Amino Group Environments and Metal Binding Properties of Carbon-13 Reductively Methylated Bovine α-Lactalbumin†

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ABSTRACT: ^{13}C NMR spectroscopy has been used to study the amino group environments and metal binding properties of ^{13}C reductively methylated bovine α-lactalbumin. Bovine α-lactalbumin is a Ca^{2+} metalloprotein containing 12 lysyl amino groups and a free amino terminus. All 13 amino groups can be ^{13}C-dimethylated without altering Ca^{2+} binding or biological activity. pH titrations (chemical shift vs. pH) of this dimethylated protein reveal unique behavior for each of the 13 amino groups. The pK_a values for the lysyl amino groups range from 9.1 to 10.8 while the pK_a for the N-terminal amino group is 8.3. This relatively high pK_a (by 1 pH unit) for the N-terminal supports its interaction in an ion pair as proposed by Warme et al. [Warme, P. K., Momany, F. A., Rumball, S. V., Tuttle, R. W., & Scheraga, H. A. (1974) *Biochemistry 13*, 768–782]. Carbon-13 NMR studies further show that the removal of Ca^{2+} from the high-affinity binding site results in a conformational change, with the disruption of the N-terminal ion pair interaction (pK_a decreased to 7.4). The study of Zn^{2+} binding to Ca^{2+}-saturated protein suggests that Zn^{2+} binds initially at a low-affinity Ca^{2+} site while maintaining the N-terminal ion pair interaction. The further addition of Zn^{2+} leads to the disruption of this ion pair forming a presumed apoprotein-like conformation. Finally on the basis of the specific effects of added Mn^{2+} on the ^{13}C NMR spectra of the methylated protein, a low-affinity divalent metal binding site is proposed about 7.5 Å from the amino terminus.

α-Lactalbumin is a major protein component of mammalian milk and by itself has no known catalytic activity. Upon its binding to galactosyltransferase, α-lactalbumin modifies the galactosyltransferase specificity toward substrate, forming the lactose synthetase enzyme system. This two-component enzyme complex catalyzes the biosynthesis of lactose from UDP–D-galactose and D-glucose in the lactating mammary gland. The α-lactalbumins are globular proteins having similar molecular weights to and sequence homologies with the avian- and mammalian-type lysozymes. Hence, both α-lactalbumin and lysozyme are thought to have similar conformations, although no X-ray crystal structure of α-lactalbumin has been reported. Structural models, however, have been proposed on the basis of the lysozyme structure (Browne et al., 1969) and further refined by using energy minimization (Warme et al., 1974).

Recently it has been reported that native bovine α-lactalbumin is a calcium metalloprotein. It contains at least one tightly bound calcium ion, whose removal decreases the stability of the protein toward heat and denaturants (Hiraoka et al., 1980). The binding of calcium to the apoprotein is thought to elicit a conformational change which can be followed by spectral changes in UV and fluorescence spectroscopy (Kronman et al., 1981; Permyakov et al., 1981a,b; Murakami et al., 1982). It has been observed that zinc ion will readily displace the tightly bound calcium ion (possibly binding at a different site), producing fluorescence spectra that are similar to the apoprotein, while manganese and lanthanide ions will bind at the Ca^{2+} site giving spectra identical with that of the native, calcium containing protein (Kronman et al., 1981; Murakami et al., 1982; Murakami & Berliner, 1983). Primarily due to the lack of an X-ray crystal structure for α-lactalbumin, the actual locations of the metal binding sites are not known. In order to detail the solution structure of and divalent metal binding to α-lactalbumin, we have used ^{13}C-labeled amino group methylation and ^{13}C NMR spectroscopy to examine the amino group environments and interactions in bovine α-lactalbumin. The results of the work presented here strongly suggest that the intramolecular ion pair interaction proposed by Warme et al. (1974), involving the NH_2-terminal amino group, exists in native Ca^{2+}-containing α-lactalbumin and that this group is highly sensitive to the removal of Ca^{2+} and to the addition of Zn^{2+} and Mn^{2+}.

Experimental Procedures

Materials. Bovine α-lactalbumin (L-4379, lot no. 50F-8105), galactosyltransferase (G-5507, lot no. 120F-8050), UDP-galactose, and Tris[1] buffer were obtained from Sigma Chemical Co. UDP–[^{14}C]galactose (302 mCi/mmol) was purchased from New England Nuclear. Gold label, 99.999% $MnCl_2 \cdot 4H_2O$ was obtained from Aldrich Chemical Co. as was 99.999% $LaCl_3 \cdot 7H_2O$ and $NaCNBH_3$. Ultrapure $CaCl_2$ and $ZnCl_2$ were obtained from Alpha Division of Ventron Corp. [^{13}C]Formaldehyde (91% enriched) was obtained from Prochem, HEPES buffer from Research Organics, Inc., and MOPS buffer from Bio-Rad Laboratories. Solutions of α-lactalbumin were dialyzed by using Spectrapor membrane tubing 1 with a molecular weight exclusion limit of 6000–8000.

Protein Purification. Initial studies began with α-lactalbumin as supplied, ca. 90% pure. For subsequent studies, in particular the metal binding studies, the protein was further purified by chromatography on DEAE-Sephadex (2.5 × 29 cm column eluted with a 0–0.3 M NaCl gradient in 5 mM $CaCl_2$ and 20 mM Tris, pH 7.8). The major protein peak was pooled, exhaustively dialyzed against distilled water, lyophilized, and stored at −20 °C prior to reductive methylation.

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[1] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; Me_4Si (TMS in figures), tetramethylsilane; T_1, spin–lattice relaxation time; T_2, spin–spin relaxation time; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

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Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the purified protein showed nearly complete removal of the original contaminating proteins.

Methylation. The reductive methylation procedure using ^{13}C-enriched formaldehyde has been described by Jentoft & Dearborn (1979). A typical α-lactalbumin methylation mixture contained 5–10 mg of protein/mL (0.3–0.7 mM), ca. 50 mM HEPES buffer at pH 7.5, and 10 mM $NaCNBH_3$ [repurified by the method of Jentoft & Dearborn (1979)] to which the desired amount of 1 M [^{13}C]formaldehyde was subsequently added. The reaction mixtures were allowed to stand overnight at 4 °C, dialyzed against 0.05–0.1 M NaCl, followed by exhaustive dialysis against distilled water, lyophilized, and stored at −20 °C. Later methylations also included a dialysis against 1–5 mM $CaCl_2$ followed by a less extensive dialysis against distilled water. The above procedures were repeated 2–3 times with 4–5-fold excess (per amino group) [^{13}C]formaldehyde to obtain fully dimethylated protein, as determined by ^{13}C NMR [see Gerken et al. (1982)]. An extinction coefficient $E_{280nm} = 2.01$ (mg/mL)$^{-1}$ cm^{-1} (20 mM Tris, pH 7.0, and 10 mM $CaCl_2$) was used to determine both native α-lactalbumin and methylated α-lactalbumin concentrations (Kronman & Andreotti 1964; Kronman et al., 1981).

Apomethylated α-lactalbumin was prepared by dialyzing protein (~1 mM, 15 mL) against ~300 mL of 20 mM EGTA and ~20 g of Chelex ion-exchange resin followed by an exhaustive dialysis (6 days, several changes per day) against deionized distilled water in the presence of Chelex. All dialyses were performed in plastic containers. No Ca^{2+}-bound protein was detected after this treatment on the basis of its ^{13}C NMR spectrum.

Metal Analysis. The calcium content of stock solutions of α-lactalbumin was determined by flame spectrophotometry using a Perkin-Elmer Model 360 atomic absorption/flame spectrophotometer. Protein samples contained 0.5% $LaCl_3$ and 0.5 or 3 N HCl and were heated (~100 °C) in sealed tubes for 0.5–6 h to fully remove bound calcium. Flame spectroscopy was also used for standardizing stock calcium and zinc solutions. Manganese chloride solutions were made fresh by the accurate weighing of previously analyzed crystalline $MnCl_2 \cdot 4H_2O$ that had been stored in a desiccator. Electron paramagnetic resonance spectroscopy (EPR) on a Varian E-112 spectrometer was used to obtain the fraction of Mn^{2+} bound to α-lactalbumin. Microliter aliquots of 1.0 mM $MnCl_2$ were added to a stock protein solution and to an identical solution lacking protein, and the EPR spectra of both solutions were obtained. The differences in the resonance intensities between the two samples were taken as the amount of manganese bound to the protein. Concentrations of manganese on the order of 1 μM could readily be determined while total manganese concentration ranged from 5 to 50 μM in these binding experiments.

