Inter- and Intramolecular Interactions of α-Lactalbumin. IV. Location of Tryptophan Groups*

Martin J. Kronman and Leo G. Holmes

ABSTRACT: The degree of "exposure" of tryptophan groups in α-lactalbumin has been determined under a variety of conditions using the solvent perturbation method of difference spectrophotometry. Thioglycolate reduction of the disulfide bonds of this protein results in complete "exposure" of the five tryptophan residues, while denaturation in 8 M urea results in an apparent exposure of 80% of these. The native protein at 25° has only two groups "exposed" with respect to the perturbants sucrose, ethylene glycol, glycerol, and D_2O, both on the alkaline side of the isoelectric zone and at low *p*H (1.8–3). The absence of a *change* in groups exposed with respect to *any* of the perturbants in going from *p*H 6 to low *p*H at 25° demonstrates that the large "denaturation blue shift" occurring in α-lactalbumin at acid *p*H (M. J. Kronman, L. Cerankowski, and L. G. Holmes, *Biochemistry 4*, 518 [this issue; preceding paper]) cannot be the consequence of an unfolding of the molecule in the region of tryptophan residues. On lowering of the temperature to 1° at *p*H 6, the two groups which are "exposed" at 25° become buried with respect to large perturbants such as sucrose and glycerol but remain accessible to heavy water. This difference appears to be owing to partial closure of the "crevices" housing two of the tryptophans. In the *p*H region 1.8–3, this "crevice" closure is more limited than at *p*H 2: one group is "buried" with respect to sucrose and glycerol but again two groups are accessible to D_2O.

In the previous paper in this series (Kronman *et al.*, 1965; hereafter referred to as paper III) we showed that the conformational change occurring on denaturation of α-lactalbumin at low *p*H is accompanied by a short-wavelength shift of the ultraviolet spectrum characteristic of changes in the environment of tryptophan groups. The magnitude of this change was both *p*H and temperature dependent, the transition actually occurring in the *p*H range 3–4.

Denaturation of proteins is usually thought of as involving an unfolding of the compact native structure to give increased accessibility of amino acid side chains to the medium. The unmasking of chromophores such as tyrosine and tryptophan has been frequently cited as the origin of the large blue shifts observed in the ultraviolet spectrum upon denaturation of many proteins.

The fact that the denatured α-lactalbumin molecule is more swollen than the native one (Kronman and Andreotti, 1964; hereafter referred to as paper I) suggests that side chains which may have been buried in the native state may now be exposed. The increased tendency of the protein to aggregate likewise suggests that the nature of groups at the molecular surface has been changed by acid treatment (Kronman *et al.*, 1964; hereafter referred to as paper II). Thus, it might be anticipated that the blue shift observed upon acid treatment is the consequence of a transfer of tryptophan groups from the interior of the molecule to contact with the medium. Considering the numerous ways in which spectral shift can arise (Wetlaufer, 1962), however, it seemed vital to verify this hypothesis directly through the use of the solvent perturbation method of difference spectroscopy (Herskovits and Laskowski, 1960, 1962a.b).

This technique allows one to measure *only* the degree of "burying" of chromophores to the exclusion of other environmental effects involving hydrogen bonding, dipole interactions, and the like, which can also give rise to spectral shifts. Results of solvent perturbation experiments to be presented here demonstrate that the "denaturation blue shift" observed with α-lactalbumin at acid *p*H values cannot be accounted for on the basis of increased "exposure" of tryptophan groups and must have other origins.

Materials and Methods

The characteristics of preparations 3088 and R49 of α-lactalbumin used in this study, as well as procedures for preparation of protein solutions, have been described in papers I and III. Spectroscopic grade glycerol and ethylene glycol were used as perturbants. Heavy water (99.5 mole %, minimum D_2O) was obtained from Matheson Coleman and Bell and Volk Radiochemical Corp. Amino acid derivatives were Mann preparations. All other chemicals were of reagent grade and glass-distilled water was used to prepare all solutions.

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TABLE I: Perturbation Results for Denatured α-Lactalbumin and Model Compound Analogs in 0.15 M KCl.

