A Distinct Zinc Binding Site in the α-Lactalbumins Regulates Calcium Binding. Is There a Physiological Role for This Control?†

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ABSTRACT: A distinct zinc binding site has been found in several α-lactalbumin species: bovine, human, guinea pig, and rabbit. Binding of Zn(II) or Al(III) to the calcium forms of these proteins causes exclusion of calcium and return of the protein to its "apo conformation" as determined by fluorescence emission spectral parameters. Zn(II) and Al(III) dissociation constants are in the low micromolar range. In addition, determinations of Zn(II) binding were made by electron spin resonance by observing free unliganded Mn(II), which was displaced upon Zn(II) binding. Co(II) and Cu(II) were also shown to bind to the zinc site while also expelling Ca(II). The most appropriate model that describes cation binding to α-lactalbumins is of two physically distinct but *mutually exclusive* sites for calcium and zinc, respectively, where the protein cannot bind cations at both sites simultaneously. Kinetic parameters for lactose biosynthesis show absolutely *no difference* between the apo or Zn(II) and Ca(II) forms of α-lactalbumin. At physiological concentrations of zinc (~50 μM) and calcium (~1 mM), a ca. 40% rate enhancement due to calcium was observed, which was totally accounted for by calcium activation of galactosyl transferase. While either conformer of α-lactalbumin [Ca(II) or Zn(II)] is kinetically equivalent, the Ca(II) form probably dominates under physiological conditions.

The protein α-lactalbumin (α-LA)[1] is the noncatalytic regulatory subunit of the "lactose synthase" complex. The association of α-LA with galactosyl transferase imparts a change in specificity of the latter enzyme from terminal *N*-acetylglucosaminyl acceptors to glucose. Our previous work with several α-lactalbumin species documented the extremely strong binding of calcium and several lanthanides ($K_{diss} \simeq 10^{-10}$–10^{-12} M) at a specific site, which caused a unique conformational change monitored by a distinct blue shift in the intrinsic fluorescence spectra (Murakami et al., 1982). This calcium site also bound Mn(II), Cd(II), and Mg(II), albeit with a weaker affinity. Since the concentration range of free calcium in milk is at least at the 1–5 mM level (Altman & Dittmer, 1971), it might seem straightforward that the calcium form of α-lactalbumin should be the physiologically important conformer. The results presented here with Zn(II), Co(II), and other metals confirm the presence of a second cation site that displays competitive behavior with the Ca(II) site for the "apo conformation" of the protein. The metals Zn(II) and Co(II) are also primary cation activators of the galactosyl transferase (O'Keefe et al., 1980a). Since the physiological concentration of Zn(II) is well above both its equilibrium dissociation constant with α-LA and its K_m value for galactosyl transferase activation, we have also examined the role or Zn(II) and Ca(II) in lactose biosynthesis.

Experimental Procedures

Proteins. Electrophoretically pure bovine α-LA (lot 50F8105) was from Sigma Chemical Co. Other α-LA species were obtained or isolated as noted earlier (Berliner & Kaptein, 1981). Apo-bovine α-LA was prepared by the procedures noted in our earlier paper (Murakami et al., 1982). Calcium α-LA species were prepared by dialyzing apoproteins or EGTA-treated proteins against excess $CaCl_2$, followed by dialysis. Galactosyl transferase was purified as reported previously to specific activities of 16–20 units mg^{-1} at 30 °C (Grunwald & Berliner, 1978).

Chemicals. Ultrapure cation salts from either Aldrich Chemical Co. or Alfa Products were used throughout (Murakami et al., 1982). All other reagents were from the same sources reported earlier (Andree & Berliner, 1980).

Methods. Fluorescence measurements were made on Perkin-Elmer MPF-44A and SLM Instruments 4800 spectrofluorometers. ESR experiments were carried out on a Varian E-4. The spectral and data analysis methods were described in an earlier paper (Murakami et al., 1982).

