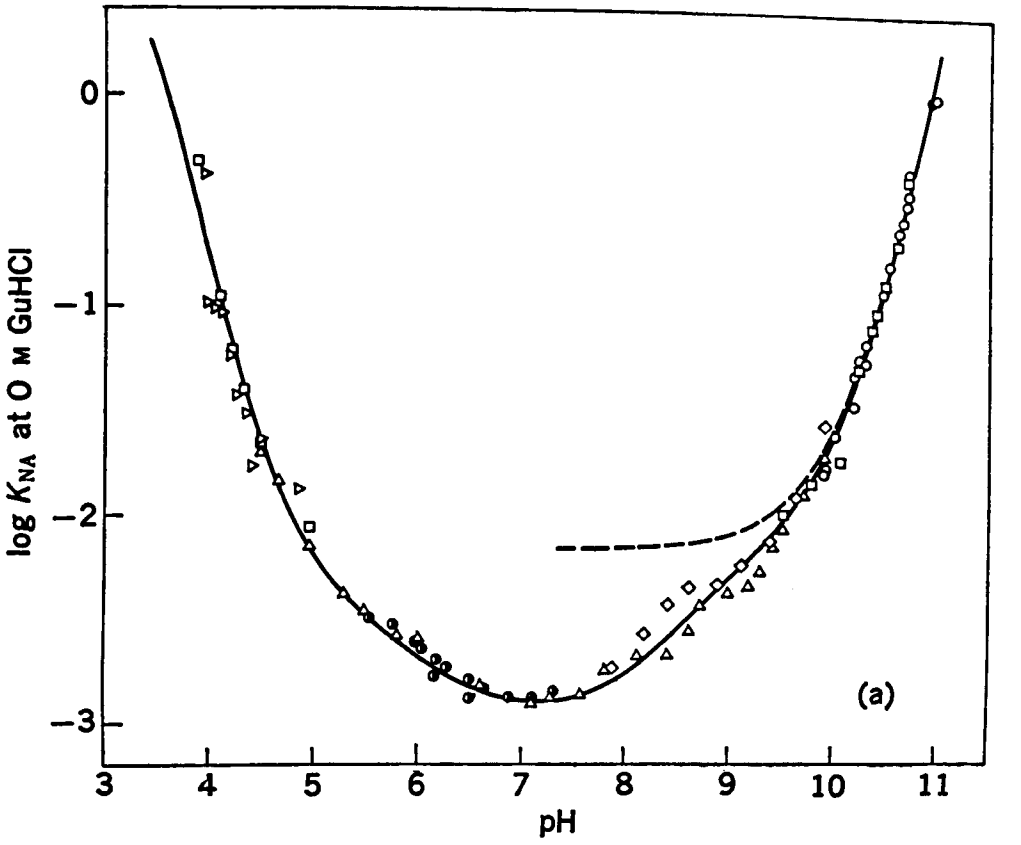


FIGURE 35. The pH dependence of the equilibrium constant for the N-A transition. The different symbols are for data obtained at different concentrations of guanidine-HCl, but are extrapolated to zero concentration of denaturant. Superposition of data curves was done by a "curve shifting" procedure. The solid line is a theoretical curve calculated using the dissociation constants given in Table 27. The dashed line is the contribution due to tyrosyl residues alone. (From Kuwajima, K., Ogawa, Y., and Sugai, S., *J. Biochem.*, 89, 759, 1981. With permission.)

3. The pH Dependence of the N-A Conformational Change

The pH dependence of the equilibrium constant, K_{NA} for the process N $\rightleftharpoons$ A, can be shown to reflect differences in the dissociation constants for specific ionizing groups in the two conformers. Kronman et al.[147] found that the low pH transition could be described by values of $pK_{diss.}$ of ca. 2.1 (N state, 3 carboxyls) and 4.4 (A state, 3 carboxyls). These ionizing groups appear to be those coordinated to calcium at site I (Section II.G). The N-A transition occurring above pH 9 could be similarly described by values of $pK_{diss.}$ of 11.4 (N state, 3 groups) and 10.6 (A state, 3 groups). Kronman et al. concluded that the latter ionizing groups were the ϵ-amino groups of lysines; however, more recent evidence indicates that they are probably tyrosyl residues.