Perturbant[a] (1)	α-Lactalbumin, Thioglycolate Reduced, 8 M Urea, 25° $\Delta E_{293}/E_{280}$[b] (2)	Model Compound Analogs $\Delta E_{293}/E_{280}$[c] 1° (3)	Model Compound Analogs $\Delta E_{293}/E_{280}$[c] 25° (4)	Denatured α-Lactalbumin, 8 M Urea, 25° $\Delta E_{293}/E_{280}$[b] (5)	Denatured α-Lactalbumin, 8 M Urea, 25° Groups[d] Exposed (6)
20% Sucrose	0.024 ± 0.002	0.030 ± 0.002	0.028 ± 0.001	0.022 ± 0.005	4.0 ± 0.8
20% Ethylene glycol	0.054 ± 0.002	0.059 ± 0.003	0.051 ± 0.003	0.037 ± 0.002	3.7 ± 0.1
20% Glycerol	0.055 ± 0.000	0.057 ± 0.001	0.051 ± 0.003	0.045 ± 0.004	4.4 ± 0.5
90% D_2O		−0.040 ± 0.002	−0.035 ± 0.003		

[a] Concentration of perturbants are on a weight per cent basis with the exception of D_2O which is a volume per cent. [b] Obtained over the *p*H range 2.6–6.0. Average of from three to five determinations. [c] Average of five determinations. [d] Based on a total of five tryptophan groups (Gordon and Ziegler, 1955).

A Cary Model 14 spectrophotometer was used for most of this work in conjunction with the thermostated tandem cell arrangement previously described by us (Holmes and Kronman, 1964). In some of the heavy water experiments a Cary Model 15 spectrophotometer was employed. The results obtained with both instruments were equivalent. Procedures for solution preparation, spectral measurements, and for preparation of reduced protein were essentially those described by Herskovits and Laskowski (1962a).

Results

Model Compounds and Denatured Protein. To provide a basis for calculating the degree of "exposure" of tryptophan groups, perturbation spectra of urea-denatured and reduced urea-denatured α-lactalbumin were compared with model compound analogs. The latter were mixtures in 5:5 molar ratio of the *N*-acetyl ethyl esters of tyrosine and tryptophan. Chemical analysis shows five tyrosine, five tryptophan, and four phenylalanine residues per molecule (Gordon and Ziegler, 1955). The latter amino acid derivative has been omitted since it makes a minor contribution to the spectrum in the wavelength region of interest (>280 mμ).

Denaturation of α-lactalbumin in 8 M urea is instantaneous and produces extensive disruption of the structure as demonstrated by a more than 2-fold increase in quantum yield of tryptophan fluorescence (M. J. Kronman, manuscript in preparation), abolition of the acid difference spectrum (paper III), and the observation of a near zero value for the rotatory dispersion parameter, b_0 (M. J. Kronman and R. Blum, 1964, unpublished experiments; Herskovits and Mescanti, 1965). Nonetheless, treatment of α-lactalbumin with 8 M urea fails to "expose" all of the tryptophans to the medium. This is illustrated by the spectra of Figure 1 obtained with 20% glycerol. Curve C (urea-denatured protein) lies significantly lower than curve A (model compound mixtures). A comparison of curves A and B (thioglycolate-reduced protein in 8 M urea) indicates that the removal of the constraints imposed by the four disulfide bridges (Gordon and Ziegler, 1955) results in greater "exposure" of chromophores.

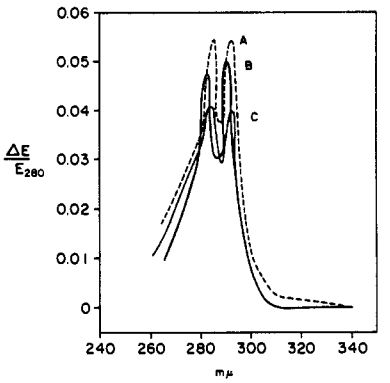

FIGURE 1: Comparison of solvent perturbation spectra of denatured α-lactalbumin and model compounds. Perturbant, 20% glycerol; temperature, 25°; protein concentration, 0.1–0.17 g/100 ml; solvent, 0.15 M KCl. (A) Model compound mixture (see text). (B) Thioglycolate-reduced protein in 8 M urea. (C) Denatured protein in 8 M urea.

