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Probing Different Conformational States of Bovine α-Lactalbumin: Fluorescence Studies with 4,4′-Bis[1-(phenylamino)-8-naphthalenesulfonate]†

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ABSTRACT: The binding of the fluorescent probe 4,4′-bis[1-(phenylamino)-8-naphthalenesulfonate] (bis-ANS) to bovine α-lactalbumin (α-LA) was investigated. A strong dependence of the K_d value with the bound calcium stoichiometry was found, with K_d values ranging from 6.2 ± 0.4 to 64.6 ± 5.9 μM for apo-α-LA and 1:1 Ca(II)–α-LA, respectively. A 350-fold enhancement of the bis-ANS emission was observed in the protein–bis-ANS complex, along with an ~33-nm blue shift. Both appeared to be related to the hydrophobicity of the binding site and were *independent* of the Ca(II) ion content. From the difference in bis-ANS affinity between apo-α-LA and Ca(II)–α-LA, we demonstrated that Zn(II) and Al(III) were able to "lock" the protein into a new "apo-like" conformation, which was *similar* to, but not *identical* with, the apo conformation. The protein could be interconverted between all three conformations in a Mn(II) titration. The first Mn(II) shifted the apoprotein to the Ca(II) conformation; at higher Mn(II) levels, binding to the second site shifted the protein toward the apo-like conformation. The same behavior was observed with calcium in *large* excess. The evidence supported a model for the mutually nonexclusive binding of metals both to site I ("calcium site") and to site II ("zinc site") simultaneously. The results suggest that α-lactalbumin possesses a hydrophobic surface that becomes somewhat less accessible upon 1:1 calcium binding in the *absence* of metals that also bind to the zinc site.

The protein α-lactalbumin (α-LA)[1] is the modifier of the "lactose synthase" complex (UDPgalactose:D-glucose 4-β-D-galactosyltransferase, EC 2.4.1.22). Upon binding to the enzyme galactosyltransferase (EC 2.4.1.38), the catalytically active complex specifies the synthesis of lactose by galactosyl transfer to acceptor glucose, instead of terminal GlcNAc residues. Although putative three-dimensional structures of α-LA have been inferred from its high primary structural homologies to that of hen egg white lysozyme (Browne et al., 1969; Warme et al., 1974), no crystal structure has been reported to date.

A primary objective of our research has been to understand how α-LA interacts with galactosyltransferase, i.e., what region of the α-LA structure is involved in this interaction, the metal ion requirements for complex formation under physiological conditions, and whether any conformational transitions occur during the binding of α-LA to galactosyltransferase. It is known from previous studies that apo-α-LA undergoes a conformational change upon calcium binding or upon zinc binding to the Ca(II) form at a different, but distinct site (Murakami et al., 1982; Murakami & Berliner, 1983). Lindahl & Vogel (1984), using phenyl-Sepharose affinity chromatography, recently suggested that a hydrophobic surface is exposed in apo-α-LA that disappears upon calcium binding,

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[1] Abbreviations: bis-ANS, 4,4′-bis[1-(phenylamino)-8-naphthalenesulfonate]; α-LA, α-lactalbumin; GlcNAc, *N*-acetyl-D-glucosamine; ANS, 1-(phenylamino)-8-naphthalenesulfonate; EDTA, ethylenediaminetetraacetic acid.

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in contrast to that of other calcium binding proteins. They used this property to selectively purify apo-α-LA from Ca(II)-α-LA and postulated that this apolar surface on α-LA might be physiologically important in its binding to galactosyltransferase. Berliner et al. (1984) also identified a hydrophobic region near the active site of galactosyltransferase that overlapped the α-LA binding locus.

Bis-ANS, the dimeric form of ANS, is a useful fluorophore that is frequently employed to probe hydrophobic sites on proteins. An increase in quantum yield and emission blue shift are generally assumed as evidence for binding to an apolar site (Brand & Gohlke, 1972; Rosen & Weber, 1969). Unfortunately, there were conflicting reports on the interaction of the monomer, ANS, with α-LA (Rawitch, 1974; Versee & Barel, 1976; Rawitch & Hwan, 1979; Mulqueen & Kronman, 1982). We have shown recently with thrombin that bis-ANS bound more effectively than the monomer, ANS, and was particularly sensitive to subtle conformational effects (Musci et al., 1985). In this work, we have investigated the binding of bis-ANS to α-LA, with particular regard to the differences between the apo and the calcium forms of the protein and to the interactions between the two principal cation sites of the protein.