Lactose biosynthesis was measured spectrophotometrically by a variation of the "UDP generation" or "split" assay of Grunwald & Berliner (1978). The following substrates were incubated with galactosyl transferase at 37°C, 0.02 M (Chelex 100 treated) Tris-HCl, pH 7.4: UDP-Gal (464 μM), glucose (19 mM), apo-BLA or Ca(II)–BLA (4.2 μM = 60 μg/mL), at varying concentrations of $ZnCl_2$. This was called the *incubation mix*. Timed 400-μL aliquots of this reaction (*incubation mix*) were measured for UDP production with a pyruvate kinase/lactate dehydrogenase coupled *assay mix* (800 μL), which contained $MgCl_2$ (5.2 mM), phosphoenolpyruvate (157 μM), and NADH (127 μM) under the same buffer conditions. The resultant "burst" of UDP was calculated by extrapolating back to zero time. This method was accurate for $ZnCl_2$ concentrations up to 60 μM. The final concentrations of Ca(II) were well below those levels for pyruvate kinase inhibition (Kwan et al., 1975).

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[1] Abbreviations: α-LA, α-lactalbumin; BLA, bovine α-lactalbumin; HLA, human α-lactalbumin; GPLA, guinea pig α-lactalbumin; RLA, rabbit α-lactalbumin; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; ESR, electron spin resonance; UDP-Gal, uridine diphosphate galactose; q_{obsd}^{360nm}, 1 – [observed α-LA intensity (360 nm)]/[apo-α-LA intensity (360 nm)]; q_{max}^{360nm}, 1 – [Ca(II)–α-LA intensity (360 nm)]/[apo-α-LA intensity (360 nm)]; % *q*, $q^{360nm} \times 100$; Tris, tris(hydroxymethyl)aminomethane; NMR, nuclear magnetic resonance.

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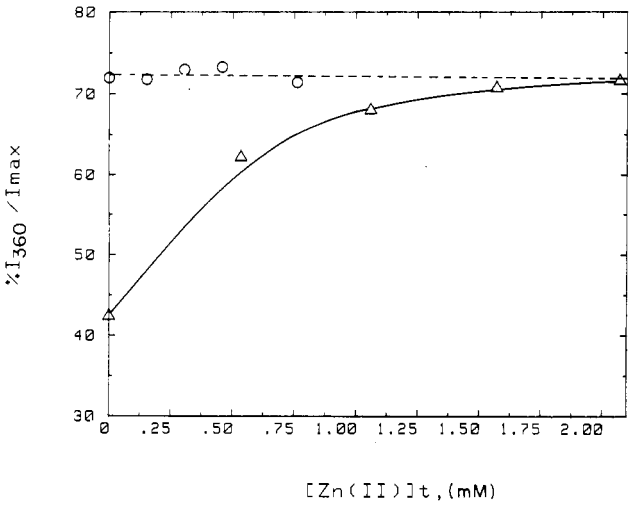

FIGURE 1: Fluorescence titration of $ZnCl_2$ binding to bovine α-LA. Zn(II) titration of 5 μM apo-BLA (○) and 2.7 μM Ca(II)–BLA (1:1) (△), K_{diss}(app) ≃ 0.4 mM. Conditions were pH 7.4, 0.02 M Tris-HCl, and 26 ± 0.5 °C. The parameter % I_{360}/I_{max}, defined in the text, was measured from the intrinsic fluorescence emission spectrum (λ_{ex} = 280 nm).

Results

Evidence for Distinct Zn(II) Binding. When calcium–BLA was titrated with $ZnCl_2$, the fluorescence line-shape parameter % I_{360}/I_{max} (which is the ratio of the emission intensity at 360 nm to the intensity at the emission maximum wavelength) returned to that of the apoprotein (see solid line, Figure 1). Furthermore, the λ^{em}_{max} shifted from the calcium-bound value of 324 nm to that of the apoprotein, 338 nm. If apo-BLA was titrated with Zn(II), absolutely no change in either the line-shape parameter % I_{360}/I_{max} or the λ^{em}_{max} (see dotted line, Figure 1) occurred. A time dependence for Zn(II) binding was observed with a $t_{1/2} \leq 1$ min. This apparent Zn(II)/Ca(II) competition is more evident in calcium titrations against fixed Zn(II)/BLA mixtures. Here, the % I_{360}/I_{max} parameter started from the apo-BLA value of 74% and approached the Ca(II)–BLA value of 37%; likewise, the λ^{em}_{max} shifted from 338 to 324 nm. The results are summarized in Table I.

Manganese cation, which also binds to the Ca(II) site of α-LA, may be monitored by both fluorescence and ESR (Murakami et al., 1982). We measured Mn(II) binding to this strong cation site in the presence of varying fixed concentrations of Zn(II). From analyses of double-reciprocal plots, a K_{diss}(Zn(II)) of 5.6 μM was calculated, based on a simple competitive binding model using K_{diss}(Mn(II)) = 31.7 μM (Murakami et al., 1982).