Enzymatic Activity. The ability of native and methylated α-lactalbumin to bind to galactosyltransferase and form the lactose synthetase enzyme complex was assayed by following the incorporation of UDP–[^{14}C]galactose into the product, lactose. The procedure of Brodbeck & Ebner (1966) as modified by Fitzgerald et al. (1970) was used. The assay contained 3 mM $MnCl_2$, 50 mM MOPS buffer, pH 8, 200 mM glucose, 4 mM UDP–[^{14}C]galactose (5×10^3 dpm/nmol), and either native or fully methylated α-lactalbumin to a total volume of 40 μL. The assay was initiated with the addition of 10 μL (6 μg) of galactosyltransferase and after various times stopped in boiling water. The product, [^{14}C]lactose, was separated from unreacted UDP–[^{14}C]galactose on small Dowex 1-X8 columns (formate form) and counted in a liquid scintillation counter (Tracor Mark III). After correcting for the initial concentrations of α-lactalbumin and for blanks with no added α-lactalbumin, it was found that exhaustively methylated α-lactalbumin had greater than 95% activity compared to that of the unmethylated protein.

NMR Spectroscopic Methods. All spectra were recorded on a Bruker WH 180/270 Fourier transform NMR spectrometer with a Nicolet 1180 computer. Most proton-decoupled carbon-13 spectra were obtained with a 10-mm diameter probe operating at 67.9 MHz. Additional spectra were obtained with a 20-mm probe operating at 45.3 MHz while using 10-mm diameter sample tubes. Sample overheating was prevented by reducing the decoupler power to 0.5 W for 1.5 s between data acquisitions of 0.7 s at a decoupler power of 2–3 W. Sample temperatures under these conditions were typically 21–25 °C. Most spectra were obtained with 3-kHz sweep widths and Fourier transformed with zero filling on 32K of memory (usually no line broadening applied). The resulting spectra had a resolution of 0.18 Hz per point.

Samples for NMR contained 10–50 mg of methylated α-lactalbumin in 2–2.5 mL of solution (0.3–1.4 mM) containing 0.05 or 0.1 M KCl, 20% D_2O, and ca. 2% methanol. Methanol was used as a secondary internal chemical shift reference taken as 49.40 ppm from tetramethylsilane.

Hydrogen ion titrations were obtained by adjusting the pH of the NMR samples with microliter amounts of 0.5 or 0.05 M KOH and 0.5 or 0.05 M HCl, pH values being obtained prior to, and directly after, acquiring spectra. The pH-dependent ^{13}C NMR chemical shifts of methylated α-lactalbumin were fit to theoretical pH curves (Dwek, 1973). pK_a values were determined to the nearest 0.01 pH unit by minimizing the errors between the experimental points and calculated theoretical curves.

Spin–lattice relaxation time data were obtained by the fast inversion recovery method of Canet et al. (1975). For each determination seven to nine different delay times were used ranging from 1.5 to 0.001 s. Relaxation times were calculated from the data by using the three-parameter least-squares method of Kovalewski et al. (1977). The errors in T_1 values range from 1 to 10%. Spin–spin relaxation times, T_2, were estimated by using the half-height line width, $\nu_{1/2}$, using $T_2 = 1/(\pi\nu_{1/2})$ after correcting for the applied line-broadening factor.

Results

The exhaustive methylation of a protein with [^{13}C]formaldehyde and $NaCNBH_3$ results in the specific ^{13}C-dimethylation of all unblocked lysyl and NH_2-terminal amino groups (Jentoft & Dearborn, 1979, 1983). Carbon-13 studies of these enriched methyl groups can provide detailed information of each amino group's environment and ionization state (Jentoft et al., 1979, 1981; Gerken et al., 1982). This is possible because the added methyl groups are relatively small, the charge on the amine is maintained, and the pK_a values of the dimethylated amines are very similar to the pK_a values of the unmodified amines (Jentoft & Dearborn, 1983). By use of this probe, the detailed titration behavior of the individual amino groups in hen egg white lysozyme has been studied, thus confirming the existence in solution of the ion pair interactions proposed by X-ray crystallographers (Gerken et al., 1982). This probe has also been used to study the metal binding of ^{13}C-methylated concanavalin A (Sherry & Teherani, 1983).

Bovine α-lactalbumin has one NH_2-terminal and 12 lysyl amino groups. Thus, one would ideally expect to observe as many as 13 dimethylamino group resonances in the ^{13}C NMR

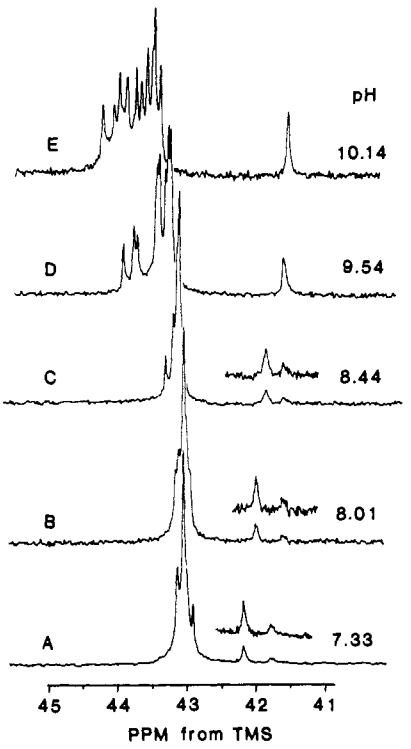

FIGURE 1: Carbon-13 NMR spectra as a function of pH for fully ^{13}C-dimethylated bovine α-lactalbumin (predominantly Ca^{2+}-bound protein). Approximately 1.2 mM ^{13}C-methylated α-lactalbumin (exhaustively dialyzed against distilled water) in 0.05 M KCl. (A) pH 7.33 (1472 scans), (B) pH 8.01 (500 scans), (C) pH 8.44 (5.08 scans), (D) pH 954 (1088 scans), and (E) pH 10.14 (548 scans).

spectra of the exhaustively methylated protein, providing these amino groups are in unique environments. The carbon-13 NMR spectra of fully methylated α-lactalbumin at various pH values are shown in Figure 1, from which one can determine that nearly all of the amino groups have unique titration behavior (Figures 2 and 3). Previous ^{13}C NMR studies of reductively methylated proteins and model peptides have identified characteristic titration behavior for both dimethylated lysyl and N-terminal amino group resonances (Jentoft et al., 1979, 1981; Gerken et al., 1982; Jentoft & Dearborn, 1983). Thus, on the basis of the pH dependence of the chemical shifts of fully dimethylated α-lactalbumin, the group of resonances that shift to lower field (higher ppm) with increased pH are assigned to dimethylated lysyl amino groups (see Figure 2) while the single resonance that shifts to higher field (lower ppm) is assigned to the dimethylated N-terminal amino group (see Figure 3). The differences in spin–spin relaxation time, T_1, between these groups of resonances (0.6–0.8 s for those assigned to lysyl amino groups and 0.31 s for the N-terminal amino group, at pH 8) further support their assignments [see Gerken et al. (1982)]. Carbon-13 NMR spectra confirm that all of the amino groups are fully dimethylated since no monomethyl resonances are observed in the 30–35 ppm region and because the relative areas between the lysyl and N-terminal amino group regions are found to be 12 to 1 (at pH $\geq$10). The titration curves for 11 of the 12 lysyl residues are shown in Figure 2. These curves were completed by titrating the fully methylated protein and several partially methylated preparations which contained reduced (or even lacking) dimethylamino group resonances. The pK_a values for these titrations are typical for lysyl residues as are the chemical shift changes that occur upon deprotonation, both of which are listed in Table I [see Jentoft & Dearborn (1983)]. As previously observed in ^{13}C-methylated lysozyme many of the dimethylamino group resonances broaden and disappear at lower pH values presumably due to an increase in the lifetime of the protonated amine and subsequent methyl group nonequivalence [see Gerken et al. (1982)].

