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High-Resolution Proton and Laser Photochemically Induced Dynamic Nuclear Polarization NMR Studies of Cation Binding to Bovine α-Lactalbumin†

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ABSTRACT: α-Lactalbumin (α-LA) is a calcium binding protein that also binds Mn(II), lanthanide ions, Al(III), Zn(II), Co(II). The structural implications of cation binding were studied by high-resolution proton (200 MHz) NMR and photochemically induced dynamic nuclear polarization (CIDNP) spectroscopy. Marked changes were observed in the NMR spectra of the apoprotein upon addition of a stoichiometric amount of calcium to yield Ca(II)-α-LA, manifested particularly in ring current shifted aliphatic peaks and in several shifts in the aromatic region, all of which were under slow exchange conditions. The CIDNP results showed that two surface-accessible tyrosine residues, assigned as Tyr-18 and -36, became inaccessible to the solvent upon addition of 1:1 Ca(II) to apo-α-lactalbumin, while Tyr-103 and Trp-104 remained completely accessible in both conformers. The proton NMR spectra of apo-α-LA and Al(III)-α-LA were extremely similar, which was also consistent with intrinsic fluorescence results [Murakami, K., & Berliner, L. J. (1983) *Biochemistry 22*, 3370–3374]. The paramagnetic cation Mn(II) bound to the strong calcium binding site on apo-α-LA but also to the weak secondary Ca(II) binding site(s) on Ca(II)-α-LA. It was also found that Co(II) bound to some secondary sites on Ca(II)-α-LA that overlapped the weak calcium site. All of the lanthanide shift reagents [Pr(III), Eu(III), Tb(III), Dy(III), Tm(III), and Yb(III)] bound under slow exchange conditions; their relative affinities for apo-α-lactalbumin from competitive binding experiments were Dy(III), Tb(III), and Pr(III) > Ca(II) > Yb(III).

α-Lactalbumin (α-LA)[1] is the noncatalytic protein subunit of the lactose synthase complex that functions by modifying the acceptor specificity of galactosyltransferase (UDP-galactose:D-glucose 4-galactosyltransferase, EC 2.4.1.22) from GlcNAc to glucose. It is also known that α-LA is highly homologous to lysozyme in primary structure (Brew et al., 1970) from which three-dimensional modeling studies were reported (Browne et al., 1969; Warme et al., 1974). Koga and Berliner (1985) found a strong structural identity between the two proteins *in solution* in the form of a hydrophobic box comprised of Trp-60, Trp-104, Tyr-103, and Ile-95. In spite of the similarities in structures, these two proteins have dramatically different biological functions. Furthermore, another significant difference between them is their respective metal ion affinities. Recently, α-LA was found to be a metalloprotein that binds Ca(II) very strongly with a dissociation constant K_d = 0.2–3.0 nM (Berliner et al., 1978; Permyakov et al., 1981a, 1987; Murakami et al., 1982; Bryant & Andrews, 1984; Van Ceunebroeck et al., 1985). On the other hand, the K_d values for lysozyme and Ca(II)-like cations are millimolar or higher (Secemski & Lienhard, 1974; Kurachi et al., 1975; Ostroy et al., 1978). We have described the ligand properties of the calcium site by NMR (^{113}Cd) and ESR [Mn(II) and Gd(III)] as essentially identical with those of parvalbumin, troponin C, and calmodulin (Berliner et al., 1983; Musci et al., 1986).

Since this specific multiple cation site binding had been well characterized by other spectroscopic methods, we were interested in attempting to characterize this cation binding from a more detailed structural perspective. The work presented here encompasses high-resolution Fourier transform and CIDNP ^{1}H NMR studies of the effects of diamagnetic and paramagnetic cation binding to bovine α-LA.

Experimental Procedures

Materials. Bovine apo-α-LA (lots 75C8110, 86C8020, 52F80751, and 50F8105) and bovine Ca(II)-α-LA (lot 33F8145) were from Sigma Chemical Co. Metal-free apo-α-LA was prepared by chromatography on tris(carboxymethyl)ethylenediamine–agarose (Pierce Chemical Co., lot 121483-87) at pH 8.0 (Koga & Berliner, 1985). Residual

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[1] Abbreviations: α-LA, α-lactalbumin; GlcNAc, *N*-acetylglucosamine; NOE, nuclear Overhauser effect; CIDNP, chemically induced dynamic nuclear polarization; ESR, electron spin resonance; EDTA, ethylenediaminetetraacetic acid; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; Tris-d_{11}, tris(hydroxymethyl)aminomethane-d_{11}; FT, Fourier transform; FID, free induction decay; bis-ANS, 4,4′-bis[1-(phenylamino)-8-naphthalenesulfonate].