The relative magnitudes of the extinction coefficients of tyrosine and tryptophan in the region above 290 mμ makes it reasonable to attribute the 293-mμ difference peak entirely to tryptophan, e.g. (at 294 mμ the ratio E_{Try}/E_{Tyr} is about 100) (Wetlaufer, 1962).

Summarized in Table I are the results obtained for urea-denatured, reduced urea-denatured α-lactalbumin and for model compound mixtures using four perturbants. Solutions of ethylene glycol, glycerol, and sucrose all have refractive indices greater than that of water and hence produce "red shifts" (indicated by

TABLE II: Summary of Solvent Perturbation Results for α-Lactalbumin at 25° in 0.15 M KCl.

Perturbant (effective radius[a]) (1)	Alkaline Side of Isoelectric Point			Low *p*H			Transition Region		
	*p*H (2)	$\Delta E_{293}/E_{280}$ (3)	Groups[b] Exposed (4)	*p*H (5)	$\Delta E_{293}/E_{280}$ (6)	Groups[b] Exposed (7)	*p*H (8)	$\Delta E_{293}/E_{280}$ (9)	Groups[b] Exposed (10)
20% Sucrose (4.7 A)	5.95–6.20	0.013[c] ± 0.001	2.3 ± 0.3	1.85–3.00	0.011[c] ± 0.001	2.0 ± 0.2	3.00–3.85	0.011[d]– 0.028	[e]
20% Glycerol (2.7 A)	5.75–5.95	0.020[d] ± 0.003	2.0 ± 0.2	1.80–3.00	0.021[c] ± 0.002	2.1 ± 0.2	3.00–3.80	0.021[c]– 0.024	[e]
20% Ethylene glycol (2.2 A)	6.26–6.37	0.017[d] ± 0.003	1.7 ± 0.2	2.06–3.15	0.015[c] ± 0.000	1.5 ± 0.0	3.15–3.81	0.014[d] ± 0.003	1.4 ± 0.3
90% D_2O (1 A)	5.8–6.2	−0.013[d] ± 0.001	1.9 ± 0.5	1.85–2.50	−0.014[d] ± 0.002	1.9 ± 0.3	3.25–3.80	"Red shift"[e]	[e]

[a] Radii (Einstein-Stokes spheres) of sucrose, glycerol, and ethylene glycol taken from Herskovits and Laskowski (1962a). Values for D_2O calculated from the diffusion constant, 1.68×10^{-5} cm^2 sec^{-1}, obtained at 25° (Wang, *et al.*, 1954). [b] Based on a total of five tryptophans per molecule of α-lactalbumin (Gordon and Ziegler, 1955). [c] Taken from Figure 3 or similar plots. [d] Average of a minimum of five values. [e] Exposure of groups not calculated; see text for explanation.

positive values of $\Delta E_{293}/E_{280}$),[1] while 90% heavy water has a lower refractive index than water and gives a "blue shift" (negative values of $\Delta E_{293}/E_{280}$). Comparison of $\Delta E_{293}/E_{280}$ for protein with that for model compounds (columns 2 and 4, Table I) reveals that within experimental error reduction of disulfide bonds gives full exposure of tryptophan groups to all perturbants employed. The values of $\Delta E_{293}/E_{280}$ for urea-denatured protein are lower than those for model compounds and correspond to about 80% exposure (average for all perturbants). Per cent "exposed" has been calculated from the relationship:

$$\% \text{ exposed} = 100 \frac{(\Delta E_{293}/E_{280}) \text{ protein}}{(\Delta E_{293}/E_{280}) \text{ model cpd}} \quad (1)$$

Implicit in the use of equation (1) is the assumption that groups will be seen as completely "exposed" or completely "buried." Actually we cannot distinguish this situation from one in which all groups are partially "buried." Bearing this possible ambiguity in mind, we shall consider "exposure" in terms of equation (1) in the subsequent presentation, making use of appropriate values of $\Delta E_{293}/E_{280}$ for model compounds (columns 3 and 4, Table I).