Kuwajima et al.[117,184] examined the pH dependence of K_{NA} in great detail in the course of their characterizing the denaturation of α LA by guanidine-HCl. Their results are summarized in Figure 35, together with a theoretical curve calculated from the parameters of Table 27. The dependence of K_{NA} on pH between 4 to 11 can be accounted for on the basis of changes in the $pK_{diss.}$ of 4 carboxyl, 1 histidyl, the α amino, and 4 tyrosyl residues in going from the N to the A state. Three of the four carboxyls almost certainly correspond to asp 82, 87, and 88 which coordinate to calcium in site I, while the fourth, whose $pK_{diss.}$ in the N state is 3.8, may correspond to asp 83, which is not coordinated to Ca^{+2}, but is

TABLE 27
Changes in the Dissociation Constants of Ionizing Groups in the N and A Conformers of BLA

Group	A State		N State		Contribution to ΔG of stabilization (kJ mol^{-1})
	No.	pK_{diss}	No.	pK_{diss}	
Carboxyl			3	3.3	
	4	4.4	1	3.8	−22.2
Histidyl	1	6.3	1	5.8	−4.2
α Amino	1	8.1	1	8.9	−3.3
Tyrosyl			1	10.5	
			2	11.8	−32.3
	4	10.3	1	12.7	

Note: The assignments are not unique; equally good fits of the data of Figure 35 were obtained with values of pK of 12.4 (2 tyrosyl groups) and 11.1 (two tyrosyl groups) and 8.6 for the amino group in the N state.

Data from Hiraoka, Y. and Sugai, S., *Int. J. Pept. Protein Res.*, 23, 535, 1984.

located in the calcium-binding elbow, or asp 84 which coordinates to the metal-ion through its peptide-carbonyl oxygen (Scheme VI). The $pK_{diss.}$ values ascribed to the four tyrosyl residues correspond to those found by Kuwajima et al.[231] for the N conformer in their stopped-flow study of tyrosine ionization (see Section III.D for discussion of the molecular environments of these tyrosyl residues in N and A states).

The minimum value of K_{NA} is observed between pH 7 and 8 (Figure 35) and corresponds to a Gibbs free energy for stabilization of the N relative to A state of ca. −17 kJ-mol^{-1}. The changes in Gibbs free energy corresponding to the differences in $pK_{diss.}$ for the residues cited here are shown in the last column of Table 26. It is apparent that changes in the dissociation constants for the α-amino and histidyl groups are too small to have a significant effect on destabilizing the N state. However, the magnitudes of the free energy changes corresponding to the alteration of the dissociation constants for the carboxyl and tyrosyl groups are significantly large to drive the equilibrium toward the A state at either extreme of pH.

4. The Thermal Stability of Various Species of α LA

The amino acid sequence of RLA differs most significantly from the other species of α LA in having a proline-rich C-terminal extension of 17 additional amino acids[10] (Figure 1). It might be anticipated that the presence of this extension of the C terminus would alter the stability of the N form of the protein, particularly in view of the putative conformational degeneracy of the C-terminal tail of BLA. However, this does not appear to be the case; the transition temperature for RLA for thermal conversion to the A state is comparable in magnitude to those found for BLA, GLA, HLA, and GPLA (Table 28).

These results are in contrast to the observations made for tryptophan synthase[218] and bacteriophage T4 lysozyme,[219] where a single point mutation results in marked changes in stability. It is striking that the incremental change in the thermal stability of RLA on addition of 17 amino residues to the C-terminal end of the molecule is not significantly different from that seen from one species of α LA to another, resulting from a number of differences at specific positions in their sequences.