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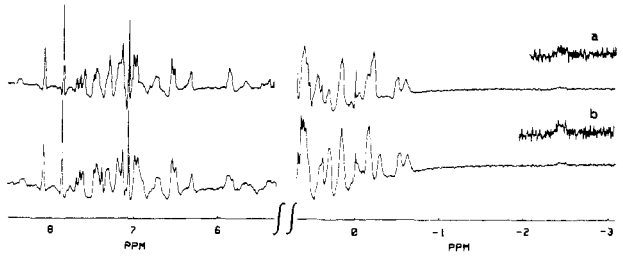

FIGURE 1: High-resolution 200-MHz proton NMR spectra of the aromatic and upfield shifted aliphatic region of bovine α-LA (50 mM Tris-d_{11}-DCl, pH 7.2). The residual HOD signal was suppressed by presaturation with a single radio frequency for 1 s, which was gated off during acquisition. Spectrometer parameters were 8K data points, 1000 transients, 3000-Hz sweep width, 3.7-μs pulse width (90° pulse), and 1.4-s acquisition time. Spectral resolution enhancement was accomplished by the convolution difference method (line broadening 0.5 Hz − line broadening 5 Hz). (a) 1.6 mM apo-α-LA; (b) 1.9 mM Ca(II)-α-LA.

calcium content was checked by fluorescence (Murakami et al., 1982) and atomic absorption and was consistently found to be less than 2%. Pure apo-α-LA was used for proton NMR experiments of Ca(II) and Al(III) binding, while untreated Sigma apo-α-LA (which contains 0.3–0.4 mol of calcium) was used in the other experiments. Protein concentration was determined with $E^{1\%}_{280} = 20.1$ cm^{-1}. Galactosyltransferase was purified from fresh bovine milk (Grunwald & Berliner, 1978). Manganese chloride (99.999%, lot 0518), cobalt chloride (99.999%, lot 0918), praseodymium chloride (99.999%, lot 1097), ytterbium chloride (99.99%, lot 0997), dysprosium chloride (99.99%, lot 0108), and thulium oxide (99.99%, lot 131184) were from Aldrich Chemical Co. Calcium chloride (ultrapure, lot 87607), terbium chloride (99.9%, lot 082878), and EDTA (99+%, lot 011581) were from Alfa Products. Aluminum chloride (analytical reagent, lot WDAP) was from Mallinckrodt. Deuterium oxide (99.95%) was from Bio-Rad Labs Inc., Wilmad Glass Co., Merck Isotopes, or Sigma Chemical Co. (99.8%). Samples for NMR experiments were dissolved in 50 mM Tris-d_{11} buffer (95% atom, lot 6037, Merck Isotopes). Protein samples were preexchanged with D_2O at room temperature and lyophilized before use. All pH values were uncorrected.

NMR Measurements. Proton NMR spectra were measured on a Bruker WP200 at 301 ± 1 K in the Fourier transform mode. The residual HOD resonance was suppressed by presaturation for 1 s, which was gated off during acquisition. Data sets were 8K or 16K with sweep widths of 3000–5000 Hz. Resolution enhancement was accomplished by convolution difference (line broadening 0.5 Hz minus 5.0 Hz) or by Gaussian multiplication. Laser photo-CIDNP difference spectra (Berliner & Kaptein, 1981) were obtained on a Bruker HX-360 (University of Groningen) or a Nicolet NT500 as described earlier (Scheffler et al., 1985). Chemical shifts were measured from internal DSS.