"Exposure" of Tryptophan Groups at 25° near pH 6. We have previously used *p*H 6 as a reference point for measurement of acid difference spectra (paper III). The degree of "exposure" of tryptophan groups in this *p*H region has been determined using four perturbants. Typical perturbation spectra obtained at 25° in sucrose and in heavy water are shown in Figure 2 together with those for the model compound mixtures. The amplitudes of the protein spectra are significantly lower than those of model compounds, indicating incomplete exposure of tryptophan groups with respect to these two perturbants.

The quantitative results obtained near *p*H 6 (columns 2 and 4, Table II) show values of $\Delta E_{293}/E_{280}$ for sucrose, ethylene glycol, glycerol, and D_2O (effective radius 4.7–1 A) which correspond within experimental error to about two exposed tryptophans.

"Exposure" of Groups at 25° at Low pH. The large "blue shift" observed in the ultraviolet spectrum of α-lactalbumin at low *p*H relative to *p*H 6 (paper III) is consistent with an unfolding of the molecule to give increased exposure of one or more of the three tryptophans found to be buried near *p*H 6 (see previous section). The perturbation spectra obtained below *p*H 3, however, negate this hypothesis. Values of $\Delta E_{293}/E_{280}$ obtained with sucrose and glycerol as perturbants (Figure 3) are essentially invariant with *p*H. Similar observations have been made with ethylene glycol and D_2O as perturbants. Values of $\Delta E_{293}/E_{280}$ for all four perturbants (columns 5–7, Table II) indicate that within experimental error two groups are exposed below *p*H 3 as was the case at *p*H 6 (columns 2–4, Table II).

"Exposure" of Groups at 1°. Tryptophan groups in α-lactalbumin are more "buried" at 1° than at 25° with respect to two larger perturbants. This is strikingly illustrated by a comparison of spectra for the protein at *p*H 5.87 and 2.90 with those for model compounds. A typical set of spectra obtained with 20% sucrose is shown in Figure 4. Within experimental error the spec-

[1] Difference peaks were found to vary in wavelength from 291 to 294 mμ. In all cases we have taken ΔE at the maximum of the curve but for convenience sake we refer to all values as ΔE_{293}.

TABLE III: Summary of Solvent Perturbation Results for α-Lactalbumin at 1° in 0.15 M KCl.

	Alkaline Side of Isoelectric Point			Low *p*H			Transition[a] Region		
Perturbant (1)	*p*H (2)	$\Delta E_{293}/E_{280}$[b] (3)	Groups[c] Exposed (4)	*p*H (5)	$\Delta E_{293}/E_{280}$ (6)	Groups[c] Exposed (7)	*p*H (8)	$\Delta E_{293}/E_{280}$ (9)	
20% Sucrose	5.75–5.95	0.0022 ± 0.0016	0.4 ± 0.3	2.03–2.90	0.0081 ± 0.0020	1.4 ± 0.3	3.20–3.44	0.017–0.030	
20% Glycerol	5.80	0.0016 ± 0.0004	0.1 ± 0.03	1.76–3.00	0.011 ± 0.0025	1.0 ± 0.3	3.00–3.61	0.012–0.025	
20% D_2O	5.85–6.09	0.014 ± 0.002	1.8 ± 0.2	1.80–2.85	0.014 ± 0.002	1.8 ± 0.2			

[a] Exposure of groups not calculated; see text for explanation. [b] Average of minimum of five values. [c] Based on a total of five tryptophan groups (Gordon and Ziegler, 1955).

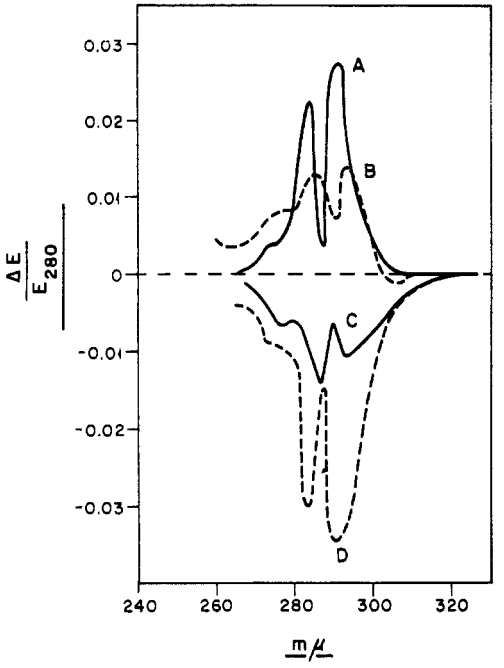

FIGURE 2: Comparison of solvent perturbation spectra of native α-lactalbumin with those for model compounds at 25°. Perturbant, 20% sucrose (curves A and B), 90% D_2O (curves C and D); other conditions as in Figure 1 (A) and (D). Model compound mixture (see text). (B) Protein, *p*H 5.96. (C) Protein, *p*H 6.05.

