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Zinc Binding in Bovine α-Lactalbumin: Sequence Homology May Not Be a Predictor of Subtle Functional Features

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ABSTRACT **α-Lactalbumin (α-LA), a calcium-binding protein, also possesses zinc-binding sites comprising a single strong site and several weaker secondary sites. The only site found by X-ray crystallography (Ren et. al., J. Biol. Chem. 1993;268:19292) was Glu 49 of human α-LA, but zinc binding had never been measured in solution for human α-LA. This residue was genetically substituted by Ala in bovine α-LA and the metal-binding properties of the resulting desMetE49A protein were compared with those for native α-LA by fluorescence methods. Surprisingly, desMetE49A α-LA and the native bovine protein had similar affinities for both Zn^{2+} and Ca^{2+}. Genetic substitution of other possible candidates for Zn^{2+} chelating residues, which included Glu 25, did not alter the affinity of bovine α-LA to Zn2+; however, substitution of Glu 1 by Met resulted in the disappearance of strong Zn^{2+} binding. A proposed site involves Glu 1, Glu 7, Asp 11, and Asp 37, which would participate in strong Zn^{2+} binding based on their propinquity to Glu 1. Human α-LA, which has a Lys at position 1 rather than Glu, binds zinc with a reduced affinity compared with native bovine α-LA, suggesting that the site identified from the X-ray structure did not correspond to strong zinc binding in solution. Proteins 2000;40:106–111.**

Key words: α-lactalbumin; site-directed mutagenesis; zinc binding; calcium binding; thermal denaturation

INTRODUCTION

Frequently, studies on homologous proteins, or between species isoforms of the same protein, are considered valid for all other members of the family. In the case of α-LA, where X-ray structures exist for five species (baboon, human, goat, guinea pig, and buffalo) and two recombinant forms (bovine and goat), the apparent commonality leads one to generalize regarding their structure and function. For example, the few amino acid replacements appear to be mostly conservative, the recombinant forms differ only by an N-terminal methionine from the native form (i.e., bovine and goat), and the number of absolutely conserved residues is impressively high. All species strongly bind calcium, promote lactose biosynthesis, and possess quite similar thermal properties. α-LA possesses one strong calcium binding site[1,2] and multiple zinc binding sites with the strongest zinc site having an affinity constant of $5 \times 10^5\ M^{-1}$.[3–5] After the initial article of Permyakov et al.[5] on zinc binding as monitored by intrinsic fluorescence, it appeared that α-LAs from many species had similar, if not identical zinc binding site(s).

We recently showed that the N-formyl methionyl residue, commonly found on recombinant α-LAs, disrupts the folding of this protein and alters metal binding properties.[6] Furthermore, we recently discovered that we can enzymatically remove the terminal methionyl residue and the protein regains the properties of α-LA isolated from milk. Alternatively, we can eliminate the terminal Glu1, leaving the methionyl as its replacement in the recombinant proteins. These modifications to the N-terminus allow recombinant bovine α-LA to fold correctly and enable the examination of metal binding properties and thermal stabilities.[7]

In the X-ray structure of human α-LA the zinc is sandwiched between Glu 49 and Glu 116 of the symmetry related subunit in the dimeric crystal unit cell.[8] For this reason, the X-ray determined Zn^{2+} site was thought to be the strongest one. Later, Permyakov and Berliner dissected this zinc binding with bovine α-LA into a single strong ($K_d \approx 1\ \mu M$) site and several secondary sites by using the hydrophobic fluorophore, bis-ANS, as a monitor of zinc induced structural changes.[9] The intramolecular distance between the strong zinc and calcium sites, as measured by Forster energy transfer studies, was 14–18 Å using Co^{2+}as acceptor and Tb^{3+}as donor, respectively.[9] This distance agreed well with the 17.5 Å found in the human α-LA X-ray structure. However, there were several other aspects that raised questions about the interpreta-

Abbreviations: α-LA, α-lactalbumin; desMetE49A and desMetE25A, the E49A and E25A mutants of bovine α-LA, respectively, in which the extra N-terminal methionine was enzymatically removed; HEPES, N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid]; EGTA, ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; bis-ANS, {4,4′-bis[1-(phenylamino)-8-naphthalene sulfonic acid]} dipotassium salt; PDB, Protein Data Bank.

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tion of the zinc site from the X-ray results. (a) A 5 mM concentration of Zn^{2+} was used in the crystal soaking solution. This concentration was several orders of magnitude greater than the measured affinity for the strong site in bovine α-LA, but this concentration corresponded well to the low affinity site(s) that lead to protein aggregation.[5] (b) No strong zinc affinity of the order found for the bovine species has been reported for human α-LA. (c) The requirement that aggregated (dimeric) human α-LA is requisite for zinc binding is inconsistent with solution measurements where bovine α-LA was in the monomeric form.[9]

In the present study we use mutagenesis to explore the location of strong Zn^{2+} binding sites in bovine α-LA. Our approach was to engineer substitutions of key side chains implied or suggested from X-ray structures of human and other α-LA species as a guide. The expected result would consequently lead to a drastically decreased affinity for Zn^{2+} ions.