NH_2-Terminal Amino Group. The pH titration of the major dimethylated N-terminal resonance is shown in Figure 3. Above pH 6.5 the data can be fit to a pK_a of 8.33 with

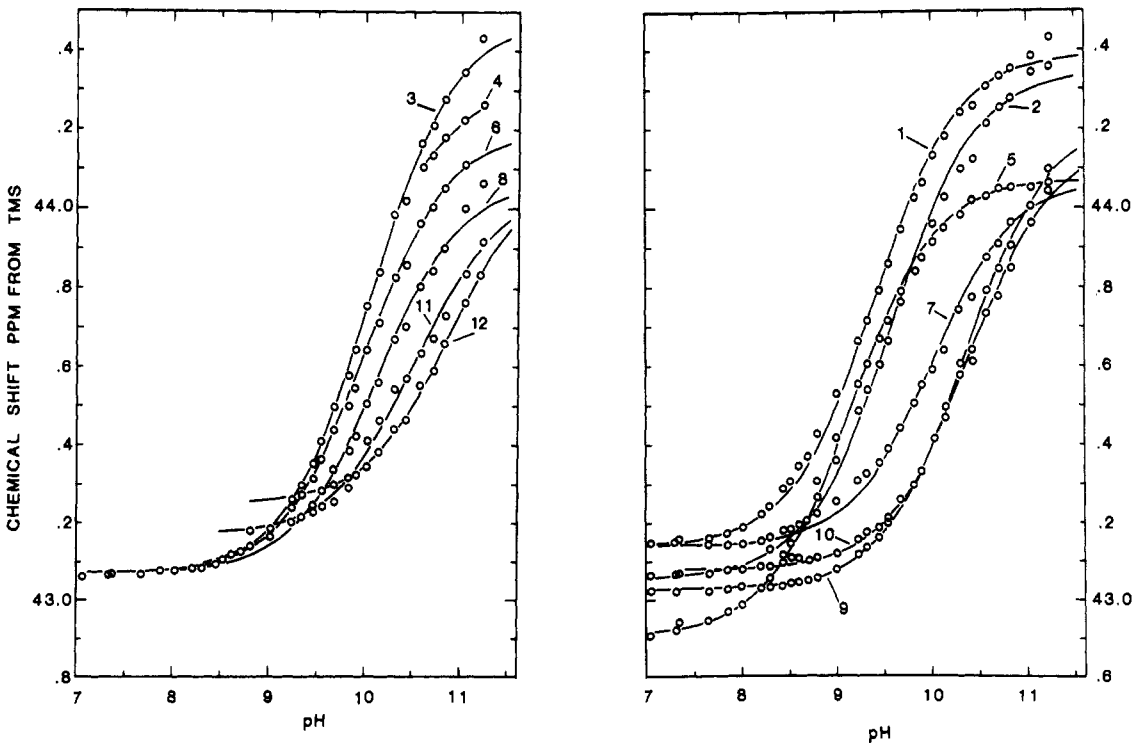

FIGURE 2: Plots of the ^{13}C NMR chemical shifts as a function of pH for the dimethyllysyl amino groups of fully ^{13}C-methylated α-lactalbumin (Ca^{2+}-bound protein). (Left panel) Resonances 3, 4, 6, 8, 11, and 12; (right panel) resonances 1, 2, 5, 7, 9, and 10. Data obtained from 0.5 to 1.3 mM protein in 0.05 or 0.1 M KCl. Solid lines through points (except resonance 4) are theoretical curves calculated from the pK_a values and chemical shift limits listed in Table I.

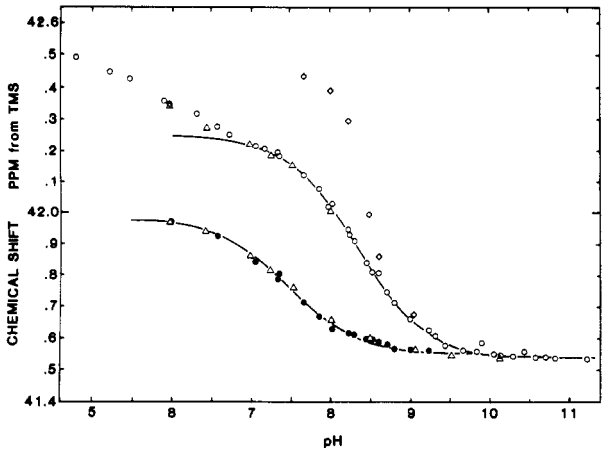

FIGURE 3: Plots of the ^{13}C NMR chemical shifts as a function of pH for the dimethyl N-terminal amino groups of fully ^{13}C-dimethylated α-lactalbumin. Circles (○) are data from the major dimethyl N-terminal resonance from 0.5 to 1.3 mM protein containing 0.05 or 0.1 M KCl. The solid curve through these points (above pH 6.5) is a theoretical curve with a pK_a of 8.33. Closed circles (●) are data for the minor resonance (when observed) from the above protein samples. Triangles (△) represent both dimethyl N-terminal resonances that are observed from ^{13}C-dimethylated protein (0.49 or 1.3 mM) in the presence of 1–10 mM EGTA and 0.1 M KCl (see Figure 4). The solid curve for the highest field resonance is a theoretical curve having a pK of 7.45. Diamonds (◇) represent data for 1.2 mM Ca^{2+} protein in the presence of 4.5 mM $ZnCl_2$.

Table I: ^{13}C NMR pH Titration Properties of the Dimethylated Amino Groups in ^{13}C-Methylated Bovine α-Lactalbumin[a]

resonance[b]	pK_a	δ(low pH) (ppm)[c]	Δ (ppm)[d]
1	9.40	43.14	1.25
2	9.58	43.06	1.28
3	10.05	43.07	1.41
4	ND[f]	ND	ND
5	9.15	42.91	1.16
6	10.00	43.07	1.13
7	10.00	43.14	0.93
8	10.12	43.07	1.00
9	10.36	43.03	1.19
10	10.40	43.08	1.08
11	10.52	43.17	0.88
12	10.88	43.25	0.86
13 (native)	8.33	42.25	−0.71
13 (apo)[e]	7.45	41.98	−0.43

[a] Data obtained from 0.5 to 1.3 mM protein in 0.05 or 0.10 M KCl. [b] Resonances identified in Figures 2 and 3. [c] Low pH chemical shift limit. [d] Chemical shift difference between nonprotonated and protonated ionization states; positive values indicate downfield shifts. [e] Data obtained from protein in the presence of EGTA; see Figure 3. [f] ND, titration parameters not determined; see Figure 2.

chemical shift limits in the range observed for dimethyl N-terminal amino groups (Gerken et al., 1982). However, below pH 6.5 and down to the isoelectric point of the protein (ca. pH 4.5) this resonance shows a nearly linear increase in chemical shift with decreasing pH. The pH titration of a minor dimethyl N-terminal resonance that can be observed in Figure 1 is also shown in Figure 3. This resonance has a pK_a value of 7.45 and titration limits typical of unperturbed dimethyl N-terminal amino groups (Gerken et al., 1982). The origins of these two resonances became clear when a more highly purified and completely Ca^{2+}-bound methylated α-lactalbumin was titrated with the calcium chelator EGTA as shown in Figure 4. The addition of EGTA causes the decrease of the major dimethyl N-terminal resonance while causing an increase in the area of the minor resonance. This process is reversible, since either the addition of Ca^{2+} or the lowering of the pH (decreasing the calcium association constant for EGTA) caused the lowest field resonance to increase in intensity at the expense of the high field resonance (data not shown). Thus, the low field resonance, pK_a = 8.3, is assigned to the dimethyl N-terminal amino group in calcium-bound "native" protein, while the high field resonance, pK_a = 7.4, is assigned to the N-terminal resonance in the apoprotein. On an electrostatic basis alone, the differences between pK_a values of the N-terminal amino groups in the native proteins and apoproteins are not in the direction expected for an amino group liganded to Ca^{2+} or another cation. The changes in the N-terminal residue are therefore most likely the result of an interaction that is coupled to the binding of calcium ion. The formation of an ion pair with a carboxyl group is one possible interaction that could easily explain the increased pK_a value. Additional changes are also observed in the dimethyl lysyl amino groups as calcium is removed by EGTA, although the changes are not as dramatic as for the N-terminal resonances, the most apparent changes being the merging of the lysyl resonances at ~43 ppm and the appearance of a broad resonance at 42.75 ppm as shown in Figure 4. These findings further suggest that the removal of calcium elicits a global conformational change.

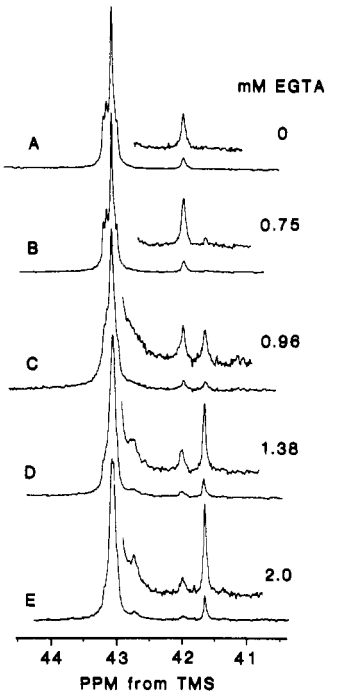

FIGURE 4: Effect of EGTA addition on the ^{13}C NMR spectra of ^{13}C-dimethylated α-lactalbumin. Protein, 0.49 mM (further purified as described under Experimental Procedures), 0.10 M KCl, and *total* calcium concentration of 1.03 mM. (A) No EGTA added, pH 8.03 (1348 scans), (B) 0.75 mM EGTA, pH 8.09 (1580 scans), (C) 0.96 mM EGTA, pH 8.01 (1296 scans), (D) 1.38 mM EGTA, pH 8.03 (1672 scans), and (E) 2.0 mM EGTA, pH 8.01 (1672 scans). Hydrogen ion concentrations were adjusted after each addition of EGTA as described under Experimental Procedures.