Materials and Methods

Proteins. Bovine α-LA was from Sigma Chemical Co. (lot 52F-8075-1), which typically contained 0.34 mol of Ca(II)/mol of protein (untreated Sigma α-LA). The apo form was prepared by repeatedly passing the protein down a column of tris(carboxymethyl)ethylenediamine. It contained less than 2% bound calcium, as estimated from atomic absorption and NMR (K. Koga and L. J. Berliner, unpublished results).

Chemicals. Bis-ANS (4,4′-bis[1-(phenylamino)-8-naphthalenesulfonic acid] dipotassium salt) was from Molecular Probes, Junction City, OR. Its concentration was estimated from the optical absorption at 385 nm, $\epsilon = 16\,790$ M^{-1} cm^{-1} (Farris et al., 1978). Ultrapure manganese chloride (99.999%, lot 0518) and zinc chloride (99.999%, lot 0208) were from Aldrich Chemical Co. Ultrapure EDTA (99+%, lot 011581) was from Alpha Products. All other reagents were of analytical grade and were used without further purification.

Methods. Fluorescence measurements were carried out on SLM Model 4800S and Perkin-Elmer Model MPF44A spectrofluorometers at 25 °C. Equilibrium binding data were fit by nonlinear regression analysis as noted earlier (Murakami et al., 1982). Protein concentration was estimated by optical absorption at 280 nm ($\epsilon = 2.01$ mg^{-1} mL^{-1}). Atomic absorption measurements were carried out on a Perkin-Elmer Model 360.

All experiments were measured at pH 7.4, 10 mM Tris buffer, in calcium-free H_2O, unless otherwise specified. All glassware, plasticware, and pipets were carefully prewashed as noted earlier (Murakami et al., 1982).

A fluorescence emission enhancement factor was defined as

$$EF = \frac{I_{max} \text{ of the } \alpha\text{-LA–bis-ANS complex}}{I_{obsd} \text{ of free bis-ANS}} \quad (1)$$

where I_{max} and I_{obsd} were the binary complex and free bis-ANS emission intensities, respectively, at the same concentration of bis-ANS.

Results

When a fixed concentration of bis-ANS was titrated with micromolar concentrations of apo-α-LA, a strong enhancement in the fluorescence emission intensity occurred along with a concomitant blue shift (~33 nm). Figure 1 depicts a typical titration [this type of titration is preferred over fixed α-LA with varying bis-ANS since inner filter effects can become severe at fluorophore concentrations above 10–15 μM (Musci et al., 1985)]. The hyperbolic curve in Figure 1 was fit to a single $K_d = 6.2 \pm 0.4$ μM. The fluorescence emission enhancement factor of the binary complex was 353 ± 6.

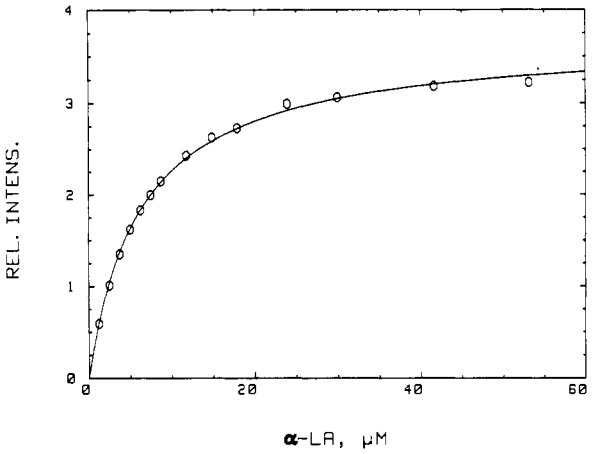

FIGURE 1: Apo-α-LA fluorescence titration of 1.05 μM bis-ANS at pH 7.4 (10 mM Tris-HCl). Fluorescence parameters were as follows: $\lambda_{ex} = 375$ nm, $\lambda_{em} = 482$ nm, 25 °C.