RESULTS

Calcium Binding to α-LA

Proton NMR Spectra. The intrinsic fluorescence results of Murakami and Berliner (1983) classified the cation ion binding sites of α-LA into two groups: a calcium site [which binds Ca(II), Mn(II), lanthanides, etc.] and a zinc site [which binds Zn(II), Al(III), Cu(II), and Co(II)]. It was also shown that cation binding to the latter site froze the protein in an apo or "apo-like" conformation whether the calcium site was occupied or not (Musci & Berliner, 1985). Figure 1 depicts the expanded aromatic and upfield aliphatic regions of the

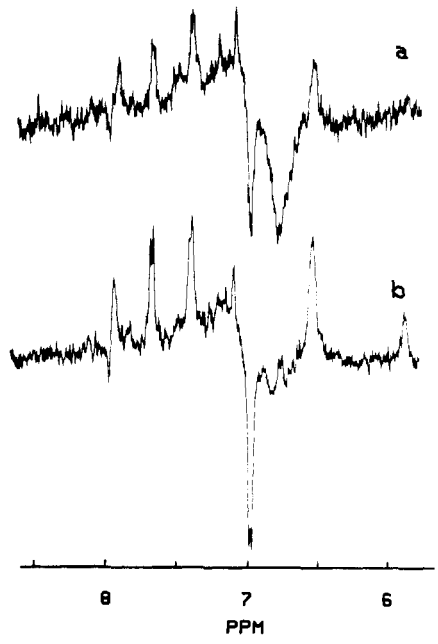

FIGURE 2: Laser photo-CIDNP difference spectra of α-LA conformers at 500 MHz. The sample contained 0.5 mM α-LA and 0.4 mM 10-*N*-(carboxyethyl)lumiflavin, pH 7.1. FID's were collected as eight alternating light (laser on) and dark (laser off) scans. Laser power was 7.0 W, and the sample was illuminated for 0.5 s by fiber optic illumination as described by Scheffler et al. (1985). Spectrometer parameters were 8K data points, 7000-Hz sweep width, and 2-μs pulse width. A 10-s delay was used between each light and dark scan. The HOD signal was presaturated for 1 s preceding each laser pulse. (a) Apo-α-LA; (b) Ca(II)-α-LA.

200-MHz ^{1}H NMR spectra of bovine apo- and Ca(II)-α-LA (pH 7.2). Several upfield ring current shifted peaks were observed; in particular, a broad line appeared at −2.45 ppm, which indicated that this proton was affected by an extremely strong ring current shift (Koga & Berliner, 1985). We have assigned this peak from NOE measurements as Ile-95 ($H_{\gamma_{12}}$) and the −0.61 ppm line to the CH_3 of this same residue, as Ile-95, comprising part of a "hydrophobic box" consisting of Trp-60, Trp-104, and Tyr-103 (Koga & Berliner, 1985). The most notable change when shifting from the apo- to the Ca(II)-α-LA conformer was the appearance of a new upfield shifted resonance at −0.30 ppm, which grew linearly in intensity with increasing Ca(II) up to a 1:1 stoichiometry. The peaks from −0.1 to −0.25 ppm also displayed altered line shapes with Ca(II) addition; the cluster of lines at ca. 0.30 ppm also increased with Ca(II) addition. While every single proton resonance could not be resolved between 0.8 and 0.35 ppm, the spectral pattern definitely changed.

In the aromatic region prominent changes were observed in the 7.25–7.4 ppm range that were very different. The resonance at 7.14 ppm was resolvable as a single line in Ca(II)-α-LA but overlapped neighboring lines in apo-α-LA; the upfield shoulder of the 6.9 ppm line was different in apo- and Ca(II)-α-LA, respectively. Of significant interest was the fact that these conformational differences between apo- and Ca(II)-α-LA were in *slow exchange* on the NMR time scale.

Laser Photo-CIDNP Studies. The laser photo-CIDNP technique is very useful in characterizing surface aromatic residues of a protein which are accessible to a photoexcited flavin dye (Kaptein, 1982). Figure 2 depicts CIDNP difference spectra of apo- and Ca(II)-α-LA at pH 7.1. As shown earlier for the apo-form (Figure 2a), the emission lines at 6.98, 6.80, and 6.71 ppm arose from three tyrosine residues (Tyr-18, -36, and -103); the resonances at 7.78 and 7.00 ppm represent the C-2 and C-4 protons of His-68 at this pH, respectively;