Direct evidence for Zn(II) displacement of Mn(II) is shown in Figure 2, where we have plotted the displacement of bound Mn(II) with increasing Zn(II) concentration. The parameter θ is a direct measure by ESR of displaced Mn(II) from the Ca(II)/Mn(II) site (Murakami et al., 1982). This Mn(II) displacement was also consistent with a K_{diss} in the same range as that obtained by fluorescence (see Table I). In other experiments (not shown), we started with Ca(II)-loaded BLA (1:1), which contained Mn(II) bound only at the secondary weaker sites (Murakami et al., 1982). The relatively small change in free Mn(II) concentration with increasing Zn(II) was consistent with minor alterations in Mn(II) weak site binding (Murakami et al., 1982).

Zn(II) Binding to Other α-Lactalbumin Species. In order to verify whether this apparent Zn(II) competition for Ca(II)–α-LA was, in fact, a general phenomenon in α-lactalbumins, we examined several species by fluorescence spectroscopy. Table I summarizes their behavior as based on emission maxima and fluorescence line-shape parameters. For human α-LA (HLA), a Zn(II) titration of Ca(II)–HLA displayed qualitatively the same behavior as that for Ca(II)–BLA in Figure 1. The parameter % I_{360}/I_{max} recovered

Table I: Zn(II) Binding to α-Lactalbumin Species[a]

form	species: bovine (BLA)	human (HLA)	guinea pig (GPLA)	rabbit (RLA)[e]
Ca(II)				
λ^{em}_{max} (nm)	324	327	323	329
% I_{360}/I_{max}	37.1	45.1	30	47
% q[b]	71.1	59.8	64.7	~0
K_{diss}(Ca(II)) (nM)	0.2	0.03	0.005	
apo				
λ^{em}_{max} (nm)	338	338	332	330.5
% I_{360}/I_{max}	74.5	70	58	49.9
Zn(II)				
λ^{em}_{max} (nm)	338	338	332	332
% I_{360}/I_{max}	74	70	57	56.1
% q[b]	0	0	64.7[c]	~0[c]
K_{diss}(Zn(II)) (μM)	5.6	1–10[d]	0.5–5[d]	

[a] pH 7.4, 0.02 M Tris-HCl, 26 °C; λ_{ex} = 280 nm. The data for Ca(II)-BLA, -HLA, and -GPLA and apo-BLA, -HLA, and -GPLA are from Murakami et al. (1982) unless otherwise noted. The zinc data were extrapolated to saturating Zn(II) when precipitation occurred at Zn(II) concentrations ≥ 1 mM. [b] Relative to the emission intensity (360 nm) of the apo form. [c] The fluorescence intensity (I_{360nm}) was identical with that of Ca(II)–α-LA while I_{max} actually decreased. [d] Approximate values, calculated from a Zn(II) titration of Ca(II)-HLA (1:1) or Ca(II)-GPLA (1:1) on the basis of $[Ca(II)]_{free}$ derived from the corresponding BLA experiments. [e] Rabbit α-LA, while interchangeable with other α-LA species and galactosyl transferases in catalyzing lactose synthesis (B. G. Conery and L. J. Berliner, unpublished results), has a sequence substitution of a His residue for Tyr-103 that results in major differences in the NMR and fluorescence behavior of Trp-104 and -60 (Berliner & Kaptein, 1981; see Table I). Furthermore, speculations about Trp-118, compared with the conformational analysis of its state in BLA, suggest that it is more mobile, exposed, and consequently less sensitive to conformational changes induced by cation (calcium) binding (Berliner & Kaptein, 1981).