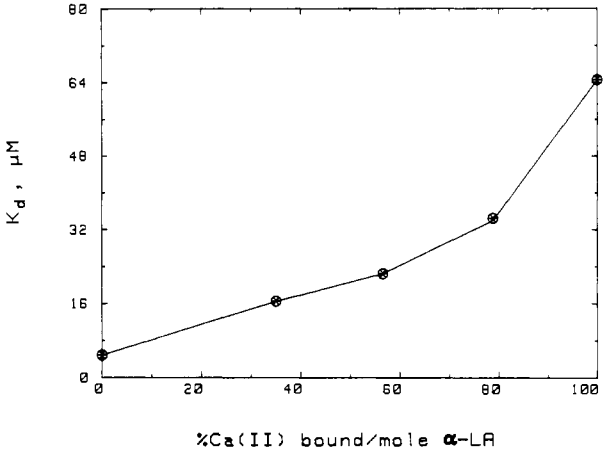

FIGURE 2: Plot of percent bound calcium vs. $K_{d(app)}$. Experimental conditions were identical with those in Figure 1.

When a similar titration was performed with calcium–α-LA (1:1), a 10-fold higher K_d (=64.6 ± 5.9 μM) value was found. As the calcium content was varied between 0% and 100% [mole of Ca(II) per mole of protein], intermediate $K_{d(app)}$ values were obtained as shown in Figure 2. The relationship is not linear since the $K_{d(app)}$ at intermediate Ca(II) values is actually a weighted average of two titrations [i.e., apo-α-LA plus Ca(II)-α-LA]. On the other hand, the enhancement factor was the same for the two forms of the protein.

In the presence of 5 mM EDTA, where the protein is metal free (Murakami et al., 1982), the K_d value was almost identical with that of the apo form.[2] A titration performed under slightly acidic conditions (pH 3.9, acetate buffer), where calcium should completely dissociate from the protein (Permyakov et al., 1982), also yielded a K_d value similar to that for the apo form at pH 7.4, suggesting that bis-ANS binding was independent of pH over this range. In order to graphically

[2] The K_d for apo-α-LA was slightly higher, due to the few percent (1–5%) of residual calcium present in the apo sample that was difficult to overcome when working at micromolar protein concentrations. This K_d value actually represents a weighted average of pure apo-α-LA and a small contribution of Ca(II)-α-LA.

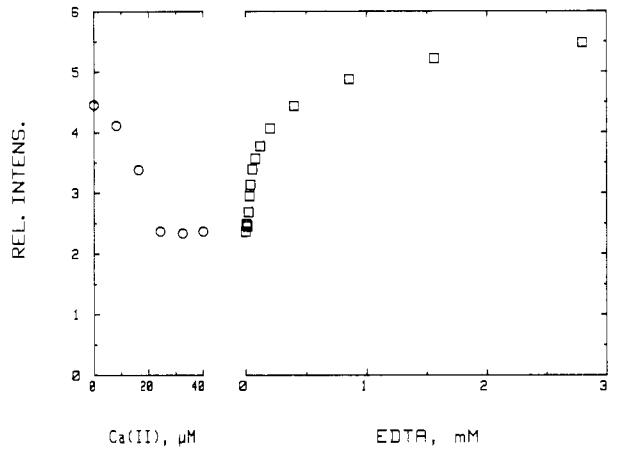

FIGURE 3: Ca(II)–EDTA fluorescence titration of bis-ANS, 1.05 μM, and Sigma α-LA (37.2 μM). Experimental conditions were identical with those in Figure 1.