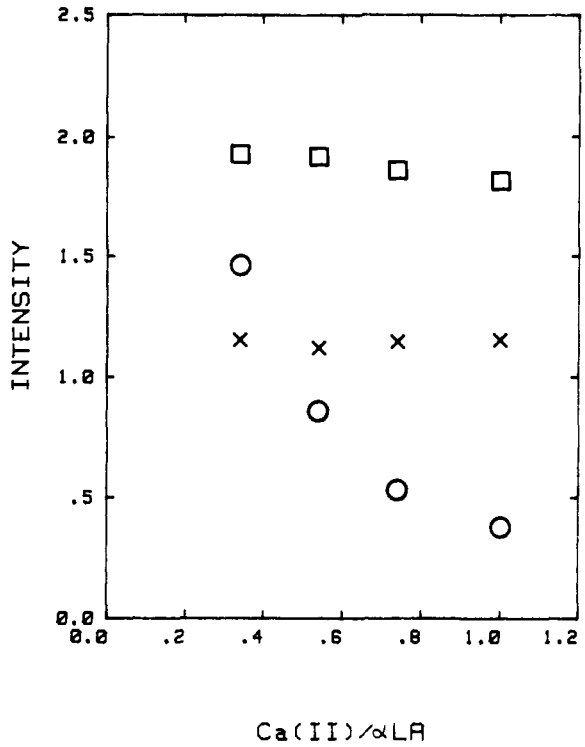

FIGURE 3: Plot of peak heights in CIDNP difference spectra vs. Ca(II)/α-LA stoichiometry. Each peak was normalized relative to the 6.51 ppm resonance line: (×) 7.36 ppm (Trp-104); (□) 6.98 ppm (Tyr-103); (○) 6.80 ppm (Tyr-18, Tyr-36).

the remaining absorption lines were from Trp-104 (7.64, 7.36, and 6.51 ppm) and a cross-polarized Trp-60 at 5.85 ppm (Berliner & Kaptein, 1981). The emission line at 6.98 ppm was assigned from NOE measurements to Tyr-103, leaving the emission lines at 6.80 and 6.71 ppm as Tyr-18 and Tyr-36. After the addition of 1:1 Ca(II), the spectrum in Figure 2b was obtained, which clearly showed the disappearance of the two Tyr emissions at 6.80 and 6.71 ppm. Figure 3 depicts a titration of the peak heights of these two Tyr peaks with increasing Ca(II) stoichiometry. Note that while the peak heights of Trp-104 [(×) 6.51, 7.36, or 7.64 ppm] and Tyr-103 [(□) 6.98 ppm] remained constant with increasing Ca(II), the 6.80 ppm (○) Tyr line decreased stoichiometrically. Note also the difference in cross-polarization to the Trp-60 line (5.85 ppm), appearing more strongly in the Ca(II)-α-LA conformer (Figure 2b), generally consistent with results found from NOE measurements earlier by Koga and Berliner (1985).

Paramagnetic Cation Binding

Manganese Ion Binding. Murakami et al. (1982) showed that Mn(II) binds to the strong calcium site in α-LA with a K_d = 30.5 μM and also to weaker, secondary binding sites of K_d = 1.1 and 5.0 mM. Since Mn(II) is a well-studied proton relaxation agent, we examined the high-resolution proton NMR of Mn(II)-α-LA complexes. In order to attempt to probe the calcium binding site, we performed Mn(II) titrations of 2.1 mM apo-α-LA followed by addition of Ca(II) as a control. As shown in Figure 4, no significant changes were observed up to 78 μM Mn(II) (Figure 4b), while some minor line broadening occurred at 180 μM (Figure 4c). On the other hand, upon titration of 2.2 mM Ca(II)-α-LA (pH 7.2), several *major* spectral changes already occurred at quite low Mn(II) concentrations, e.g., 41–88 μM (Figure 5a–c). Most pronounced was the broadening of the aliphatic resonance at 0.30 ppm. All of the His C-2 lines, His-107 (8.08 ppm), His-68 (7.84 ppm), and His-32 (7.59 ppm), were severely broadened; the C-4 line of His-68 (7.06 ppm) also broadened, as well as