Since separate N-terminal resonances are observed for the apoprotein and calcium-bound protein, it is evident that the exchange of Ca^{2+} and/or the accompanying conformational change that occurs upon binding Ca^{2+} is slow (≪20/s based on the frequency difference). From the relative areas of both N-terminal resonances, the mole fraction of Ca^{2+}-bound protein and apoprotein can be obtained as a function of EGTA and used to estimate a single high-affinity calcium binding constant of $\sim 6 \times 10^7$ M^{-1} (Figure 5). This fit is not altered when the molar excess of calcium in the sample is considered to be either free in solution or bound to the protein at a second and weaker binding site that exhibits no spectral changes upon calcium removal. Further confirmation that the changes in

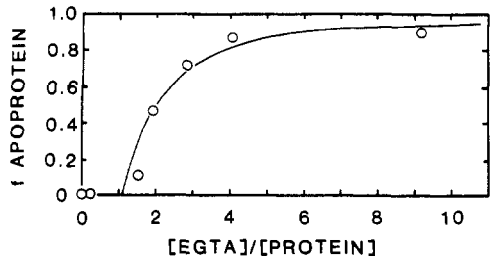

FIGURE 5: Analysis of the EGTA titration of ^{13}C-dimethylated α-lactalbumin at pH 8. The fractions of native (calcium-bound) protein and apoprotein were determined from the relative areas of the low- and high-field ^{13}C-dimethyl N-terminal resonances, respectively (see Figure 3). The solid curve is a theoretical curve calculated by using a calcium binding constant for α-lactalbumin of 6×10^7 M^{-1} with the following experimental conditions: 0.49 mM protein and 1.03 mM total calcium. A calcium binding constant of 2.5×10^8 M^{-1} was used for EGTA at pH 8 (Schwarzenbach & Flaschka, 1969).

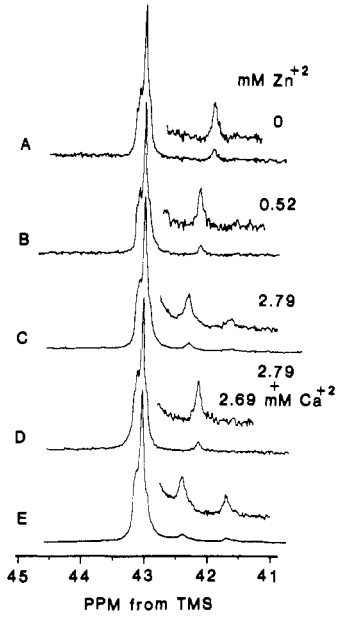

FIGURE 6: Effects of $ZnCl_2$ addition on the ^{13}C NMR spectra of ^{13}C-dimethylated α-lactalbumin. Protein, 0.49 mM (further purified as described under Experimental Procedures), 0.10 M KCl, and initial total calcium concentration of 1.03 mM. (A) Native, no Zn^{2+} added, pH 8.03 (256 scans), (B) 0.52 mM Zn^{2+}, pH 7.99 (286 scans), (C) 2.79 mM Zn^{2+}, pH 7.94 (1860 scans), (D) sample C, 2.79 mM Zn^{2+}, plus 2.69 mM total calcium, pH 8.00 (776 scans), and (E) 1 mM protein (purified), 1.4 mM Ca^{2+}, and 1.9 mM Zn^{2+}, pH 7.8 (1348 scans). Hydrogen ion concentrations were adjusted after each addition of Zn^{2+} as described under Experimental Procedures.

the N-terminal resonance are due to a single Ca^{2+} binding to the high-affinity binding site arise from our Ca^{2+} titration of the apoprotein. At a 1:1 mole ratio of Ca^{2+} to apoprotein the apoprotein N-terminal resonance was nearly gone and the native Ca^{2+}-bound resonance was at full intensity (data not shown). Similar findings have been reported for the fluorescence, UV, and CD studies of unmethylated bovine α-lactalbumin, from which it was determined that the binding of one Ca^{2+} to a high-affinity binding site produced all of the observed spectral changes (Hiraoka et al., 1980; Kronman et al., 1981; Permyakov et al., 1981a; Murakami et al., 1982). In these studies high-affinity Ca^{2+} binding constants of 2.7 $\times 10^6$ M^{-1} (Kronman et al., 1981), (3–6) $\times 10^8$ M^{-1} (Permyakov et al., 1981a), and 4×10^9 M^{-1} (Murakami et al., 1982) were reported. Recently it has been suggested that the presence of EDTA (or EGTA) can modify the equilibrium between the apo and native bovine α-lactalbumin conformations (Kronman & Bratcher, 1983). These workers concluded

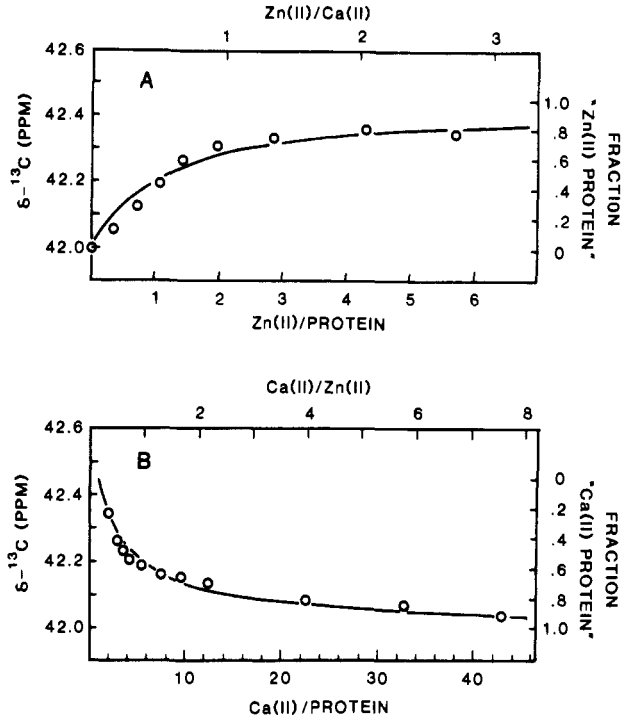

FIGURE 7: Analysis of the Zn^{2+} and Ca^{2+} titrations of ^{13}C-dimethylated α-lactalbumin at pH 8. The fractions of "Zn^{2+}-bound" and "Ca^{2+}-bound" protein were determined from the chemical shift of the dimethyl N-terminal amino group by using 42.00 and 42.45 ppm, respectively, as the Ca^{2+}- and Zn^{2+}-bound protein chemical shift limits (see Figure 6). Protein, 0.49 mM (further purified as described under Experimental Procedures), 0.10 M KCl, and initial total Ca^{2+} concentration of 1.03 mM. (A) The addition of Zn^{2+} to Ca^{2+}-bound protein; total $[Ca^{2+}] = 1.03$ mM. The solid curve is plotted by using an equilibrium constant, $K_{Zn/Ca}$, of 0.8 and assuming a second Ca^{2+} is bound to protein which does not exchange with Zn^{2+}. (B) The addition of Ca^{2+} to the Zn^{2+}-bound protein; total $[Zn^{2+}] = 2.79$ mM [last point from (A) above]. The solid curve is plotted by using an equilibrium constant, $K_{Zn/Ca}$, of 0.6 and assuming a second Ca^{2+} is bound to protein which does not exchange with Zn^{2+}. Hydrogen ion concentrations were adjusted after each addition of divalent metal ion as described under Experimental Procedures.

that this will result in an increase in the apparent Ca^{2+} binding constant obtained in the presence of EDTA and therefore may be the source of the wide range of the above reported K_a values.