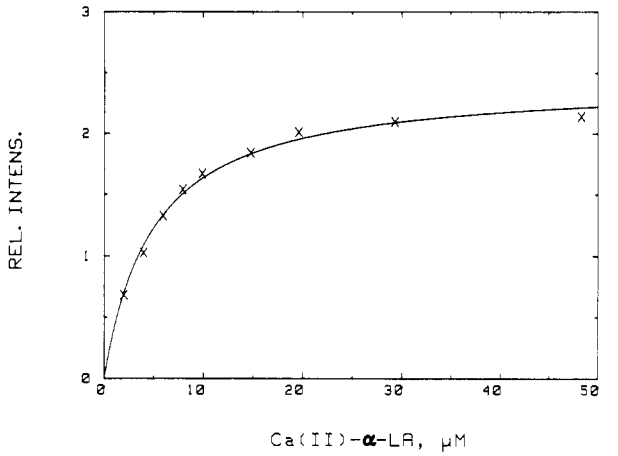

FIGURE 4: Ca(II)–α-LA fluorescence titration of 1.05 μM bis-ANS in the presence of 0.4 mM Zn(II). Experimental conditions were identical with those in Figure 1.

demonstrate the reversibility of the bis-ANS binding to α-LA, we took a partially saturated protein–dye complex with untreated Sigma α-LA (~34% calcium bound) and followed the change in fluorescence intensity upon addition of Ca(II) (up to 1:1) followed by EDTA addition to remove the bound metal ion. As shown in Figure 3, addition of calcium caused a sharp decrease in the observed intensity; upon subsequent EDTA addition, the fluorescence intensity increased to a final intensity that was *greater* than the starting point, since about 0.3 equiv of calcium/mol of α-LA was present in the starting sample. In all cases, λ_{em}^{max} and λ_{ex}^{max} were identical.

In order to verify that bound calcium affects only the *affinity* of the protein for bis-ANS, but not the enhancement factor, we measured the emission fluorescence intensities of the apo and the Ca(II) forms at the same protein and dye concentrations. These intensities may be measured from the titrations shown in Figure 3. The EDTA titration was fit to give a maximum intensity value for the calcium-depleted (apo) α-LA. For a fixed bis-ANS concentration the expression for observed fluorescence intensity, I_{obsd}, is

$$I_{obsd} = \frac{I_{max}[\alpha\text{-LA}]}{K_d + [\alpha\text{-LA}]} \quad (2)$$

where α-LA refers to either apo-α-LA or Ca(II)–α-LA; I_{max} and I_{obsd} were as defined earlier. For the apo and Ca(II) forms, we thus have

$$\frac{I_{obsd}^{apo}}{I_{obsd}^{Ca(II)}} = \frac{I_{max}^{apo}[\text{apo-}\alpha\text{-LA}]}{K_d^{apo} + [\text{apo-}\alpha\text{-LA}]} \frac{K_d^{Ca(II)} + [\text{Ca(II)-}\alpha\text{-LA}]}{I_{max}^{Ca(II)}[\text{Ca(II)-}\alpha\text{-LA}]} \quad (3)$$

When also [apo-α-LA] = [Ca(II–α-LA] = [α-LA], rearranging the expression simplifies to

$$\frac{I_{max}^{apo}}{I_{max}^{Ca(II)}} = \frac{I_{obsd}^{apo}}{I_{obsd}^{Ca(II)}} \frac{K_d^{apo} + [\alpha\text{-LA}]}{K_d^{Ca(II)} + [\alpha\text{-LA}]} \quad (4)$$

For the experiment shown in Figure 3 ([α-LA] = 37.2 μM), we measured an experimental $I_{obsd}^{apo}/I_{obsd}^{Ca(II)}$ value of 2.3. From the calculated K_d values for apo-α-LA and calcium–α-LA, we obtained $I_{max}^{apo}/I_{max}^{Ca(II)} = 1.0 \pm 0.05$. Since the bis-ANS concentration was the same in both cases, this ratio also represents the ratio between the enhancement factors of apo-α-LA and Ca(II)–α-LA. Actually, one can calculate the calcium content of any α-LA sample in the presence of a fixed concentration of bis-ANS using these relationships above.