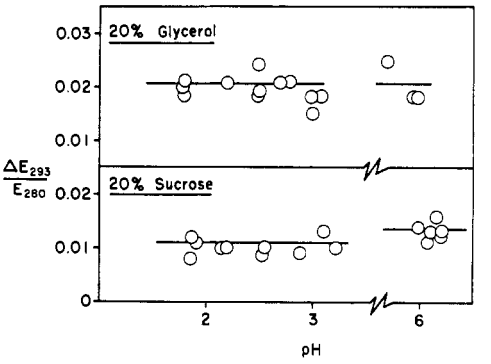

FIGURE 3: *p*H dependence of the perturbation spectral data. Conditions as in Figure 1. Perturbant, 20% glycerol (upper curve), 20% sucrose (lower curve).

trum for the *p*H 5.87 protein is flat and devoid of the characteristic maxima, while the amplitude of the *p*H 2.90 curve is significantly reduced as compared to 25° (compare curves B in Figures 2 and 4). The results of the perturbation experiments at 1° are summarized in Table III. For both 20% sucrose and 20% glycerol, within experimental error, no tryptophan groups are exposed near *p*H 6, while at low *p*H a single group is accessible to perturbant. For reasons which are not clear, perturbation experiments with 20% ethylene glycol yielded data of very poor reproducibility at 1° and consequently have not been tabulated.

In contrast with these observations made with sucrose and glycerol, perturbation experiments with heavy water indicated the same degree of exposure at 1° and 25°. At low temperature, at low *p*H, and near *p*H 6 (columns 4 and 7, Table III), two groups remain exposed (see also columns 4 and 7, Table II).

Perturbation Experiments in the Transition Region. The *p*H range 3–4 is of particular interest since it is within this region that the molecular changes actually occur for α-lactalbumin reflected as alterations in solubility (paper II), rotatory dispersion (M. J. Kronman and R. Blum, unpublished experiments), ultraviolet fluorescence (M. J. Kronman, manuscript in preparation), and in acid difference spectra (paper III). Unfortunately, as has been pointed out by Herskovits and Laskowski (1962a), it is just within such transition regions that solvent perturbation data cannot be interpreted in a straightforward manner. They found that in the neutral *p*H region in the presence of sucrose or glycerol, urea-

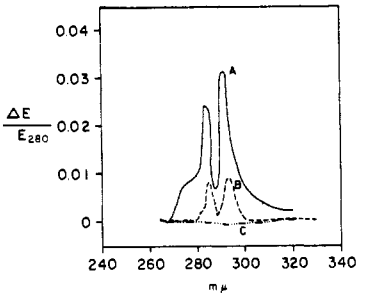

FIGURE 4: Comparison of solvent perturbation spectra of model compounds with those for native α-lactalbumin at 1°. Perturbant, 20% sucrose. (A) Model compound mixture (see text). (B) α-Lactalbumin, *p*H 2.90, (C) α-Lactalbumin, *p*H 5.87.

denatured bovine serum albumin and ribonuclease exhibit values of $\Delta E/E$ higher than the theoretical upper limit. They attributed this anomaly to preferential stabilization of the native conformation by the perturbant. Similar observations have been made in this study with α-lactalbumin. Values of $\Delta E_{293}/E_{280}$ for sucrose at 1° and 25° (column 9, Tables II and III) were equal to or greater than those observed with reduced denatured protein or with model compounds (Table I).

Further evidence for the stabilization of the native form of α-lactalbumin by sucrose and glycerol can be directly demonstrated by comparison of acid difference spectra (paper III) obtained in the presence and absence of perturbant. Reference and acid solutions containing perturbant yielded ΔE_{293} values, the absolute values of which were about 14% higher than those of the corresponding perturbant-free solutions and in the anticipated direction, i.e., shifted toward the red.