Zinc Addition. Carbon-13 NMR spectra showing the effects of the addition of zinc chloride to Ca^{2+} saturated ^{13}C-methylated α-lactalbumin are shown in Figure 6. At constant pH, the addition of Zn^{2+} caused the resonance of the dimethyl N-terminal amino group to shift to lower field (Figure 6A–C), while at high Zn^{2+} values a peak for the apo N-terminal appears. The apoprotein resonance further increased as the relative Zn^{2+} concentration increased (see Figure 6E). The addition of Ca^{2+} reversed this shift and reduced the apo N-terminal resonance (Figure 6D). Since the initial addition of Zn^{2+} primarily shifts the N-terminal resonance, the initial binding of zinc to the protein is in fast exchange with Ca^{2+} while the formation of the "apoprotein" at higher Zn^{2+} concentrations is in slow exchange. The addition of Zn^{2+} to prepared apoprotein produced no readily observable change in either the dimethyl lysyl or N-terminal resonances at pH 7–8. More complete data for the rapid Zn^{2+} to Ca^{2+} and Ca^{2+} to Zn^{2+} exchanges are shown in parts A and B of Figure 7, respectively. The theoretical curves were obtained by using the equilibrium protein–Ca^{2+} + Zn^{2+} $\rightleftharpoons$ protein–Zn^{2+} + Ca^{2+}, with $K_{Zn/Ca}$ representing the equilibrium constant in the forward direction. The value of $K_{Nn/Ca}$ represents the ratio of the Zn^{2+} to Ca^{2+} association constants for dimethylated α-

lactalbumin. Best fits to the data at pH 8 were obtained by using 42.00 and 42.45 ppm for the chemical shift limits of the presumed Ca^{2+} and Zn^{2+} "bound" proteins, respectively. The mole fractions (f) of Zn^{2+}- or Ca^{2+}-bound protein were estimated from the chemical shift of the dimethylated N-terminal resonance: $f_{Ca} = (42.42 - \delta_{obsd})/(42.45 - 42.00)$ and $f_{Zn} = 1 - f_{Ca}$. From these data $K_{Zn/Ca}$ is approximately 0.7; hence, the Zn^{2+} binding constant is similar to the binding constant of the Ca^{2+} with which it exchanges. The best agreements between equilibrium constants (calculated separately for Figure 7) were obtained when a second Ca^{2+} binding site was assumed which did not participate in the Zn^{2+} exchange, thus effectively lowering the concentration of free calcium that is able to participate in the exchange process.[2] This accounts for the differential chemical shift values of the N-terminal amino group in Figure 7 at equivalent Zn^{2+} to Ca^{2+} ratios. Preliminary studies of the Zn^{2+} exchange at lower ratios of Ca^{2+} to protein (i.e., 2:1 to 1:1) appear to give best fits to the above equilibrium constant when 1 mol of Ca^{2+} remained inaccessible to Zn^{2+} exchange for each mole of protein. Thus, it appears the tightly bound Ca^{2+} may not be in rapid exchange with Zn^{2+}; rather a separate low-affinity Ca^{2+} site may be in fast exchange with Zn^{2+}. Hiraoka et al. (1980) have suggested the presence of additional weaker Ca^{2+} binding sites, and Kronman et al. (1981) have obtained a binding constant of 3.1×10^4 M^{-1} for a weak Ca^{2+} binding site in native bovine α-lactalbumin. Calcium binding to this second site did not produce observable fluorescence changes (Kronman et al., 1981). Using the competition between Zn^{2+} and Mn^{2+} (at a 1:1 Mn^{2+}:protein ratio), Murakami & Berliner (1983) report a binding constant for Zn^{2+} of 1.8×10^5 M^{-1}. These workers suggest the Zn^{2+} binding site is removed from the high affinity Ca^{2+}/Mn^{2+} site and that Zn^{2+} binding and Ca^{2+} (or Mn^{2+}) binding are mutually exclusive (see below).

The pH titration of the N-terminal amino group of fully dimethylated α-lactalbumin in the presence of 4-fold excess of Zn^{2+} is shown by the diamonds in Figure 3. The apparent pK_a of this titration (~8.5) is similar to the pK_a of the native Ca^{2+}-bound protein (pK_a = 8.3), although the data do not fit to a simple theoretical protonation–deprotonation titration curve. This lack of fit may be the result of a pH dependence on the binding of Zn^{2+} to the protein, perhaps due to the formation of zinc hydroxide, which has a pK_a of ~8.8 (Basolo & Pearson, 1967). Interestingly the apparent low pH chemical shift limit of the initial Zn^{2+}-bound protein may be similar to the chemical shift limit of the native Ca^{2+}-bound protein near its isoelectric point (pH ~4.5) (see Figure 3). The initial binding of Zn^{2+} may therefore promote a conformation that may be similar to that of the native protein at low pH. Indeed our above results suggest the high-affinity Ca^{2+} remains bound at this stage of Zn^{2+} addition. The appearance of a separate apo N-terminal resonance with increased Zn^{2+} concentration suggests the initially formed Zn^{2+}-bound conformation may be in slow exchange with an apoprotein-like conformation. An alternate explanation that presently has not been ruled out is the existence of a second Zn^{2+} site that upon binding zinc produces an apoprotein-like conformation. This site could be associated with the high-affinity Ca^{2+} site.

Manganese Addition. The use of the paramagnetic manganese ion as an NMR probe is well established for the study of metal binding sites in proteins (Dwek, 1973; Mildvan et al., 1980). When small amounts of $MnCl_2$ are added to the

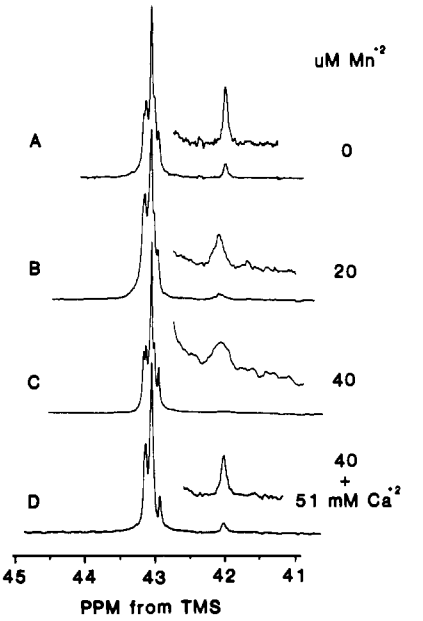

FIGURE 8: Effect of $MnCl_2$ addition on the ^{13}C NMR spectra of ^{13}C-dimethylated α-lactalbumin. Protein, 0.49 mM (further purified as described under Experimental Procedures), 0.10 M KCl, and total calcium concentration of 1.03 mM. (A) Native protein, no Mn^{2+} added, pH 7.99 (1120 scans), (B) 20 μM Mn^{2+}, pH 7.75 (3000 scans, expansion line broadened 1 Hz), (C) 40 μM Mn^{2+}, pH 7.78 (4200 scans, expansion line broadened 2 Hz), and (D) sample C plus 51 mM total Ca^{2+}, pH 7.80 (1528 scans).

Ca^{2+}-saturated α-lactalbumin (Figure 8), the dimethyl N-terminal amino group resonance is the only resonance that is broadened. Spin–lattice relaxation time measurements confirm that this dimethyl N-terminal resonance is the only resonance perturbed by Mn^{2+} binding. The T_1 values for the dimethyl lysyl amino group resonances in Ca^{2+}-bound protein vary by less than 10% at pH 8 in the presence of up to 40 μM Mn^{2+} while the N-terminal resonance T_1 is decreased by 80% at 40 μM Mn^{2+}. Evidence that Mn^{2+} exchanges with Ca^{2+} is demonstrated by the narrowing of the Mn^{2+}-broadened N-terminal resonance (Figure 8C) after the addition of excess Ca^{2+} (Figure 8D). (The T_1 of this resonance was restored to the original value upon the addition of Ca^{2+}.) These results indicate that a Mn^{2+}/Ca^{2+} binding site is located near the NH_2 terminus.

Kronman et al. (1981) and Murakami et al. (1982) report high-affinity Mn^{2+} binding constants for bovine α-lactalbumin of 3.5×10^5 Mn^{-1} and 3.2×10^4 M^{-1}, respectively, while additional lower affinity Mn^{2+} binding sites with binding constants of 1×10^3 M^{-1} and 5×10^2 M^{-1} are reported by Murakami et al. (1982). These later workers have also shown that the Ca^{2+} in the high-affinity site does not exchange with added Mn^{2+}. For Ca^{2+}-saturated methylated α-lactalbumin a Mn^{2+}–Ca^{2+} exchange equilibrium constant, K_{Mn}/K_{Ca}, of 0.04 is obtained on the basis of the EPR measurements of free Mn^{2+} as a function added Mn^{2+}. This equilibrium constant was calculated by assuming that one Ca^{2+} per protein is unable to participate in the exchange (i.e., the high-affinity Mn^{2+} site is blocked by Ca^{2+}).[3] With methylated apoprotein a single high-affinity Mn^{2+} site with a binding constant of 5×10^4 M^{-1} is obtained by using a Scatchard analysis of the EPR binding data (0.1 M KCl and 0.2 mM protein, pH 8). No attempts were made to detect the several lower affinity Mn^{2+} sites due

[2] Equilibrium constant calculations not taking into account a second nonexchanging Ca^{2+} binding site give $K_{Zn/Ca} = 2.0$ by using the data in Figure 7A and by use of Figure 7B, $K_{Zn/Ca} = 0.7$.