The finding of an apparent difference in the hydrophobic nature of apo-α-LA and calcium–α-LA, as suggested by their different K_d values, led us to question whether the apoprotein was the only conformer of the protein that exhibited strong bis-ANS binding (i.e., K_d = 4–7 μM). Specifically, we were interested in the effect of Zn(II) on the bis-ANS–Ca(II)–α-LA or bis-ANS–apo-α-LA fluorescence. In Figure 4 we show a titration at fixed bis-ANS with Ca(II)–α-LA (1:1) in the presence of (saturating) 0.4 mM Zn(II) (Murakami & Berliner, 1983). The curve fit to a $K_d = 4.8 \pm 1.2$ μM, a value very close to that of the apo form (Figure 1). This result could be due to calcium release induced either by zinc binding, yielding the Zn(II)–apo form, or by shifting the calcium-bound protein toward an apo-like conformation, where the bis-ANS binding site was in some ways comparable to that of the apo conformer. In order to ascertain whether or not the calcium was bound in the presence of zinc, we exhaustively dialyzed a sample of Ca(II)–α-LA (1:1) against ultrapure 0.4 mM $ZnCl_2$ (pH 7.4) and then checked the calcium content by atomic absorption. A 1:1 Ca(II):α-LA stoichiometry was found before and after dialysis, which was consistent with a model where Zn(II) may bind simultaneously with Ca(II).

Table I: Fluorescence Parameters for Bis-ANS Complexes with Different α-LA Conformers[a]

species	K_d (μM)	EF
apo-α-LA	6.2 ± 0.4	353 ± 6
α-LA, pH 3.9	4.9 ± 1.6	320 ± 45
α-LA-EDTA	3.9 ± 0.2	239 ± 5[b]
Ca(II)–α-LA (1:1)	64.6 ± 5.9	353 ± 6
Ca(II)–Zn(II)–α-LA	4.8 ± 1.2	237 ± 17[c]
Zn(II)–α-LA	4.8 ± 1.8	440 ± 50[c]
Al(III)–α-LA	2.5 ± 0.3	237 ± 7[d]
Mn(II)–α-LA	18.9 ± 4.3[e]	234 ± 18[e]

[a] Titrations were carried out by varying the protein at a fixed concentration (1.05 μM) of bis-ANS, pH 7.4, and 10 mM Tris, 25 °C. [b] This value is probably underestimated since EDTA has been shown to be a weak quenching agent at concentrations above 1–2 mM (E. A. Permyakov, K. Murakami, and L. J. Berliner, unpublished results). [c] $[ZnCl_2]$ = 0.4 mM. [d] $[AlCl_3]$ = 0.12 mM. [e] Since at a concentration of 1.6 mM the second Mn(II) site was only 59% saturated (Murakami et al., 1982), the K_d value represents an average of Ca(II) form and apo-like form.

The K_d values for bis-ANS binding to apo-α-LA, Zn(II)–α-LA, or Ca(II)–Zn(II)–α-LA are listed in Table I. While these values were indistinguishable (K_d = 4.8–6.2 μM), subtle *differences* were found in their enhancement factors. This parameter ranged from ~350 for apo-α-LA to ~440 for Zn(II)–α-LA to ~240 for Ca(II)–Zn(II)–α-LA. These differences were significant as they were well outside of the error range of the binding curve fits and may reflect differing degrees of solvent access to the bound fluorophore (Lakowicz,

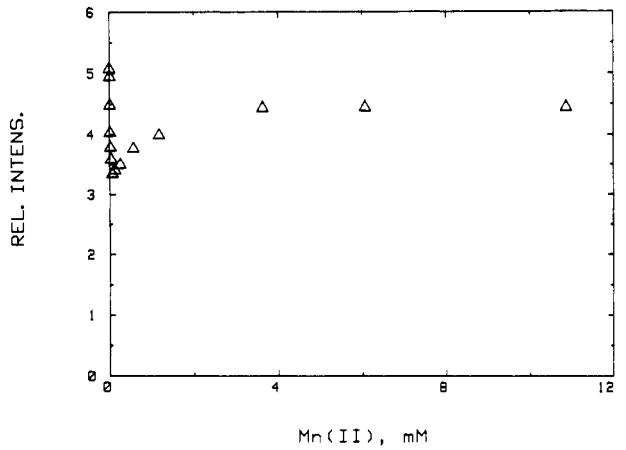

FIGURE 5: Effect of Mn(II) on the fluorescence intensity of the bis-ANS–α-LA complex. Bis-ANS, 1.05 μM; Sigma α-LA, 24.9 μM. Experimental conditions were identical with those in Figure 1.