Similar anomalous behavior has been observed in the transition region using 90% D_2O as perturbant. Under these conditions a red-shifted perturbation spectrum is observed in contrast with the normal blue shift (see Tables I and II and Figure 2, for example), suggesting that stabilization of the native form has occurred. The effect of D_2O on the *pK* values of carboxyl groups of the protein may also be of importance with this perturbant.

These anomalous results obtained in the transition region are to be contrasted with those obtained with ethylene glycol (column 10, Table II) where within experimental error the number of groups exposed in the transition region is equal to that observed at low *p*H and near *p*H 6. Furthermore, in contrast with our findings for sucrose and glycerol, ethylene glycol had no effect on acid difference spectra. These observations are in accord with previous observations which show that ethylene glycol has little or no effect on the conformation of β-lactoglobulin and γ-globulin (Tanford *et al.*, 1962) and bovine serum albumin (Herskovits and Laskowski, 1962a) except at very high concentrations.

Effect of Perturbant on Conformation of α-Lactalbumin outside the Transition Region. While the anomalous perturbation results indicate that sucrose, glycerol, and heavy water have an effect on the conformation of α-lactalbumin in the transition region, these effects seem to be absent near *p*H 6 and at low *p*H. Values of b_0 and a_0 obtained with sucrose, glycerol, ethylene glycol, and D_2O are not significantly different from those obtained in the absence of perturbant (columns 1 and 2, Table IV). Likewise, values of $\Delta E_{293}/E_{280}$ obtained from acid difference spectra in the presence of 20% sucrose and 20% ethylene glycol are, within experimental error, equal to those obtained with perturbant-free protein solutions (column 3, Table IV). We conclude from these data that the four perturbants employed in this study do not alter the conformation of α-lactalbumin near *p*H 6 nor at low *p*H, and the $\Delta E_{293}/E_{280}$ values obtained in perturbation experiments are a reflection of the degree of exposure of tryptophan residues.

TABLE IV: Effect of Perturbants on Conformation of α-Lactalbumin.

Conditions	Rotatory Dispersion Parameters[a] $-a_0$ (1)	$-b_0$ (2)	Acid Difference Spectra[b] $-\Delta E_{293}/E_{280}$ (3)
0.15 M KCl, 25°, *p*H 5.7–6, perturbant free	273	215	
90% D_2O	278	222	
20% Ethylene glycol	290	206	
20% Glycerol	285	199	
0.15 M KCl, 25°, *p*H 2.00, perturbant free	359	151	0.065
90% D_2O	361	146	
20% Ethylene glycol	372	166	0 068
20% Glycerol	363	165	
20% Sucrose			0.070

[a] Parameters a_0 and b_0 obtained in usual way from the Yang-Moffitt equation. A more detailed description of the rotatory dispersion changes will be published subsequently (M. J. Kronman and R. Blum, work in progress). [b] Procedure for acid difference spectra described in paper III. Where perturbants were employed *both* *p*H 6 reference and *p*H 2 solution contained identical concentrations of perturbant.

Possible Effects of Association on "Burying" of Tryptophan Groups. Previous studies (paper I) have shown that α-lactalbumin is capable of undergoing association on the acid side of the isoelectric point and perhaps to

some degree near *p*H 6. This suggests that "buried" groups might be located at the subunit interfaces of such polymerized species and hence be inaccessible to perturbant. This possibility can be ruled out on several counts: First, over the limited protein concentration available to us for accurate spectral measurements (0.1–0.17 g/100 ml), $\Delta E_{293}/E_{280}$ is independent of concentration. Second, measurements of weight-average molecular weights ($\overline{M}_w$) at protein concentrations near 0.2 g/100 ml indicate that 75–95 mole % of the protein is found in monomeric form at *p*H 2, 3, and 6 (paper II). In addition, the change in molecular weight with temperature cannot account for the significant changes in groups exposed at 1° as compared to 25°, e.g., $(\overline{M}_w)_{1°}/(\overline{M}_w)_{25°}$ was found to be 1.07 ± 0.1 at *p*H 6 and 1.10 ± 0.10 at *p*H 2 at protein concentrations of about 0.2 g/100 ml. The possible enhancement of association by the perturbant can likewise be ruled out since molecular weights obtained in the presence of perturbant were identical with those obtained in its absence. Thus, the "burying" of groups observed in this study is not caused by polymerization of the protein but is rather a reflection of the conformation of the monomeric unit.