TABLE 28
Species Differences for the Transition Temperature for the Thermally Induced N-A Conformational Change of α LA at Near-Neutral pH

Protein	T_{tr} (deg.)
BLA	55[a], 51[b], 50[c], 53[d], 58[f]
GLA	55[a], 55[b]
HLA	55[a], 55[b], 57[c]
GPLA	63[a], 62[b]
RLA	58[e]

[a] Tryptophan fluorescence measurements, 310 nm.[146]
[b] Tryptophan fluorescence measurements, 350 nm.[146]
[c] Optical rotation dispersion measurements.[203]
[d] UV absorption spectral measurements.[81]
[e] CD measurements.[204]
[f] CD measurements.[150]

V. SUMMARY AND PROSPECTIVES

A. INTRODUCTION

The binding of metal-ions to α LA and the attendant transconformational changes have proven to be of extraordinary complexity. In spite of the wealth of information available about these processes, there are still a number of unresolved questions:

1. Our model of three adjacent-interacting metal-ion-binding sites on the α LA molecule, while very attractive in explaining a wide range of observations, must be regarded as tentative. While the location of the high affinity calcium-binding site is now known from the crystal structure, the location of the other two putative sites, one for Mn^{+2} and the other for Zn^{+2} and Tb^{+3}, have been deduced from experimental observations and low resolution structure of the protein. We anticipate that refinement of the crystal structure of α LA and extension of such studies to the Mn^{+2}-liganded form of the protein will address this issue, as well as the possibility of the presence of a third site adjacent to ones that bind calcium and manganese.
2. Binding of Co^{+2} to α LA is of particular significance in view of its role as an activator of GT. Its binding to the former protein, therefore, needs to be substantiated, and the characteristics of the conformational state which Murakami and Berliner[106] deduce from their spectal studies need to be examined in depth. The multiplicity of metal-ion-binding sites found on the α LA molecule, as well as the complexity of their associated conformational changes, suggests that the *sole* use of spectral changes or *indirect* procedures (see Section II.B) in characterizing binding to this protein is less than adequate. Use of radioisotopic Co^{+2} in conjunction with techniques such as gel filtration, ultrafiltration, or equilibrium dialysis can provide more reliable information about the stoichiometry and affinity of binding of this cation.
3. The significance of the activation of the lactose synthase reaction by zinc ion likewise calls for further investigation of the molecular properties of Zn^{+2} liganded α LA. It

has been generally assumed that the molecular characteristics of this state and of the metal-free protein are comparable. However, little is known about the properties of metal-ion-liganded A states, other than the fact that the molecular environments of tryptophan residues are comparable to those of the metal-free protein. Considering the variation in fluidity found for the various N-like states, a more detailed investigation of the properties of A states should be made.

4. The dependence of the pattern of binding of calcium and manganous ions on their order of mixing with BLA demonstrates that the characteristic conformational states promoted by their binding are not in equilibrium with each other. The generality of this kinetic phenomenon needs to be assessed for other cations which bind to α LA.

The number of different metal-ions which bind to α LA (Tables 6 to 8), the relatively large number of binding sites (Table 13), and the apparent multiplicity of conformational states of α LA promoted by such binding (Scheme VII and Table 23) are unusual in so small a protein molecule. These molecular states, with some exception, have not been well characterized, but it is likely that they exhibit considerable differences in internal fluidity, i.e., varying degrees of spatial correlation of side chains, ranging from that of the calcium-liganded protein, where the molecule appears to have a well-defined tertiary structure, to the metal-free state, where tertiary structure appears to be absent. Conformational states have been classified as N or A states, based on a rather simple-minded criterion, the molecular environment of relatively few, probably identical, tryptophan residues. Since the "fluidity" of the α LA molecule, based on reactivity of amino groups, is different for different N conformers, it is apparent that classification of states as N or A is not sufficiently discriminatory to be useful. Criteria other than the molecular environment of tryptophan residues and reactivity of amino groups are needed for a more satisfactory characterization of the various N- and A-like states.

Multiplicity of conformational states for α LA implies a high degree of "conformational adaptability" of this protein by which the spatial relationships of its structural moieties are markedly influenced by interactions with ions and other molecules in the milieu. Such interactions would include those with moieties in other protein molecules such as those of GT. This is a rather attractive feature for a protein having a regulatory function.