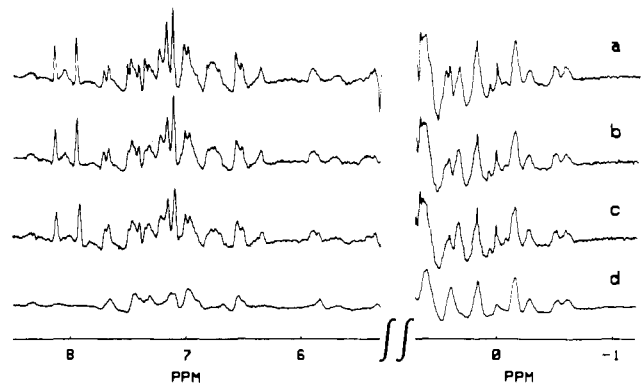

FIGURE 4: Mn(II) titration of 2.1 mM apo-α-LA as monitored by proton NMR. All other spectrometer parameters were identical with those in Figure 1 except that 1100 transients were collected and 4K data points were acquired (acquisition time 0.7 s) and zero filled to 8K before FT: (a) 0 μM Mn(II); (b) 78 μM Mn(II); (c) 180 μM Mn(II); (d) 180 μM Mn(II) plus 2.1 mM Ca(II).

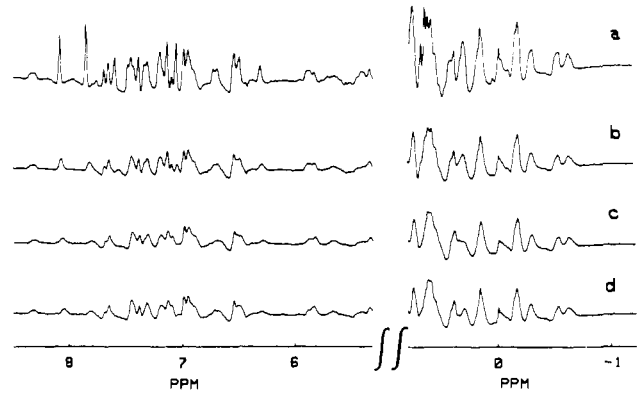

FIGURE 5: Mn(II) titration of 2.2 mM Ca(II)-α-LA as monitored by proton NMR. All other conditions were identical with those in Figure 4: (a) 0 μM Mn(II); (b) 41 μM Mn(II); (c) 88 μM Mn(II); (d) 88 μM Mn(II) plus 20 mM Ca(II).

several other aromatic peaks, especially at 6.71 and 6.31 ppm. In the control experiment in Figure 4d, similar changes occurred in the Mn(II) titration of apo-α-LA after the addition of 2 mM Ca(II) to 180 μM Mn(II):2.1 mM apo-α-LA. Figure 5d depicts the spectra after addition of a large excess (20 mM) of Ca(II) to the sample in Figure 5c. Here, the 0.30 ppm line "recovered" while several of the aromatic lines retained substantial paramagnetic broadening.

Parallel CIDNP measurements also revealed a slow exchange preferential broadening of His-68 (C-2 and C-4 protons) up to Mn(II) concentrations of 100 μM (L. J. Berliner and R. Kaptein, unpublished results). Upon addition of a stoichiometric amount of Ca(II), the His-68 resonances were completely broadened out (as in Figure 4d), and the two Tyr emissions at 6.71 and 6.8 completely disappeared, as observed in Figure 2b.

Cobalt Ion Binding. Murakami and Berliner (1983) showed by intrinsic fluorescence spectroscopy that Co(II) binds to the zinc (*site II*) of α-LA. Figure 6 depicts proton NMR spectra showing the results of a Co(II) titration of the calcium conformer of α-LA. A marked effect was the shift of the −0.52 ppm line to −0.54 ppm [0.5 mM Co(II), Figure 6b], to −0.58 ppm [1.0 mM Co(II), Figure 6c], and to −0.62 ppm [1.5 mM Co(II), Figure 6d]. Several other lines were also effected but to a lesser extent in chemical shift changes. The other notable change was a broadening of the 0.30 ppm line, which was the same resonance that was broadened by Mn(II) (Figure 5b–c). There were also several paramagnetically broadened peaks in the aromatic region. In addition, a peak at 6.88 ppm appeared