[3] This value of K_{Mn}/K_{Ca} may be in agreement for exchange at the weaker (3.1×10^4 M^{-1}) Ca^{2+} site of Kronman et al. (1981) and the low-affinity (1×10^3 M^{-1}) Mg^{2+} binding site of Murakami et al. (1982).

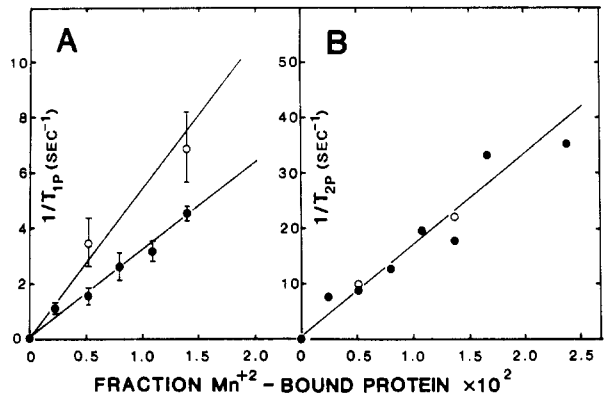

FIGURE 9: Carbon-13 NMR paramagnetic-induced spin–lattice and spin–spin relaxation rates for the [^{13}C]dimethyl N-terminal amino group of ^{13}C-dimethylated α-lactalbumin as a function of bound Mn^{2+}. Protein, 0.49 mM (further purified as described under Experimental Procedures), 0.10 M KCl, and total Ca^{2+} concentration of 1.03 mM, pH 8. (A) Mn^{2+}-induced spin–lattice relaxation rates ($1/T_{1P}$) at carbon-13 frequencies of 45.3 (□) and 68.9 (○) MHz as a function of bound Mn^{2+}. (B) Mn^{2+}-induced spin–spin relaxation rates ($1/T_{2P}$) at carbon-13 frequencies of 45.3 (□) and 68.9 (○) MHz as a function of bound Mn^{2+}.

to the high Mn^{2+} concentrations that would be required.

Estimation of the N-Terminal Mn^{2+} Intramolecular Distance. The data presented above indicate that Mn^{2+} specifically alters the relaxation parameters of the dimethyl N-terminal amino group resonance and that Mn^{2+} binds at a weak Ca^{2+} binding site in the Ca^{2+}-saturated methylated bovine α-lactalbumin. On the basis of these data, the dipolar form (simplified for Mn^{2+}) of the Solomon–Bolembergen equation was employed [see Dwek (1973), Mildvan et al. (1980), and Komoroski et al. (1976)] to estimate the intramolecular distance between the bound Mn^{2+} and the dimethyl-N-terminal methyl groups:

$$r = C[T_{1M}[3\tau_c(1 + \omega_1^2\tau_c^2)^{-1}]]^{1/6} \quad (1)$$

The experimental parameters needed to solve for the distance, r, are T_{1M} and T_{2M} (the paramagnetic contributors to the ^{13}C spin–lattice and spin–spin relaxation time for the protein–Mn^{2+} complex) and τ_c (the isotropic correlation time that modulates the Mn^{2+}–^{13}C dipolar interaction). For the ^{13}C–Mn^{2+} dipolar interaction, C is 512 Å/s$^{1/3}$ (Mildvan et al., 1980), and ω_1 is the Larmor angular frequency for the ^{13}C nuclear spin. To determine T_{1M} and T_{2M}, the Mn^{2+}-induced paramagnetic contributions to the spin–lattice and spin–spin relaxation rates ($1/T_{1P}$ and $1/T_{2P}$) were obtained as a function of the fraction, f, of total protein bound to Mn^{2+} (see Figure 9). Relaxation rates could be obtained only for the Ca^{2+}-saturated protein to which Mn^{2+} has been added due to the extensive Mn^{2+} broadening found in the presence of Mn^{2+} alone. The Mn^{2+}-induced relaxation rates were obtained as the difference between the relaxation rates of native Ca^{2+}-saturated protein and the relaxation rates in the presence of Mn^{2+}. The observed paramagnetic-induced relaxation rates, $1/T_{1P}$ (or $1/T_{2P}$), were related to $1/T_{1M}$ (or $1/T_{2M}$) by

$$1/T_{1,2P} = f[1/(T_{1,2M} + \tau_M)] \quad (2)$$

where f is the ratio of Mn^{2+}-bound protein to total protein and τ_M is the residence time of the Mn^{2+}–protein complex [see Dwek (1973) and Mildvan et al. (1980)]. If it can be shown that the $1/T_{1P}$ values are dependent on the ^{13}C NMR observation frequency (i.e., $T_{1M} > \tau_M$), then eq 2 simplifies, and T_{1M} can be directly determined from T_{1P} and f. Since a frequency dependence is observed for the $1/T_{1P}$ values of the dimethylated N-terminal amino group (Figure 9A), T_{1M} values can be directly obtained. At 45.3 MHz $1/T_{1M}$ is 500 ± 90 s^{-1} while at 67.9 MHz $1/T_{1M}$ is 310 ± 20 s^{-1}.

The frequency dependence of $1/T_{1M}$ can be used further to determine the correlation time, τ_c, for the ^{13}C–Mn^{2+} dipolar interaction (Dwek, 1973; Mildvan et al., 1980). For data obtained at ^{13}C frequencies of 45 and 68 MHz

$$\frac{T_{1M}(68\text{ MHz})}{T_{1M}(45\text{ MHz})} = \frac{1 + \omega_{I(68MHz)}{}^2\tau_c{}^2}{1 + \omega_{I(45MHz)}{}^2\tau_c{}^2} \quad (3)$$

which yields a correlation time of (4 ± 2) × 10^{-9} s with the above $1/T_{1M}$ values. The parameters used in eq 1 give a value of 7.4 ± 0.3 Å for the Mn^{2+}–^{13}C intramolecular distance (assuming Mn^{2+} binds to a single site under these conditions).

Values for τ_c can also be obtained from the ratio T_{1M}/T_{2M} at constant frequency. To be rigorous, T_{2M} should be calculated from T_{2P} by using eq 2. At the correlation time determined above, however, T_{2M} is theoretically frequency independent [see Dwek (1973)]. This is shown experimentally by comparing the 45.3 and 67.9 MHz data in Figure 9B. Therefore, the relative importance of τ_M with respect to T_{2M} cannot be determined from the data obtained at different frequencies. Nevertheless, if it is assumed that T_{2M} is large compared to τ_M (as is the case for T_{1M}), then T_{1P}/T_{2P} is equivalent to T_{1M}/T_{2M} from which values for τ_c may be obtained (Dwek, 1973) by using

$$\frac{T_{1M}}{T_{2M}} = \frac{4 + 3/(1 + \omega_1^2\tau_c^2)}{6/(1 + \omega_1^2\tau_c^2)} \quad (4)$$

from which a correlation time of (6 ± 1) × 10^{-9} s is obtained, in reasonable agreement with the correlation time obtained by using the frequency dependence of the T_{1M} values. The distance calculated by using this latter value of τ_c is 7.3 ± 0.2 Å.

Further information about the nature of τ_c can be gained by comparing its value to the rotational correlation time for the protein, τ_r, calculated by using the Stokes–Einstein equation (Mildvan et al., 1980). By use of this equation, the rotational correlation time for α-lactalbumin is estimated to be 3.9 × 10^{-9} s. Since $1/\tau_c = 1/\tau_r + 1/\tau_s + 1/\tau_M$ [see Dwek (1973) and Mildvan et al. (1980)], it appears that τ_c is dominated by τ_r and not by the electron spin relaxation time, τ_s, or by the Mn^{2+} residence time, τ_M.

Discussion

Methylated α-lactalbumin maintains full biological activity with galactosyltransferase, binds Ca^{2+} with high affinity, and undergoes a calcium-induced conformational change that is observable by both fluorescence (data not shown) and ^{13}C NMR spectroscopy. The effects of Mn^{2+} and Zn^{2+} binding to methylated protein were also monitored by fluorescence (not shown) and ^{13}C NMR spectroscopy, giving results consistent with the effects reported by others using fluorescence spectroscopy of the native protein. The function and presumably the structure of bovine α-lactalbumin is thus apparently unaltered upon the dimethylation of its amino groups.