Another important consequence of the multiplicity of conformational states for α LA is that its ligand-binding properties are likely to be altered on transformation of the protein from one state to another. Examples of this given in Section II include the increase in binding affinity of zinc and terbium on transformation of the protein to the "expanded A" state. Similarly, although binding of monosaccharides to *isolated* native α LA has never been demonstrated (Section I.F), the conformation of the protein *within* the lactose synthase complex may permit tight binding of the sugar *to the α LA moiety*. It would be of interest, therefore, to investigate binding of monosaccharides to apo-α La and to apo-α LA plus metal-ions other than calcium. Likewise, where binding of metal-ions to sites of *isolated* α LA may be too weak to be "physiologically significant", incorporation into a complex with GT may induce a conformational state with much tighter binding at these sites.

B. METAL-ION BINDING AND LACTOSE SYNTHASE ACTION

Although α LA binds Mn^{+2}, Zn^{+2}, and perhaps Co^{+2}, cations which activate GT in the synthesis of lactosamine and of lactose (Section I.F), no role for metal-ions bound to the α LA moiety in the lactose synthase complex has ever been *established*. It is apparent that the distance from the region of contact of the α LA and GT molecules in the complex to metal-ion-binding sites I, II, and III (Table 13) is too great (Figure 4) for the cations bound at these sites to participate *directly* in formation of the lactose synthase complex or in subsequent events in the catalysis. Thus, any functional significance of the binding of

Ca^{+2}, Mn^{+2}, or Zn^{+2} at these sites must be an indirect one, e.g., maintenance of a biologically active conformational state. It should be emphasized that cation binding at sites I to III gives rise to a global change in conformation, encompassing virtually the entire protein molecule.

Musci and Berliner[247] have proposed that the apo-α LA is more active than Ca^{+2}-α LA in lactose synthase action and that Zn^{+2} serves physiologically to maintain the protein *in vivo* in the more active apo-form. This explanation can be rejected on two counts: (1) their conclusion that the "apo-form" of the protein is more active than the calcium form of the protein seems dubious since their assays were carried out under less-than-optimal conditions, i.e., in the presence of ca. 9 μ*M* Mn^{+2}, where site II of GT is not occupied by Mn^{+2}; under these conditions activation of GT itself is low and formation of the lactose synthase complex is impaired; (2) their assumption that the conformation of *native* α LA is shifted to an "apo-like" state in the presence of 53 μ*M* Zn^{+2} is not valid (see Figure 19 and the discussion in Section II.E.3). While the stabilization of a biologically active molecular conformation of α LA by bound metal-ions may be a resonable hypothesis, there is no evidence to date to substantiate their conclusion of the role of zinc ion.

Attempts to establish a role of metal-ions in the lactose synthase system have been thwarted by a lack of knowledge of the characteristics of metal-binding of α LA *complexed* with GT and indeed the metal-binding properties of the enzyme itself. The involvement of metal-ions in both the synthesis of lactose and lactosamine has been deduced from the kinetics of catalysis and are consistent with a *minimum* stoichiometry of two bound metal-ions/GT molecule.[69] However, there is no corroborative evidence to support the conclusions drawn from these kinetic experiments. Experiments aimed at determining the binding of Mn^{+2} to GT and to the cross-linked lactose synthase complex proved to be technically impossible to carry out because of extensive self-association of the enzyme at the *minimum* concentrations of enzyme required for the Hummel-Dryer procedure.[261]

While the stoichiometry and the affinity of binding of metal-ions to α LA or GT *in the lactose synthase complex* are uncertain, the observation of partial inhibition of lactose synthesis at high concentrations of K^+ *in vitro* indicates that binding of cations has significant influence on this reaction (Figure 36).[261] While K^+ has no effect on the catalysis by GT in the absence of α LA, inhibition of the lactose synthase reaction occurs in the concentration range where this cation binds (Figure 36). These preliminary observations, while not pinpointing a specific functional role for bound cations, suggest a number of alternative explanations of the inhibition involving either GT or α LA:

1. A conformational change of the *GT* molecule on formation of the lactose synthase complex might alter the cation-binding affinity and/or specificity at one or the other of its metal-ion sites, permitting the binding of potassium ion and the concomitant alteration of its catalytic properties or its ability to form the lactose synthase complex. Alternatively, such a structural change might promote the formation of a new site for binding of the monovalent cation with a similar effect on its catalytic or complex-forming properties.
2. The binding of potassium ion to the α LA moiety in the lactose synthase complex might alter its conformational state, thereby making it less effective in promoting the binding of glucose or forming the complex with GT.
3. A third mechanism, very different from the others proposed, is based on the hypothesis that the lactose synthase complex is stabilized by a metal-ion bridge from α LA to the GT molecule; inhibition by potassium ion would result from competitive displacement of the bridging cation. As indicated previously, metal-ion-binding sites I to III (Table 13) are too distant from the α LA-GT contact region to be directly implicated in such a process. However, one of the secondary sites, IV to VI, might serve as the

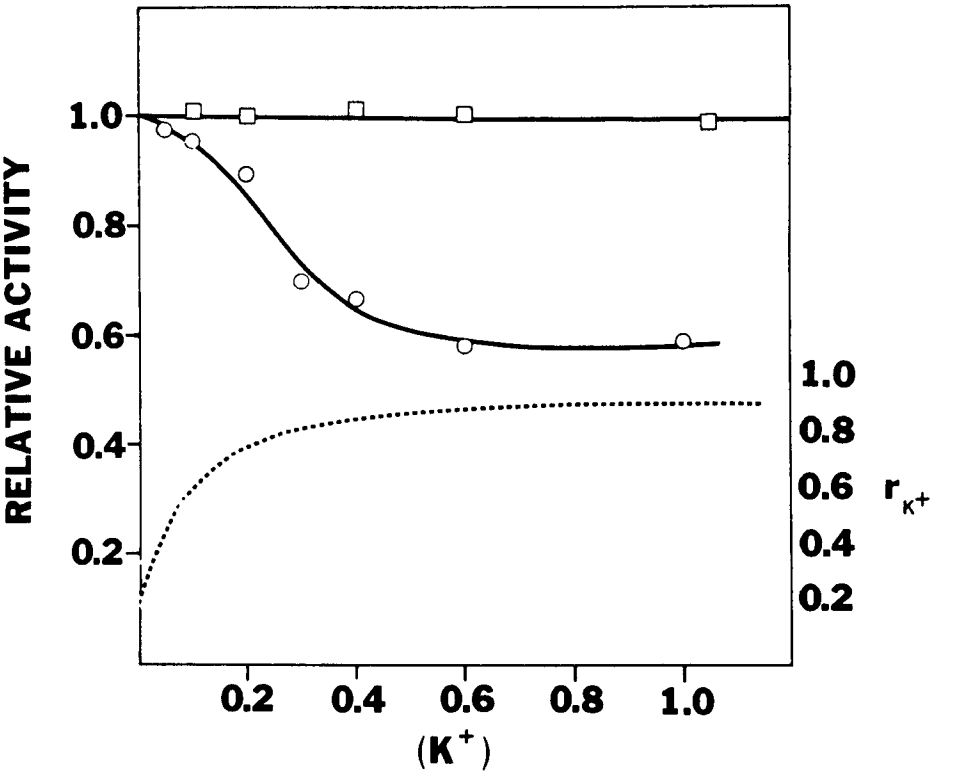


FIGURE 36. Inhibition of lactose synthase action by potassium ion. For assay conditions, see Figure 3. □, synthesis of lactosamine in the absence of α LA; ○, synthesis of lactose - - - binding of potassium ion.[107,261]

locus for such a bridge with GT. A very attractive possibility is that his 32 of α LA, which is essential for lactose synthase activity, lies in such a bridging site. As we have indicated in Section I.F.3, studies of chemical modification of this residue suggest that a very specific interaction with its N-1 nitrogen is essential for lactose synthase activity. We propose that this interaction coordinates to the metal-ion which in turn coordinates to moieties on the GT molecule.

Two possibilities suggest themselves for the coordination site on the GT molecule; (1) a third metal-ion-binding site distinct from the two deduced from the kinetic studies; (2) the supposedly essential low affinity metal-binding site. As discussed in Section I, Navaratnam et al.[71] proposed that the natural activator of GT *in vivo* is not a metal-ion bound at site II, but rather is a basic secretory protein presumably binding in the same region of the enzyme molecule. This conclusion was based on their observation that a wide range of polycationic substances were as effective in activation of GT as relatively high concentrations of manganous ion. Activation by a wide range of cationic substances is consistent with the findings that a large number of different metal-ions having widely different coordination properties activate GT on binding at site II (Table 5).[69,70] In extending the hypothesis of Navaratnam et al., we propose that α LA, having metal-ion coordinated at his 32, is the cationic substance activating GT in the lactose synthase complex. This hypothesis requires verification.