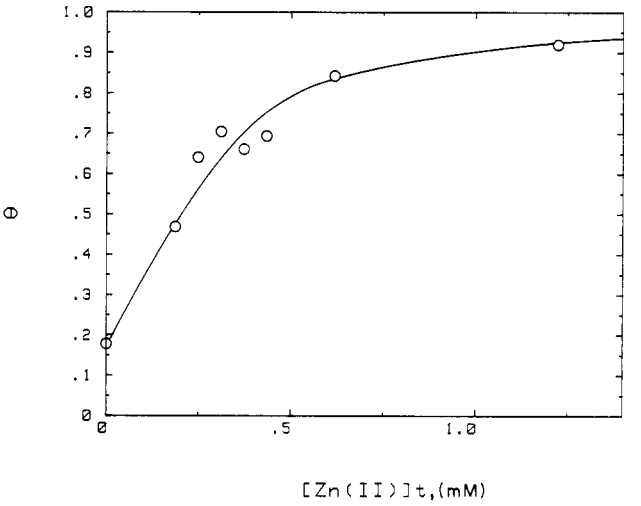

FIGURE 2: Zn(II) titration of Mn(II) displacement by ESR with Mn(II)-loaded BLA: [Mn(II)] = 0.4 mM; [BLA] = 0.37 mM. The parameter θ, which has been described earlier (Murakami et al., 1982), represents the free fraction of total $MnCl_2$. Any residual bound Mn(II) at saturating Zn(II) must reside in one of the secondary weak sites (K_{diss} = 1.1 or 5.0 mM) since the calcium site cannot bind cations when the zinc site is occupied (Murakami et al., 1982). All other conditions were identical with those in Figure 1.

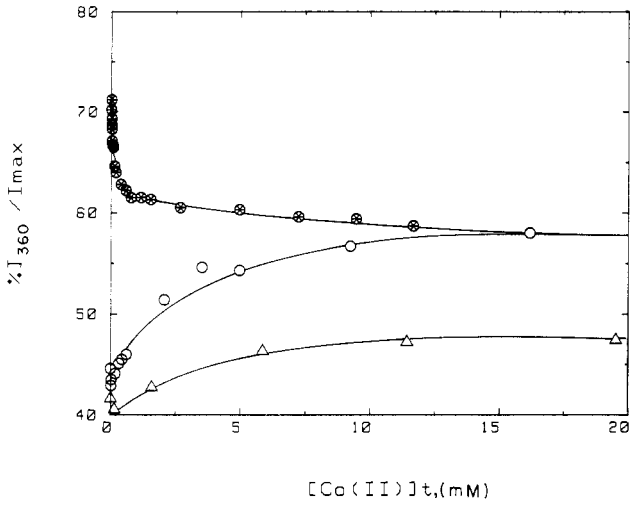

FIGURE 3: Fluorescence titration of $CoCl_2$ binding to bovine α-LA. All conditions were identical with those in Figure 1 unless otherwise noted. Co(II) titration of 9 μM apo-BLA (⊗), 7 μM Ca(II)–BLA (1:1) (○), and Mn(II)-loaded BLA([Mn(II)] = 90 μM, [BLA] = 7 μM) (△). A K_{diss} for Co(II)–BLA of 0.14 ± 0.02 mM was calculated. We also measured Co(II) displacement of Mn(II) from BLA by ESR where a similar K_{diss} for Co(II) binding was obtained.

from the Ca(II)-bound value to that of the apoprotein. In the case of guinea pig α-LA, the line-shape parameter returned toward the apoprotein value, but the maximum fluorescence intensity, I_{max}, actually *decreased*, while the 360-nm band remained constant (see Table I). This was the first evidence reflecting the fact that the Zn(II)-bound forms of these proteins are by definition *not* apoproteins, even though the fluorescence monitors (λ^{em}_{max}, % I_{360}/I_{max}) suggested an "apo-α-LA" conformation. We also examined rabbit α-LA, which displayed small but significant changes in quantum yield upon Ca(II) or Zn(II) binding, respectively, but essentially undetectable changes in emission maxima (see Table I).

Binding of Other Metals to the Zn(II) Site. We examined several other cations with bovine α-LA that might exhibit similar ligand binding behavior to that of Zn(II). When monitoring Al(III) binding to BLA by fluorescence spectroscopy, we observed identical behavior with that exhibited by Zn(II) in Figure 1. The Al(III)-bound protein exhibited precisely the same λ^{em}_{max}, % I_{360}/I_{max}, and % q values as observed for Zn(II)–BLA or apo-BLA (see Table II). The dissociation constant for Al(III) was not measured by as many methods (e.g., see Figures 1 and 2) but appeared to be *smaller* than the value of 5.6 μM determined for Zn(II). A much slower time dependence, $t_{1/2} \simeq 30$ m, was observed for Al(III)–BLA binding.