On the basis of the pH dependence of the ^{13}C NMR chemical shifts of the ^{13}C-dimethylated amino groups, the 12 dimethylated lysyl residues and single dimethylated N-terminal amino group have been unambigously identified. The pK_a values and chemical shift parameters describing the individual (resolvable) amino group resonances are listed in Table I and plotted in Figures 2 and 3. It is obvious that each amino group has a unique titration behavior, indicating the presence of many different environments and interactions. Several lysyl amino group interactions have been suggested on the basis of

the model structures of bovine α-lactalbumin by Browne et al. (1969) and Warme et al. (1974) although presently these interactions cannot be confirmed without an unambiguous assignment of the individual dimethylated lysyl residues or the X-ray structure. On the basis of chemical modification studies, Richardson & Brew (1980) found that Lys-5, Lys-16, and Lys-98 have increased reactivity toward acetic anhydride (relative reactivities Lys-5 ≈ Lys-16 < Lys-98), suggesting that these residues have lower than normal pK_a values. In the dimethylated protein three dimethyl lysyl residues (resonances 1, 2, and 5) have pK_a values 0.5–1 pH unit lower than the typical value of 10.2 [see Gerken et al. (1982)]. The pK_a values of these three lysyl resonances follow a pattern similar to the reactivities reported by Richardson & Brew (1980) (pK_a of resonance 1 ≈ resonance 2 > resonance 5). Thus, resonance 5 tentatively can be assigned to Lys-98 and resonances 1 and 2 to Lys-5 and Lys-16. Likewise on the basis of the decreased activity toward acetic anhydride and on a higher than typical pK_a value, Lys-114 is tentatively assigned to resonance 12 (see Table I).

Three possible conformations (T2, T3, and T4) have been calculated by Warme et al. (1974) for the COOH-terminal region of bovine α-lactalbumin. Conformations T3 and T4 allow Lys-5 to interact with the terminal carboxyl group of Leu-123, in an ion pair, while in conformation T2 these groups cannot interact. As discussed by Richardson & Brew (1980) the low pK_a (increased reactivity) of Lys-5 supports the T2 conformation. The fact that the removal of the C-terminal Leu-123 (by carboxypeptidase A) does not significantly alter the pK_a values and chemical shift parameters of any of the ^{13}C-dimethylated amino groups provides additional evidence indicating that the Lys-5–Leu-123 ion pair does not exist (T. A. Gerken, unpublished results).

The dimethylated N-terminal amino group in native Ca^{2+}-saturated α-lactalbumin has a pK_a value of 8.3. This pK_a value is approximately 1 pH unit higher than what we have observed for this group in model peptides and proteins [see Gerken et al. (1982)], suggesting that in α-lactalbumin the N-terminal is involved in an ion pair interaction. On the basis of their energy-minimized model for α-lactalbumin, Warme and co-workers (1974) have proposed that the N-terminal amino group favorably interacts with the carboxyl groups of both Asp-37 and Glu-39 (see Figure 10). Thus, the abnormally high pK_a obtained for the N-terminus supports its proposed ion pair interactions in the Ca^{2+}-bound protein. Additional evidence for an abnormally high pK_a value for the NH_2-terminal amino group of α-lactalbumin is inferred from the low reactivity of this amino group toward methylation with formaldehyde and $NaCNBH_3$ (data not shown). Unperturbed NH_2-terminal amino groups typically methylate very rapidly under these conditions (Gerken et al., 1982).

The removal of Ca^{2+} from ^{13}C-dimethylated α-lactalbumin is shown to greatly alter the ^{13}C NMR pH titration behavior of the N-terminal amino group and to a lesser but significant extent the chemical shift of several of the lysyl residues (see Figure 4). These results further document the conformational change that occurs upon calcium ion binding or removal. In the dimethylated apoprotein the N-terminal amino group titrates with a pK_a value of 7.4 with chemical shift limits that, on the basis of model studies, are consistent with an amino group in a relatively unperturbed environment. On the basis of electrostatic considerations, the increase in pK_a that is observed in the presence of Ca^{2+} cannot be attributed to direct Ca^{2+} chelation at the N-terminal amino group. Thus, the removal of Ca^{2+} elicits a conformational change that disrupts

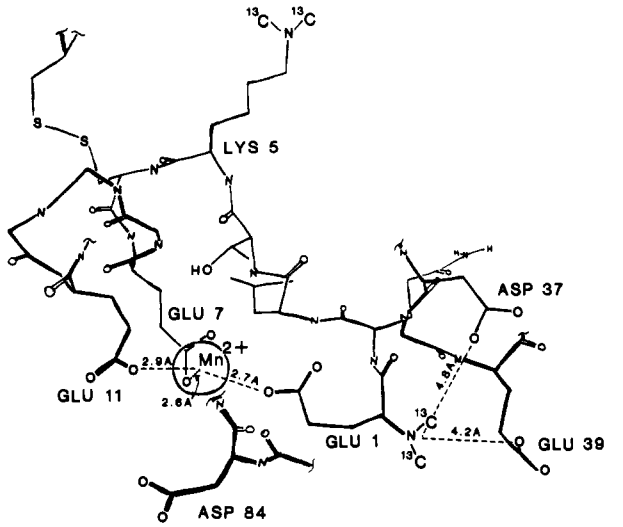

FIGURE 10: Proposed structure of the NH_2-terminal region of bovine α-lactalbumin based on the crystal structure of hen egg white lysozyme (Warme et al., 1974). The NH_2-terminal ion pair interactions and a likely low-affinity Ca^{2+}, Mn^{2+}, and perhaps Zn^{2+} binding site are indicated. The peptide and side chain of Leu-85 are directly above the metal ion in this figure and are not shown for clarity.

the presumed N-terminal ion pair interaction. Since separate N-terminal resonances are observed for both the apoprotein and Ca^{2+}-bound protein the exchange of Ca^{2+} and/or the associated conformational change that occurs upon binding Ca^{2+} is relatively slow. The continuous change in the chemical shift of the N-terminal resonance in the Ca^{2+}-bound protein at low pH (see Figure 3) may reflect the occurrence of additional pH-dependent conformational changes that are not present for the apoprotein. These changes may be due to the titration of one or more carboxyl groups that may compose the high-affinity calcium binding site. On the basis of the fluorescence titrations of Ca^{2+}-saturated bovine α-lactalbumin, Permyakov and co-workers (1981a) have suggested the existence of three carboxyl groups in the Ca^{2+} binding site with pK_a values of ~5. Groups titrating with pK_a values of ~5 could readily produce the changes in chemical shift that are observed in the N-terminal resonance of the Ca^{2+}-saturated protein. As shown in Figure 3 no differences are observed in the high pH chemical shift limits of the N-terminal resonance between apoprotein and the Ca^{2+}-bound protein. This suggests that the N-terminal amino group of the Ca^{2+}-bound protein when not interacting in its ion pair (i.e., when deprotonated) is relatively unperturbed and becomes insensitive to Ca^{2+} binding.

The addition of zinc ion to the Ca^{2+}-saturated protein causes the dimethyl N-terminal amino group resonance to shift to lower field. Upon further addition of Zn^{2+} the N-terminal resonance approaches a limiting chemical shift, and a resonance appears at the chemical shift of the N-terminal amino group in the apoprotein. These findings are in general agreement with previously reported fluorescence and EPR studies of α-lactalbumin (Kronman et al., 1981; Murakami & Berliner, 1983) where Zn^{2+} has been shown to displace Ca^{2+} (or Mn^{2+}) from the native metal bound protein giving an apoprotein-like conformation. Our results suggest that initially Zn^{2+} binds competitively at a weak Ca^{2+} binding site and that Zn^{2+} and Ca^{2+} are in rapid exchange at this site. It is the zinc binding to this site that shifts the N-terminal amino group resonance toward lower field, while the presence or absence of calcium in this weak binding site apparently does not alter the N-terminal amino group resonance. The findings that the apparent pK_a of the N-terminal resonance in the presence of

low Zn^{2+} remains relatively unchanged, that the resonance is shifted to lower field (similar to the low pH Ca^{2+}-bound protein), and that the equilibrium constant calculations require a bound Ca^{2+} suggest the Ca^{2+} in the high-affinity site may initially remain bound to the Zn^{2+}-bound protein intermediate. This supposes the chemical shift of the N-terminal resonance reflects its ion pair interaction which in turn is the result of a nativelike Ca^{2+}-bound protein conformation. The appearance of an N-terminal resonance at the chemical shift of the apoprotein in the presence of high Zn^{2+} may suggest the initially formed Zn^{2+}-bound protein is unstable and readily converts to an apoprotein-like conformation. Hence, only when sufficient Zn^{2+} is present (or if there is sufficiently low Ca^{2+} in the weak site), and the lifetime of the intermediate Zn^{2+} bound conformation becomes long, does an apoprotein-like conformation appear. The fact that the chemical shift limit of the initial Zn^{2+}-bound protein (Figure 3) is similar to that of the Ca^{2+}-bound protein at pH 5 may suggest that Zn^{2+} binding alters the titration behavior of the carboxyl groups of the high-affinity Ca^{2+} binding site, thereby destabilizing the Ca^{2+}-bound conformation. Significantly it has been shown that α-lactalbumin does not bind calcium below its isoelectric point, pH 4.5 (Kronman & Bratcher, 1983).