1983). Also, addition of 1 equiv of $CaCl_2$ to Zn(II)–α-LA resulted in a *decrease* of the fluorescence emission intensity, consistent with the difference in quantum yield between Zn(II)–α-LA and Ca(II)–Zn(II)–α-LA–bis-ANS complexes (Table I). On the other hand, zinc addition to apo-α-LA–bis-ANS resulted in an enhanced fluorescence intensity of the complex. In parallel experiments (not shown), the apparent K_d for Zn(II) binding to α-LA was measured by monitoring bis-ANS fluorescence; K_d values were in the range of 12–14 μM, regardless of the calcium content, in good agreement with the previous value measured by intrinsic fluorescence (Murakami & Berliner, 1983). Aluminum ion at saturating concentrations (Murakami & Berliner, 1983) was found to behave similarly to zinc; however, the Al(III)–α-LA–bis-ANS complex fluorescence enhancement factor was different from other apo forms or apo-like forms of the protein.

The results with manganese binding to the bis-ANS–α-LA complex demonstrably confirm the above model. Mn(II) is known to bind to the calcium site with high affinity (K_d = 31 μM) and to a second site (possibly the zinc site) with moderate affinity (K_d = 1 mM), and to two additional very weak sites (Murakami et al., 1982). Figure 5 depicts a titration of an untreated Sigma [34% Ca(II)] α-LA–bis-ANS complex with increasing amounts of Mn(II). Note the sharp decrease in the fluorescence intensity in the first phase (where manganese saturated the calcium site) and then a gradual increase in intensity as the Mn(II) binds to the second low-affinity (zinc) site (and presumably to other sites). That is, sequential occupancy of site I (calcium site) and then site II (zinc site) would shift the protein from apo- to Ca(II)- to apo-like conformations as manifested first in a decrease and then in an increase in the α-LA–bis-ANS fluorescence. We also measured the bis-ANS binding constant to Sigma α-LA in the presence of 1.6 mM Mn(II). The intermediate K_d value of 18.9 ± 4.3 μM and enhancement factor (=234 ± 18) suggested that, at the higher manganese concentrations, the binding to sites other than the two principal metal binding sites (Murakami et al., 1982) had some effect on the conformational state of α-LA. An experiment similar to that above, using 2 mM calcium instead of manganese, yielded similar K_d and EF values to those with manganese. In other words, both calcium and manganese are able to *reverse* their effect on α-LA–bis-ANS affinity at higher cation concentrations where they bind to site II (and possibly other low-affinity sites).

Discussion

The remarkable utility of bis-ANS fluorescence to discover and discriminate between different conformers of α-LA has allowed us to draw several conclusions from these experiments. Consistent with previous reports and suggestions of a very hydrophobic locus on α-LA (Lindahl & Vogel, 1984; Mulqueen & Kronman, 1982), we found evidence for a site whose affinity and solvent exposure depend on the binding of specific metal ions to the protein. Specifically, the affinity for bis-ANS changed by 10-fold upon binding 1 equiv of Ca(II) to demetallized apo-α-LA. Since the enhancement factor remained unchanged, bis-ANS fluorescence may be utilized as a convenient analytical method to assess the extent of Ca(II) substitution in an α-LA sample. While we did not examine other cations, e.g., lanthanides, which bind strongly and stoichiometrically to the calcium site [other than Mn(II)], it is reasonable to expect that their K_d and EF parameters would be identical with that for Ca(II)–α-LA (1:1), paralleling their identical effects on α-LA intrinsic fluorescence (Murakami et al., 1982). The results presented here for cation binding to the zinc site clearly suggested that not only was occupation of this site possible with simultaneous occupation of the calcium site but also binding to this site alters the calcium-bound protein to a new apo-like conformation that differs from apo-α-LA in the bis-ANS complex enhancement factor (Table I). Binding to the zinc site, in contrast to calcium site binding, appeared to depend upon the specific cation, as shown earlier by intrinsic fluorescence (Murakami & Berliner, 1983). This was evident here by differences in enhancement factors between various cations that bind (or presumably bind) to this site, such as Zn(II), Al(III), and Mn(II) (see Table I). Reports of subtle variations in metalloprotein conformation with the ligand preference and ionic radius of the cation have been made for other systems. For example, Kwan et al. (1978) correlated differences in pyruvate kinase activity upon the *extent* to which each particular cation binding "adjusted" the protein conformation. Thus, it would appear for α-LA that zinc binding "fine tunes" the conformation of the apo or Ca(II) forms to slightly different degrees, as manifested in the enhancement factors of the various species as the bis-ANS complexes (see Table I). The aluminum-bound conformation, on the other hand, differed slightly as evidenced by the much smaller enhancement factor for Al(III)- vs. Zn(II)–apo-α-LA. Overall, these results suggest that, while the binding of a metal ion to site II shifts the protein to an apo-like conformation, this conformation is not exactly *identical* with the apo form. In fact, the apo-like structure retains a small but still detectable sensitivity to calcium binding, as the differences between Ca(II)–Zn(II)–α-LA and Zn(II)–α-LA enhancement factors indicate.