When titrating either apo-BLA or Ca(II)–BLA with Co(II), the fluorescence line-shape parameter % I_{360}/I_{max} approached 58%, a value intermediate between the apo or Ca(II)-bound conformation (Figure 3). The λ^{em}_{max} of 332 nm was also in between either conformation (see Table I). When Co(II)-loaded BLA was titrated with Zn(II) (not shown), a complete return to the apo conformation was observed, suggesting competition between Zn(II) and Co(II). The other notable feature of the Co(II)-bound protein was the decreased quantum yield as monitored by % q_{360nm}, which is 62.2% of the apoprotein emission intensity at this wavelength.

Cu(II) was also found to quench BLA fluorescence upon binding and, like Co(II), shifted the % I_{360}/I_{max} ratio to an essentially intermediate value. The estimation of λ^{em}_{max} for saturated Cu(II)–BLA was less accurate for this cation due to substantial quenching as well as absorption and inner filter effects.[2] The results for these other cations are summarized in Table II.

Table II: Binding of Other Metals to Bovine α-Lactalbumin[a]

	Al(III)	Co(II)[b]	Cu(II)
λ^{em}_{max} (nm)	338	332	333.5
% I_{360}/I_{max}	74.5	58	60
% q[c]	0	37.8[d]	68 ± 3[e]
K_{diss} (μM)	1–7	137 ± 18	6.8 ± 0.8

[a] pH 7.4, 0.02 M Tris-HCl, 26 °C; λ_{ex} = 280 nm. The parameters were extrapolated to saturating cation when precipitation occurred, e.g., with Cu(II). [b] We also titrated Ca(II)–GPLA (1:1) with Co(II). At 12.5 mM Co(II), the following parameters were measured: λ^{em}_{max} = 323 nm, % I_{360}/I_{max} = 32, and % q = 74 [57% due to "Zn(II) site" binding, 17% due to Co(II) absorption/inner filter effects and to Co(II) energy transfer or contact quenching]. [c] Relative to the emission intensity (360 nm) of the apo form. [d] Since Co(II) also absorbs at 280 nm, this value is somewhat overestimated due to a Co(II) inner filter effect. For 0.99 mM $CoCl_2$, 12% of the observed quenching was due to absorption/inner filter effects. [e] Since Cu(II) also absorbs at 280 nm, this value is somewhat overestimated due to a Cu(II) inner filter effect. For 83.6 μM $CuCl_2$, 19% of the observed quenching was due to absorption/inner filter effects.