Discussion

Environment of Tryptophan Groups in α-Lactalbumin. The number of "exposed" tryptophan groups in α-lactalbumin is strikingly constant at 25° with respect to both larger and small perturbants (Table II). The slightly lower values of 1.5–1.7 obtained with ethylene glycol, while possibly a reflection of a shorter-range effect (Herskovits and Laskowski, 1962a), are probably not significantly different than values obtained with other perturbants. This is lent support by Herskovits' (1965) observation that dimethyl sulfoxide, a perturbant of short range, "sees" 2.1 tryptophan groups in α-lactalbumin under comparable conditions. These observations permit a crude division of the five tryptophans of α-lactalbumin into two classes: (1) three groups which are completley "buried," and (2) two groups whose accessibility depends upon *p*H and temperature.

The three groups of the first class remain "buried" even below *p*H 3 where changes in the frictional ratio (paper II) and in the electrostatic factor, *w*, obtained from the titration curve (F. M. Robbins, R. E. Andreotti, and M. J. Kronman, work in progress) indicate that the α-lactalbumin molecule exists in a more swollen state. Full exposure of these three groups (relative to model compounds) could be effected only by denaturation in 8 M urea and reduction of the four disulfide bridges (Table I). Sufficient structure is maintained by the S—S bridges in the presence of 8 M urea to continue to insulate the tryptophan residues to some degree from perturbants as small as ethylene glycol. The apparent number of groups "exposed" (column 6, Table I) could be owing either to four completely "exposed" groups, or to two completely "exposed" groups (columns 4 and 7, Table II) and three partially "exposed" groups. Similar observations have been reported by Herskovits and Laskowski (1960, 1962a,b; personal communications from Dr. T. T. Herskovits) for a variety of tyrosine- and tryptophan-containing proteins.

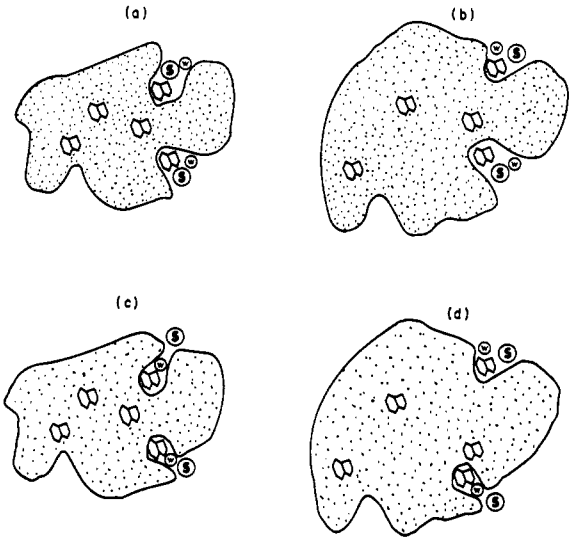

FIGURE 5: Schematic representation of the "exposure" of tryptophan residues (⌂) in α-lactalbumin. (a) *p*H 6, 25°, "exposed" groups freely accessible to sucrose (Ⓢ) and water (⊙) molecules. (b) *p*H 1.8–3, 25°, swollen protein molecule with two groups still "exposed." (c) *p*H 6, 1°, both groups accessible to water molecules but not to sucrose. (d) *p*H 1.8–3, 1°, swollen molecule with one group fully "exposed" and one group accessible only to water molecules.

Exposed Tryptophans of α-Lactalbumin. Our observations that two out of five tryptophans are "exposed" at 25° (Table II) is in remarkable agreement with studies of enzymatic degradation of α-lactalbumin (Weil *et al.*, 1959). They found that treatment of the protein with chymotrypsin followed by addition of carboxypeptidase A (protaminase) liberated only 2 moles of tryptophan per mole of protein. While the failure to release the other three tryptophan groups might be ascribed to a variety of phenomena, in light of our observations the most reasonable explanation is that these groups are sterically inaccessible to chymotrypsin.