The sensitivity to subtle differences in cation binding was evident only after utilizing bis-ANS as a probe. It is now understandable why the conclusions made earlier from monitoring α-LA intrinsic fluorescence could not discriminate between various cations that bind to the second (zinc) site and why Murakami & Berliner (1983) suggested mutually *exclusive* binding at sites I and II as the simplest model to explain their data. While it is clear from the experiments reported here that both calcium and zinc site specific cations may bind simultaneously (apo-like conformation), it is likely that their respective K_d values are larger in the doubly bound species.[3]

[3] While the intrinsic fluorescence titrations of Murakami & Berliner (1983) at high Mn(II) or Ca(II) never revealed a reversal toward apo conformation fluorescence parameters, careful examination of these data showed that the protein approached 95–98% rather than a 100% "calcium" conformation. This would be consistent with altered K_d values for sites I and II and a small (2–5%) concentration of site II only bound species.

What was also remarkable here was the observation that calcium (or manganese), at concentrations high relative to site I binding, also occupied a secondary site(s)—quite likely the zinc site—and mimicked Zn(II) by "reversing" the effect on bis-ANS affinity and quantum yield caused by the initial mole of Ca(II); i.e., the protein shifted toward the apo-like conformation. This was also consistent with effects of Mn(II) or various lanthanides at very high concentrations on the elution behavior of α-LA from a phenyl-Sepharose column (Lindahl & Vogel, 1984). In analogy with the retention of a calcium ion in site I after addition of zinc (as confirmed by atomic absorption), in the case of Mn(II) binding to α-LA we may assume that site I remained occupied at higher Mn(II) concentrations where the secondary site(s) became saturated. It is also important to note that bis-ANS proved to be both a more sensitive and less troublesome probe than the monomer fluorophore, ANS. While ANS probably binds only to α-LA aggregates (Rawitch & Hwan, 1979; Mulqueen & Kronman, 1982), bis-ANS was a sensitive probe of monomeric α-LA. (At the protein concentration and pH conditions of our experiments, the presence of any soluble aggregates may be excluded.)

Lastly, it is interesting to speculate on the physiological state and possible function of these respective α-LA species. If we extrapolate the secreted concentrations of unliganded Zn(II), ~50 μM, and Ca(II), 1–5 mM, in milk (Altman & Dittmer, 1971) to the site of lactose biosynthesis in the mammary cell Golgi apparatus (Hill & Brew, 1975), we may conclude that both the free Zn(II) and Ca(II) levels contribute to maintaining α-LA in an apo-like conformation. It will be interesting to learn whether this latter, more apolar-like conformation of the protein is related to the hydrophobic α-LA binding locus on the galactosyltransferase.

Added in Proof

The cation Cd(II) was most demonstrative of metal ion binding to both site I and site II as evidenced by first a decrease in α-LA–bis-ANS emission intensity (site I saturation) followed by a *complete* reverse to that of the apo-like α-LA complex (site II saturation).

Acknowledgments

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Registry No. Bis-ANS, 63741-13-9; Ca, 7440-70-2; Zn, 7440-66-6; Al, 7429-90-5; Mn, 7439-96-5.

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