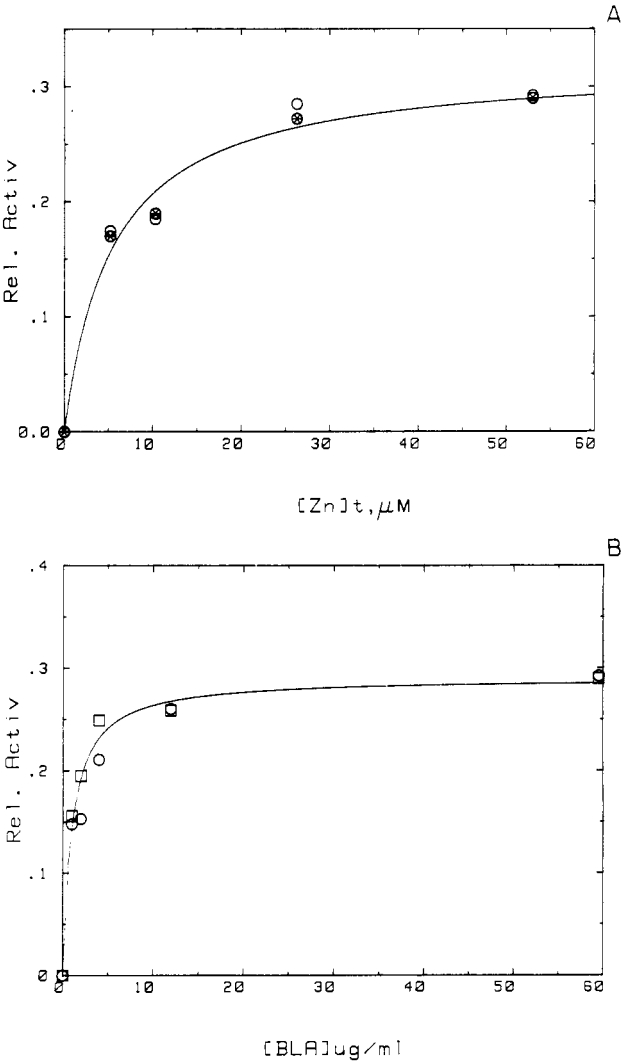

FIGURE 4: Kinetics of lactose biosynthesis. (A) Zn(II) dependence with 60 μg/mL apo-BLA (○) or Ca(II)–BLA (⊗). The theoretical curve is fit to a K_m(app) = 5.6 μM. (B) α-Lactalbumin dependence at 53 μM $ZnCl_2$ for either apo-BLA (□) or Ca(II)–BLA (○). The theoretical curve is fit to a K_m(app) = 1 μg/mL. Conditions were 0.02 M Tris-HCl, pH 7.4, and 37 °C. See Experimental Procedures for details of the assay.

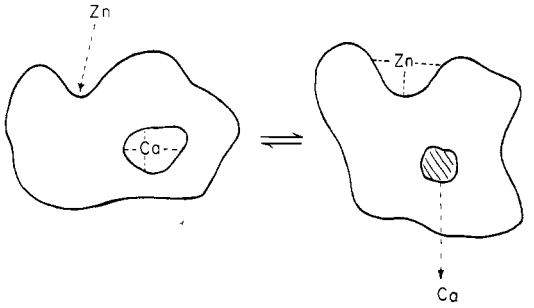

FIGURE 5: Two-site competitive binding model with distinct (but not independent) sites for Ca(II) [Mn(II), Cd(II), Mg(II), Tb(III), Dy(III), Yb(III), Gd(III), Eu(III)] and Zn(II) [Al(III), Co(II), Cu(II)], respectively. As indicated, binding at one site excludes binding at the other via conformational changes.

Cation Effects on Lactose Biosynthesis. Figure 4 depicts the effects of Zn(II)- and Ca(II)-BLA on lactose synthesis. The data in Figure 4A indicate that with either apo-BLA (○) or Ca(II)-BLA (⊗), Zn(II) has the identical K_m(app) and V_{max}. In Figure 4B, where apo-BLA (□) or Ca(II)-BLA (○) are varied at fixed $ZnCl_2$ concentration, it is obvious that both α-LA conformers have the same K_m(app) = 0.07 μM in the lactose synthase complex. It should also be noted that the Ca(II)-BLA in either experiment (Figure 4) does *not* shift to the Zn(II) form at the levels of $ZnCl_2$ used (see Figure 1). In order to more closely mimic physiological conditions, we compared rates of lactose synthesis in the ranges Zn(II) = 50 μM (Murthy, 1974) and Ca(II) = 0, 0.1, and 1.0 mM, where we obtained relative velocities of 1.00, 1.25, and 1.38, respectively. While soluble ionic Ca(II) concentrations in milk are *at least* in the 1–5 mM range, it is pertinent to note that even at Ca(II) = 0.1 mM, all α-lactalbumins are totally in the calcium form (see Figure 1 and Table I).

Discussion

Evidence for a Distinct Zinc Site. The model in Figure 5, which depicts a zinc site that is physically distinct from the calcium site, is supported by several pieces of evidence from this work. When a cation binds at one site, the resultant conformational change excludes binding to the other site (i.e., competitive inhibition where the two sites are distinct). All metals that bound to apo-α-LA at the calcium site [Ca(II), Mn(II), Cd(II), Mg(II), Tb(III), Dy(III), Yb(III), Gd(III), and Eu(III)] caused *precisely* the same fluorescence blue shift in λ^{em}_{max}, % I_{360}/I_{max}, and % q.[3] These cations were also competitive with Mn(II) by ESR (Murakami et al., 1982). Furthermore, Ca(II), Mn(II), and several of the lanthanides impart specific effects on the ^{1}H NMR of His-68 in BLA (H. Nishikawa and L. J. Berliner, unpublished results).

Zn(II) or Al(III) titrations of Ca(II)- or Mn(II)-protein caused a complete recovery to apo-α-LA fluorescence parameters: λ^{em}_{max}, % I_{360}/I_{max}, and % q (see Table I). Titrations of Zn(II) binding to apo-BLA caused no change in fluorescence parameters (Figure 1).[4] The same results were observed with Al(III) in place of Zn(II).