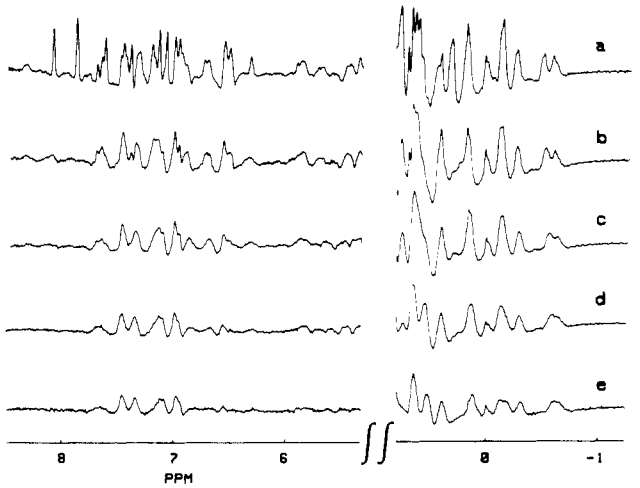

FIGURE 6: Co(II) titration of 2.0 mM Ca(II)-α-LA as monitored by proton NMR, 50 mM Tris-d_{11}-DCl, pH 7.2. All other conditions were identical with those in Figure 4 except that 1000 transients were collected and the pulse width was 3.0 μs. Co(II) concentration was (a) 0, (b) 0.5, (c) 1.0, (d) 1.5, and (e) 2.0 mM.

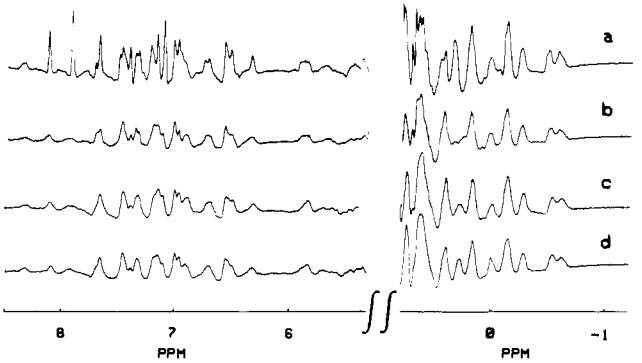

FIGURE 7: Competition between Co(II) and Ca(II) on Ca(II)-α-LA. All other parameters were identical with those in Figure 4 except that 1500 transients were collected and the pulse width was 3.0 μs: (a) 2.3 mM Ca(II)-α-LA alone; (b) sample a plus 0.5 mM Co(II); (c) sample b plus 2.7 mM Ca(II); (d) sample c plus 7.7 mM Ca(II).

Table I: Chemical Shift Changes of the Aliphatic Resonance at 0.3 ppm by Co(II) Addition

[α-LA] (mM)[a]	[Co(II)] (mM)	[Ca(II)] (mM)	chemical shift, ppm
2.3	0	$\geq$2.3	0.3
2.3	0.5	0	
2.3	0.5	4	0.27
2.3	0.5	10	0.28

[a] pH 7.2, 50 mM Tris-d_{11}-DCl.

to be shifted out from the shoulder at 6.9 ppm. Figure 7 depicts a competition experiment between Co(II) in the presence of excess Ca(II). Here again, as with the Mn(II) experiments above, a "recovery" of the "0.30 ppm" resonance was observed. This recovery was, however, accompanied by an upfield chemical shift change toward that of the original position of this line. Some typical data are listed in Table I.

Lanthanide Ion Binding. Murakami et al. (1982) also showed that lanthanide ions bind strongly to the Ca(II) site of α-LA. The effects of lanthanides on the NMR of proteins as paramagnetic or shift reagents are well documented [for a review, see Lenkinski (1984)]. Figure 8 depicts the effects of the lanthanide shift reagent Tb(III) on the proton NMR of α-LA. There were several substantially shifted lines (Figure 8b) compared to the spectrum of apo-α-LA (Figure 8a). However, *all* of the new lines grew proportionally with the *stoichiometric* addition of lanthanide shift reagent; i.e., all were