C. A PROSPECTIVE VIEW OF α LACTALBUMIN AS A MODULATOR OF BIOLOGICAL PROCESSES IN MAMMALS

The biological activity of α LA has generally been regarded as being restricted to a

rather specialized function as a promoter of lactose synthase action in the mammary gland. More recently, however, studies have been published which point to the possibility that "α lactalbumin-like" proteins participate in reproductive and developmental processes as well. These observations are briefly outlined here.

1. While GT is widely distributed in a variety of tissues, it was generally assumed that since lactose is synthesized only in the mammary gland α LA would likewise be present solely in that organ. However, α LA activity has been detected in human semen and rat, rabbit, and mouse epididymal fluid.[233, 234, 237] Epididymal and milk α LA appear to be very similar molecules: they have comparable amino acid compositions;[237] epididymal α LA cross-reacts with an antibody to the milk α LA[236] and its mRNA hybridizes with cDNA of the milk protein.[236,237] Interestingly, epididymal α LA utilizes glucose *and* inositol equally well as galactose acceptors, in contrast to the milk protein which is restricted to use of the former monosaccharide.[237] The amino acid sequence of the epididymal protein has not as yet been determined and no information is available concerning its metal-binding properties.
2. Bovine mammary α LA inhibits neural crest cell migration on basal lamina, a process which involves participation of GT.[239]
3. Bovine mammary α LA specifically interrupts uterine epithelial cells adhesion *in vitro*, presumably by interaction with a cell surface GT.[240]
4. Bovine mammary α LA acts as a bimodal regulator of rat parotid acinar cell growth.[241]

Presumably, the functions *in vivo* in the latter three cases are mediated by tissue-specific α LAs, as was true in the male reproductive system.

Based on the appearance of α LA in the mammary gland early in gestation, prior to lactose synthesis, Nakhasi and Qasba[235] proposed that the protein might have a more general function in mammary and other tissues "in the modification of cell surface carbohydrates involved in cell-cell interactions and cell differentiation." As pointed out by Byers et al.,[237] α LA can be either a peripheral or an integral membrane protein depending on its molecular conformation. Incorporation of α LA into membranes occurs more readily with A-like conformers and leads to an increase in their fluidity[248-253,257,258] and can also lead to fusion of phosphotidyl vesicles.[246] The latter observation is of particular interest since membrane fusion is an essential process in a broad range of biological phenomena.

It is likely then that the biological effects mediated by α LA *in vivo* will have their origins in two types of processes: (1) direct interaction of the protein with GTs typified by formation of the lactose synthase complex *and* (2) modification of membrane properties by the incorporated protein with concomitant alteration of the catalytic properties of membrane-bound enzyme molecules and changes in their mobility in the membrane. The complex modulation of the conformational states of α LA by binding of metal-ions demonstrated in the studies reviewed here, therefore, is likely to be of paramount significance in the regulation of processes mediated by this protein.

ACKNOWLEDGMENTS

The research reported from my laboratory has been supported by grants from the Institutes of General Medical Sciences and of Child Health and Human Development, USPHS and from the Hendricks Fund. Thanks are also due to a number of individuals who provided manuscripts of their papers prior to their publication. I also wish to thank Professor K. Brew for helpful discussions in reexamining certain aspects of previously reported observations; any errors or failures in such interpretation, however, are entirely mine. Special thanks are due to Professor C. F. Van Cauwelaert, who has generously kept me informed of the progress

of research in his laboratory. I am also pleased to acknowledge my debt to Dr. Sugai and the members of his laboratory in the Department of Polymer Science, University of Hokkaido, who for many years have kept me informed in a timely manner of their research with α lactalbumin. Finally, I thank Debra Knapp for her patience and her intelligent and expert efforts in preparing the manuscript of this review.

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