The evidence presented for Co(II) and Cu(II) binding suggests that these two cations also bind to the zinc site and displace Ca(II) as in Figure 5. Co(II) or Cu(II) binding to Ca(II)-BLA shifted λ^{em}_{max}, % I_{360}/I_{max}, and % q toward the apo-α-LA conformation (see Figure 3). Co(II) binding to apo-BLA approached precisely the same fluorescence parameters reached in titrating Ca(II)-BLA (see Table II), supporting the model that Ca(II) was excluded from binding to the protein upon Co(II) binding. Zn(II) titrations of Co(II)-saturated BLA restored the protein to the apo-BLA fluorescence parameters, displaying Zn(II)/Co(II) competition (Table II). Co(II) binding to BLA quenched fluorescence by some (energy transfer?) mechanism unique to the bound Co(II). On the other hand, cations that bind at the calcium site quench intrinsic fluorescence *solely* through a conformational change (Murakami et al., 1982). While Cu(II) binding to proteins was generally less discriminating than other cations, the fluorescence behavior and strong K_{diss} (see Table II) indicated a more specific Cu(II) binding in this case, its behavior quite similar to that for Co(II).[5]

Further physical confirmation of the differences between the calcium and zinc sites comes from the elution behavior of BLA on phenyl-Sepharose columns when bound with metals that prefer the calcium site vs. apo-BLA or metals that prefer the zinc site (H. J. Vogel, private communication of unpublished results).

Physiological Roles of Zn(II) and Ca(II) in Lactose Biosynthesis. The results of Figure 4 demonstrated that there was no difference in K_m(app) and V_{max} for Zn(II) due to the state of α-LA [apo or Ca(II) conformer]. Furthermore, the K_m(app) and V_{max} of both apo-BLA and Ca(II)-BLA were identical with those of Zn(II) as the metal ion substrate. The K_m(app) of 5.6 μM for Zn(II) was somewhat smaller than its reported K_m of 37 μM in the *N*-acetyllactosamine reaction (O'Keefe et al., 1980a); however, a similar difference was noted by O'Keefe et al. (1980b) with Co(II) as substrate, where a value of 6 μM was derived in the lactose synthase reaction vs. a K_m = 68 μM in the *N*-acetyllactosamine reaction (O'Keefe et al., 1980a).

While it was clear that the rate activation in the presence of 1 mM Ca(II) was ca. 1.4 times the rate in the absence of calcium, this was not due to "kinetic" differences between apo-α-lactalbumins and Ca(II)-α-lactalbumins (see Figure 4). Instead, this enhancement resulted from direct Ca(II) activation of *galactosyl transferase* as found earlier by O'Keefe

[2] We were unable to measure an accurate extinction coefficient in the 320–400-nm range for Co(II)- or Cu(II)-BLA complexes, which must have been in the range of ca. 0–10 M^{-1} cm^{-1}. Consequently, the distance between the Zn(II) [Co(II), Cu(II)] and those Trp that are quenched may be small. In the case of Co(II) or Cu(II), there may comprise two mechanisms: a conformational change toward the apo form plus a small contribution of energy transfer quenching. In both cases, the λ^{em}_{max} was blue shifted with respect to apo-BLA (see Table II), which could be accounted for by specific (energy transfer) quenching of a red tryptophan (i.e., Trp-118).

[3] In fact, the calcium site must be quite distant from all of the Trp in this molecule to account for the total lack of energy transfer quenching by Mn(II) and the lanthanides.

[4] While this was perhaps a fortuitous coincidence with BLA and HLA, the clearest evidence that the Zn(II)-bound protein differed from the apo form was in the case of GPLA. Although λ^{em}_{max} and I_{360}/I_{max} were identical with those for apo-GPLA, the overall emission intensity was quenched relative to that for the apoprotein (Table I). It is pertinent to note that, relative to all other α-LA species, GPLA has an amino acid substitution of a Phe for Trp-60, a residue that resides close enough to Trp-104 for nuclear spin cross polarization and intertryptophan fluorescence energy transfer (Berliner & Kaptein, 1981). Similarly, RLA, which was quenched by Zn(II), has substituted a His at Tyr-103, which has major consequences to the environment of Trp-104 (Berliner & Kaptein, 1981).