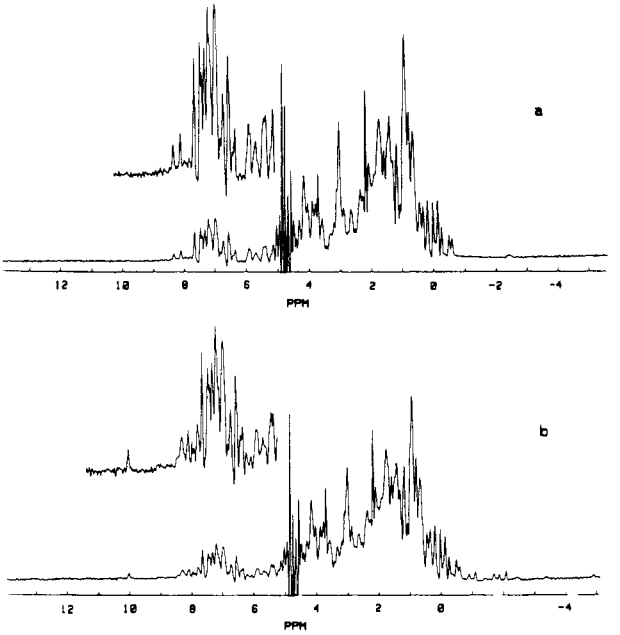

FIGURE 8: Proton NMR spectra of 2 mM Ca(II)-α-LA with or without added Tb(III). All other conditions were identical with those in Figure 1 except that 16K data points were used and the sweep width was 4000 Hz. Gaussian multiplication was employed for resolution enhancement: (a) 2 mM Ca(II)-α-LA alone; (b) 2 mM Ca(II)-α-LA plus 0.5 mM Tb(III). Insets represent higher gain plots of the downfield regions of (a) and (b).

under slow exchange. Several other lanthanide–α-LA complexes were examined (spectra not shown). Although we were unable to extract any accurate assignment or distance information between the bound shift reagent and the protein (see Discussion), we were able to estimate relative affinities for several lanthanide ions compared to that of Ca(II) from competition binding experiments as monitored by NMR (the K_d values between α-LA and the lanthanides were so small that it was difficult to determine them by direct methods). The results from the strongest to weakest were Dy(III), Tb(III), and Pr(III) > Ca(II) > Yb(III). In the case of the relaxation reagent Gd(III), the slow exchange restriction resulted in an essentially uniformly broadened spectrum, which could not be resolved nor accurately assigned.

Al(III) Binding to α-LA. In order to examine the conformation of Al(III)-bound α-LA, we compared the apo-α-LA 200-MHz ^{1}H spectra before and after addition of Al(III). Remarkably, there were essentially no significant differences resolvable at this frequency between apo-α-LA and Al(III)-α-LA, which correlated well with the fluorescence results (Murakami & Berliner, 1983; Musci & Berliner, 1985).

Multiple Conformations of His-68. Although our α-LA samples were fully active in the lactose synthase assay (Fitzgerald et al., 1970), we frequently found NMR evidence for minor conformational differences in pure α-LA preparations. Specifically, two His-68 C-2 resonances in apo-α-LA were observed at 7.86 and 7.93 ppm, respectively, at pH 7.2 (Figure 4a). A pH titration of these two "His-68" resonances fit almost identical pK_a values; the maximum chemical shift difference between the two lines was 0.10 ppm and frequently less (data not shown). Upon stoichiometric addition of Ca(II) to the apo-α-LA sample, the broader His-68 line at 7.93 ppm completely disappeared (Figure 5a).

Discussion and Conclusions

It is clear from the ^{1}H NMR results that cation binding to bovine α-LA may be detected by conformationally sensitive

methods. Calcium binding resulted in small, but detectable, shifts in many resonances with a particularly diagnostic "new line" at −0.3 ppm as a result of large ring current shifts originating from a line in a more downfield cluster of resonances. It is interesting to speculate that these chemical shift changes above corresponded to the changes in the local environment of Trp residues upon Ca(II) addition to apo-α-LA, as detected previously by intrinsic fluorescence measurements (Murakami et al., 1982; Permyakov et al., 1982). A ^{13}C NMR study of dimethylated lysyl and N-terminal amino α-LA derivatives also showed evidence for a conformational change upon Ca(II) addition where the effect was most pronounced at the amino terminus (Gerken, 1984). On the other hand, the overall *similarities* between the apo- and Ca(II)-α-LA spectra in Figure 1 show that the two conformers are not *drastically* different in their three-dimensional conformations.

While at 200 MHz the overall spectral pattern is not drastically shifted upon calcium binding, several subtle changes, such as surface Tyr exposure, occur upon shifting from the apo to the Ca(II) conformer. The results show that the solvent accessibilities of Tyr-103 and Trp-104, which comprise in part the hydrophobic box of α-LA, were identical in both apo and Ca(II) forms. On the other hand, the accessibilities of Tyr-18 and Tyr-36 were completely obliterated upon addition of 1:1 Ca(II). It is interesting to compare these results above with CIDNP studies of other Ca(II) proteins. Hincke et al. (1981) observed that Tyr-5, -111, and -150 were exposed in apo bovine cardiac troponin C but were buried in the Ca(II) form. They also found similar behavior with Tyr-10 and -109 in rabbit skeletal troponin C and Tyr-99 in bovine brain calmodulin. Perhaps the Ca(II)-induced changes in Tyr accessibility and the similarities in the structure of the Ca(II) binding sites (Berliner et al., 1983) are related.