Herskovits and Laskowski have demonstrated that for ovamucoid (1962b) and serum albumin (1962a) the use of perturbants of different size allows one to estimate "crevice" size. According to this view, chromophores which are partially "buried" are located near the surface of the molecule in channels or "crevices" which are penetrable to solvent to a lesser or greater degree, depending upon the surface geometry in that region.

The relative constancy of groups exposed at 25° with respect to sucrose, ethylene glycol, glycerol, and D_2O (Table II) suggests that the two "exposed" groups are *fully* "exposed" to the medium or lie in "crevices" having as a critical dimension a distance greater than 4.7 A, the effective radius of the sucrose molecule. This type of model applied to α-lactalbumin is depicted in schematic form in Figure 5. The "exposed" groups at

25° (Figure 5a,b) are either completely accessible or lie in "crevices" having a critical distance greater than 4.7 A (the effective radius of the sucrose molecule, column 1, Table II). Indeed, the accessibility of these two groups to chymotryptic attack (Weil *et al.*, 1959) suggests a much larger value than 4.7 A.

"*Crevice*" *Size at Low Temperature.* At 1° near *p*H 6 neither of the two groups "seen" at 25° is "exposed" with respect to sucrose or glycerol but both are fully accessible to D_2O (Table III). As illustrated in Figure 5, this implies that the "crevice" housing these two groups has become so reduced in size as to exclude the larger perturbants. It would appear that the "crevice" size must lie between 1 A (radius of D_2O molecule) and 2.7 A (radius of glycerol molecule).

This contraction of the "crevice" with decrease in temperature appears to be less complete in the swollen denatured molecule (*p*H 1.8–3), where a single group is exposed to sucrose and glycerol but a pair is seen by D_2O (Table III). This appears to be the result of partial "contraction" of both crevices or to more drastic closure of one of them. We have depicted the latter case in Figure 5d.

Additional evidence for this subtle conformational change at *p*H 6 is seen in the low-wavelength shift of the tryptophan fluorescence maximum as the temperature is lowered (M. J. Kronman, manuscript in preparation). The relationship between "crevice contraction," absorption, and emission spectra of tryptophan in α-lactalbumin will be considered in a later publication.

It is apparent that the "burying" of groups at *p*H 6 with decrease in temperature is the result of a conformational change involving only limited regions of the α-lactalbumin molecule. Both the rotatory dispersion parameters (M. J. Kronman and R. Blum, unpublished experiments) and the frictional ratio (paper I) remain unchanged as the temperature is lowered. This was also true of the rotatory dispersion parameters at *p*H 2. Unfortunately, the presence of association and aggregation reactions (papers I and II) precludes an unambiguous statement about the effect of temperature on the hydrodynamic properties of the protein at low *p*H.

*The Low-p*H *Denaturation.* The observations made in this study demonstrate that the denaturation of α-lactalbumin occurring below *p*H 4 does not involve enhanced exposure of tryptophan groups. Indeed, as the heavy water experiments indicate (Tables II and III), the molecular alteration does not even involve an increased permeability of the protein molecule to water. Thus, the large blue shift observed in acid difference spectra must have other origins (see paper III). It might be expected that at low *p*H the high molecular charge would tend to drastically disrupt the structure. In spite of the fact that the denatured molecule at *p*H 2 is more swollen or elongated than the native protein (paper II), sufficient structural integrity is maintained on denaturation to continue the insulation of some of the tryptophans from the medium. The resistance of α-lactalbumin to gross molecular disruption at acid *p*H values may well be the reflection of a more general property of the molecule. We have previously reported (papers II and III) that α-lactalbumin, isolated by the procedure of Aschaffenburg and Drewry (1957), which involves precipitation of the protein at *p*H 2.0 with concentrated HCl in the presence of 2.1 M sodium sulfate, is indistinguishable with respect to aggregation-association properties from that obtained under milder conditions. Wetlaufer (1961) likewise has noted the high resistance of α-lactalbumin to thermal denaturation. The molecular basis for this high stability of α-lactalbumin is at present far from clear and will certainly require more detailed information about amino acid sequence and conformation.

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