The fluorescence studies of Kronman et al. (1981) and Murakami & Berliner (1983) were made under conditions employing large excesses of Zn^{2+} or protein containing only a single Ca^{2+} or Mn^{2+}; hence, only the apoprotein-like conformation was observed. Their reported time dependence ($t_{1/2} \approx 30$ s) for the fluorescence change observed after the addition of Zn^{2+} supports the ideal of a slow Zn^{2+}-bound nativelike protein to apo-like protein conformational change. The NMR results tend to corroborate the work of Murakami & Berliner (1983) which suggests the Zn^{2+} binding site is distinct from the high-affinity Ca^{2+} site. Their conclusion that the binding of both Zn^{2+} and the high-affinity Ca^{2+} (or Mn^{2+}) is mutually exclusive is not readily supported by our results unless the appearance of the apoprotein N-terminal resonance is due to Zn^{2+} occupying a second binding site that is associated in some manner with the high-affinity Ca^{2+} site. Additional studies need to be done to resolve these points and to further determine the origins of the changes that are observed upon Zn^{2+} binding. This is especially interesting since both the Ca^{2+}-saturated protein and Zn^{2+}-bound apo-like protein have been shown to be identical in their activation of galactosyltransferase to lactose synthetase (Murakami & Berliner, 1983).

On the basis of the broadening of the dimethyl N-terminal amino group resonance in the presence of Mn^{2+}, it has been concluded that a Mn^{2+}/Ca^{2+} binding site is located near the N-terminus of bovine α-lactalbumin. Evidence suggests that this Mn^{2+} site is at a low-affinity Ca^{2+} site and not at the high-affinity Ca^{2+}/Mn^{2+} site that is associated with the global conformational changes observed by both fluorescence and NMR spectroscopies. On the basis of the paramagnetic contributions of Mn^{2+} binding to the relaxation rate of the dimethyl N-terminal resonance, a distance of 7.4 Å is estimated for the ^{13}C–Mn^{2+} intramolecular distance. Since the N-terminal residue is a glutamic acid, it is possible that this binding site is composed in part of the carboxyl group of this residue. By use of the model for bovine α-lactalbumin based on the structure of lysozyme (Warme et al., 1974), a possible binding site can be located that includes the carboxyl groups of Glu-1, Glu-7, and Glu-11. By use of this model, the distance from this site to the N-terminal methyl groups is 8.4 Å. As shown in Figure 10 one oxygen from each carboxyl group is less than 3 Å from the center of the proposed Ca^{2+}/Mn^{2+} binding site, and this site is surrounded by additional potential liganding groups, i.e., the peptide carbonyls of Asn-83, Thr-4, and Glu-2 and the hydroxyl of Thr-4. Hydrophobic groups also potentially important may be the methylene of Leu-3 and the methyls of Leu-85. The carboxyl groups comprising this proposed binding site are not conserved in other α-lactalbumin species that are shown to have very high affinities for Ca^{2+} and that undergo similar fluorescence changes upon binding metal ion. This provides further evidence that the divalent metal binding site described here is not the same as the high-affinity Ca^{2+} binding site. It is interesting, however, that the chicken and human lysozymes have been shown to have a Glu-7–Lys-1 N^{ϵ} ion pair link (Browne et al., 1969; Artymiuk & Blake, 1982) while the Ca^{2+}-saturated bovine α-lactalbumin may have a similar structural link in the proposed Glu-7–Ca^{2+}–Glu-1 interaction.

This work demonstrates the potential of ^{13}C-reductive methylation and ^{13}C NMR spectroscopy as an approach that can provide specific information detailing the solution structure, intramolecular interactions, and metal binding properties of bovine α-lactalbumin. This method is ideally suited for further studies of bovine and other species of α-lactalbumin, in particular, to compare and contrast their NH_2-terminal environments and their metal binding properties. Studies of methylated α-lactalbumin, furthermore, will be useful in the study of α-lactalbumin's interaction with galactosyltransferase.

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Registry No. Lys, 56-87-1; Ca, 7440-70-2; Zn, 7440-66-6; Mn, 7439-96-5.

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A Two Sodium Ion/D-Glucose Symport Mechanism: Membrane Potential Effects on Phlorizin Binding†

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ABSTRACT: Apical membrane vesicles isolated from a continuous renal cell line, LLC-PK$_1$, catalyze electrogenic Na^+-stimulated hexose transport and Na^+-dependent binding of ^{3}H-labeled 1-[2-(β-D-glucopyranosyloxy)-4,6-dihydroxyphenyl]-3-(4-hydroxyphenyl)-1-propanone ([^{3}H]phlorizin), a competitive ligand of this transport system. Phlorizin was not itself transported across the membrane and thus can serve as a probe of the binding step. The stoichiometry of Na^+-dependent phlorizin binding in vesicles was 1:1, whereas Na^+/hexose cotransport in vesicles exhibited a 2:1 stoichiometry. Na^+ increased the affinity of phlorizin binding without affecting the total number of binding sites. An increased number of Na^+-dependent phlorizin binding sites was observed under conditions of interior-negative membrane potential. These results are consistent with a model of the Na^+/glucose cotransport cycle in which the unloaded transporter is negatively charged and its orientation influenced by membrane potential. Glucose and one sodium ion interact with the transporter, resulting in an uncharged complex. Binding of a second sodium ion triggers translocation of glucose and both sodium ions via formation of a loaded carrier complex bearing a single positive charge.

Electrogenic Na^+-stimulated hexose cotransport is expressed in apical membrane vesicles isolated from confluent cultures of LLC-PK$_1$, an established kidney epithelial line (Lever, 1982; Moran et al., 1982). The Na^+/hexose symporter expressed in this cell line exhibits a sugar and inhibitor specificity similar to that of the corresponding renal cortical proximal tubule transporter but differs in one important aspect. The stoichiometry of Na^+/glucose cotransport in LLC-PK$_1$ cells is 2:1 on the basis of studies using apical membrane vesicles (Lever, 1982; Moran et al., 1982) and short-circuit current measurements of intact cell monolayers grown on filters (Misfeldt & Sanders, 1981, 1982). By contrast, a Na^+/glucose stoichiometry of 1:1 has been estimated for the renal cortical proximal tubule symporter (Beck & Sacktor, 1978; Turner & Moran, 1982a).

The stoichiometry of the coupled species in a symport mechanism is of fundamental importance in determining the energetics of the transport mechanism, since the ability to accumulate substrate is proportional to the power of the number of sodium ions cotransported per substrate. Evaluation of the individual contributions of membrane potential and the coupled ionic species to the binding step must also be considered in order to understand the energetics and mechanism of the transport cycle.

1-[2-(β-D-Glucopyranosyloxy)-4,6-dihydroxyphenyl]-3-(4-hydroxyphenyl)-1-propanone (phlorizin)[1] is a competitive inhibitor of Na^+/glucose symport across the renal proximal tubule brush border membrane (Frasch et al., 1970). The binding of phlorizin to the glucose carrier is Na^+ dependent, but several lines of evidence indicate this inhibitor is not translocated across the membrane (Toggenburger et al., 1982). As a consequence, this nonpenetrating ligand has been used as a probe in studies of the mechanism of Na^+/glucose symport in brush border preparations from intestine and renal cortex

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[1] Abbreviations: αMeGlc, methyl α-D-glucopyranoside; $TPMP^+$, triphenylmethylphosphonium ion; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; phlorizin, 1-[2-(β-D-glucopyranosyloxy)-4,6-dihydroxyphenyl]-3-(4-hydroxyphenyl)-1-propanone; phloretin, 3-(4-hydroxyphenyl)-1-(2,4,6-trihydroxyphenyl)-1-propanone; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; MES, 2-(*N*-morpholino)ethanesulfonic acid; BES, 2-[bis(2-hydroxyethyl)amino]-ethanesulfonic acid.

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