The apo- to Ca(II)-α-LA transition is in slow exchange on the NMR time scale, in contrast to cation binding at secondary calcium sites. For the reasons outlined below, we suggest that manganese and cobalt (which also bind to other sites as well) showed overall fast exchange when binding to the weak secondary calcium site. The results may be explained as follows: Mn(II) binding to apo-α-LA (i.e., to the strong calcium site) was under slow exchange on the NMR time scale, resulting in less overall broadening. On the other hand, the potent effects of small concentrations of Mn(II) on Ca(II)-α-LA were consistent with Mn(II) binding to a second site(s) under *fast exchange* conditions [while the "first" metal site was occupied by Ca(II)]. The extraordinary spectral changes observed after addition of 1:1 Ca(II) to 180 μM Mn(II):2.1 mM apo-α-LA (Figure 4d) reflected the Ca(II) ion *displacing* Mn(II) from the first site (calcium site) to the weaker faster exchanging secondary site(s). Lastly, the recovery of the 0.30 ppm line upon addition of excess Ca(II) revealed that Ca(II) also binds, albeit weakly, to a secondary site common to Mn(II) as verified earlier by ESR experiments (Murakami et al., 1982). It was also clear from these experiments that there were probably multiple secondary sites for Mn(II) since only the 0.30 ppm line recovered upon excess Ca(II) addition. Unfortunately, the possibility that multiple Mn(II) binding occurs at the fast exchange sites precludes any accurate meaningful distance determinations between, e.g., the various His protons and the secondary cation site(s).

These effects were remarkably similar with Co(II) in this respect since this line also showed "recovery" with excess Ca(II). The results suggest that *at least* one of the Co(II) binding sites is the same as the secondary Mn(II) and Ca(II) sites. [Note that we concluded above that the first and second sites for Mn(II) were the same as those for Ca(II), respectively, since the behavior of the 0.30 ppm line was clearly sensitive to secondary site binding By Mn(II) or calcium]. Lastly, the −0.52 ppm line noted above must be one of the protons closest to the Co(II) binding site by virtue of the substantial Co(II)-induced shifts (Figure 6).

Several cations in the lanthanide series bind strongly to the strong calcium site of bovine α-LA as shown from competition studies by monitoring the ^1H spectral shifts (or broadening). Since cation binding to the strong Ca(II) site was always under slow exchange conditions, it was difficult to observe selective effects with relaxation agents. Shift reagents can be useful in both the slow and fast exchange regions as reported for several calcium binding proteins by Lee and Sykes (1981, 1982). However, we could not identify nearby residues to the calcium (lanthanide) site since the relative contact shift contributions appeared to be quite large [especially with Dy(III)] and not precisely known. Unfortunately, we could not determine accurate assignments nor distances since the contact and dipolar contributions to the spectral changes are not well enough understood at present for adequate resonance assignments or distance calculations. On the other hand, the smaller ionic radii of Mn(II) and Yb(III) vs. Ca(II) may account for their relatively higher K_d values. One case where a correlation was found between ionic radius and binding affinity was in Kwan et al. (1975).

The multiple resonance lines for the C-2 resonance of His-68 were difficult to understand for quite a long time by ours as well as other laboratories (H. J. Vogel, personal communication). We now realize that the minor His line is closely related to differences between apo- and Ca(II)-α-LA. In particular, the two "apo conformers" represent a partitioning between thermally unfolded (7.93 ppm) and native (7.86 ppm) conformers in Figure 4a (L. J. Berliner and K. Koga, unpublished results). Another example where two His lines corresponded to two conformers of a protein was reported by Birdsall et al. (1984) for *Lactobacillus casei* dihydrofolate reductase.

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Registry No. Al, 7429-90-5; Mn, 7439-96-5; Ca, 7440-70-2; Co, 7440-48-4; Dy, 7429-91-6; Tb, 7440-27-9; Pr, 7440-10-0; Yb, 7440-64-4.

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