[5] In the case of Co(II) and Cu(II) binding, one could also propose distinct cobalt (and/or copper) binding sites. However, such a complex model requires "three-way" interactions between the cobalt (or copper) site and the zinc and calcium sites. Both the unnecessary complexity of such a three- (or more) site model and the many similarities between Zn(II)-, Al(III)-, Co(II)-, and Cu(II)-BLA fluorescence behavior lend strong support to the two-site model in Figure 5.

et al. (1980a) with Zn(II), Mn(II), or Co(II) as the essential cation. Since both Mn(II) and Co(II) are present at submicromolar to micromolar levels in milk, only Zn(II) exists at physiological levels of the order of its K_m (Murthy, 1974). Of course, without exact knowledge of the concentrations of these cations in the Golgi apparatus of the lactating mammary cell, extrapolation of these cation levels in colostrum or milk to the site of biosynthesis cannot be made precisely. However, Zn(II) and Ca(II) are the only two cations at satisfactorily high *overall* levels that are both known to effect galactosyl transferase (and lactose synthase) activity. Under the physiologically relevant conditions studied here, the Ca(II) form of α-LA was the dominant conformer; thus, the physiological role or Zn(II) is *only* its catalytic activation of the galactosyl transferase.

Some Generalizations about the Calcium and Zinc Binding Sites. A precise description of the two distinct cation sites in the *solution* structure of α-LA requires correlative and complementary studies by a variety of techniques including NMR, ESR, and fluorescence spectroscopy. However, we can make some generalizations at this point that will be refined by our magnetic resonance results in progress (K. Murakami, H. Nishikawa, and L. J. Berliner, unpublished experiments). Calcium binding induces a conformational change that causes distinct Trp fluorescence shifts and quenching (Murakami et al., 1982; see also Table I), a slow exchange conformational ^{1}H NMR shift of His-68, and a distinct paramagnetic broadening of His-68 protons upon Mn(II) binding at the calcium site (H. Nishikawa and L. J. Berliner, unpublished results). The nature of the calcium site itself has been established by ^{113}Cd NMR and Mn(II) ESR, which correlates with an octahedral, totally oxygen liganded binding site, thus identical by these criteria with the well-classified binding proteins such as troponin C (L. J. Berliner, P. D. Ellis, and K. Murakami, unpublished results). When comparing λ_{max}^{em} for apo-GPLA against apo-BLA, apo-HLA, or apo-goat α-LA (Murakami et al., 1982), GPLA is clearly "blue shifted" relative to the latter species (see Table I). Considering also that in GPLA a substitution at Trp-60 by a Phe resulted in a substantial increase in overall Trp quantum yield (Sommers & Kronman, 1980), it follows that the shifted λ_{max}^{em} for GPLA was due either to a lack of red tryptophans (i.e., Trp-60) or to a dominance in quantum yield of blue vs. red tryptophans. The latter explanation appears more plausible since structural considerations dictate internal energy transfer quenching from Trp-26 and -104 to Trp-60 (Sommers & Kronman, 1980) as confirmed by nuclear spin cross polarization between Trp-26 and -104 (Berliner & Kaptein, 1981). Thus the fluorescence changes upon Ca(II) binding were consistent with the conformationally induced quenching of a common (red) tryptophan, i.e., Trp-118, yielding a net blue-shifted spectrum (Table I).

Zinc binding was associated with fluorescence parameters that mimicked almost exactly those for the apo conformer; the only exceptions were in % q for GPLA and RLA (Table I). These exceptions were in fact the first pieces of evidence that indicated that there must indeed be some (local) structural change accompanying Zn(II) binding, which may later lend some insight into the location of this site. Since Zn(II) is essentially ineffective as an energy transfer acceptor, while Co(II) and Cu(II) are effective contact or energy transfer quenching agents, it is probable that the mechanism of Zn(II)-induced fluorescence changes is the result of conformational changes, especially in light of the complete *reversal* of Ca(II) binding to BLA or HLA. In the case of GPLA or RLA, overall fluorescence intensity *decreased* upon Zn(II) binding. The associated conformational change must have quenched higher energy (blue-shifted) tryptophans, which would quench the entire spectrum. The only candidates would be Trp-26 and/or Trp-104.

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Registry No. Zinc, 7440-66-6; calcium, 7440-70-2; manganese, 7439-96-5; aluminum, 7429-90-5; cobalt, 7440-48-4; copper, 7440-50-8; lactose, 63-42-3; galactosyltransferase, 9031-68-9.

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