MATERIALS AND METHODS

Materials

Bovine α-LA (lot 128F-8140) was purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were reagent grade or better. Solutions were prepared from double-distilled, demineralized water. Protein concentrations were evaluated spectrophotometrically by using an extinction coefficient of $E_{1\%,\ 280\ nm} = 20.1$.[10] Bis-ANS was purchased from Molecular Probes (Eugene, OR); concentrations were estimated from optical absorption at 385 nm, $\epsilon = 16{,}790\ M^{-1}cm^{-1}$.[11]

Recombinant bovine α-LAs were prepared as described previously.[12] Mutations (E25A, E49A, and E1M) were prepared by the method of Kunkel.[13] In previous publications E1M was called ΔE1, where the E1 residue of the native sequence is removed, leaving the N-terminal methionine in its place.[6] The proteins were characterized for conformation and purity by absorption spectroscopy,[12] intrinsic fluorescence,[1–3] and SDS gel electrophoresis under reducing/nonreducing conditions[14] and size-exclusion HPLC on a Millipore Protein PAK 125 column. The N-terminal Met of E25A and E49A bovine α-LAs was removed by treatment with *A. proteolytica* aminopeptidase as previously described.[7] Briefly, aminopeptidase (6 μL of 0.37 U/μL solution) was added to a 1 mL solution of protein (1 mg/ml) in 10 mM HEPES, pH 8.0, and incubated for 2 h at 37°C. Excess EDTA was added to stop the reaction. The post-transitionally cleaved recombinant proteins were separated by gel filtration on Sephadex G-50 eluted with 10 mM HEPES, 1 mM $CaCl_2$, pH 8.1. The protein-containing fractions were collected and dialyzed against 10 mM ammonium bicarbonate and subsequently lyophilized. The completeness of the enzymatic removal of the N-terminal methionine and deformylation was previously confirmed by mass spectrometry.[7]

Instrumentation

Fluorescence measurements were performed on a Perkin-Elmer LS-50B and a homemade spectrofluorimeter as described earlier.[15] Protein fluorescence was excited at 280 nm. All spectra were corrected for instrument spectral sensitivity. Temperature scans were performed stepwise, allowing the sample to equilibrate at each temperature for at least 5 min. The temperature was monitored directly inside the cell. The fractional conversion from the native to the thermally denatured state was calculated from plots of the temperature dependence of emission intensity at a fixed wavelength as previously described.[16]

Calcium-binding affinity was measured spectrofluorimetrically by EGTA titrations as described earlier.[7] The binding affinity for zinc ions was determined by monitoring the fluorescence of a bis-ANS:α—LA complex, which is a sensitive reporter of strong zinc binding.[5] Bis-ANS was excited at 365–385 nm. The binding of Zn^{2+} results in an increase of bis-ANS fluorescence emission intensity. Typically, a zinc titration curve is fit to (at least) two steps, which appear to correlate with a sequential complexation of zinc ions at different affinity sites. The fluorescence data are fit to the following scheme:

$$P + Zn^{2+} \overset{K_1}{\Leftrightarrow} P \cdot Zn$$

$$P \cdot Zn + nZn^{2+} \overset{K_2}{\Leftrightarrow} P \cdot Zn_{n+1} \quad (1)$$

where P is protein and K_1 and K_2 are best fit apparent binding constants for two classes of zinc binding sites.[5,9,17]

RESULTS

Recombinant α-LAs contain an N-terminal methionine residue.[7] Previous work has shown that these methionyl proteins have a lower affinity for calcium and lower thermal stability than the native protein. Enzymatic removal of this N-terminal Met increases both the calcium binding affinity and thermal stability. Therefore, by providing a "native-like" N-terminus, we can prepare mutant α-LAs that have properties similar to the milk-isolated protein.

desMetE49A Bovine α-LA

Because the Zn^{2+} binding site of human α-LA specifically involves chelation by the side chains of Glu 49 (and Glu 116), we constructed E49A bovine α-LA, removed the N-terminal methionine, and tested the ion-binding properties of the resultant desMetE49A protein. DesMetE49A bovine α-LA binds zinc at both the strong site and secondary site(s) with similar affinities to native α-LA (Fig. 1). This result implies that the unusual zinc site suggested by X-ray crystallography of human α-LA[8] is not a zinc binding site in bovine α-LA. Moreover, this also suggests that high-affinity zinc binding is not a consequence of protein dimerization.

In addition, the protein intrinsic fluorescence spectra of both apo- and calcium-loaded desMetE49A bovine α-LA were 2–3 nm red shifted compared with the native protein before the thermal transition. This probably means that some tryptophan residues in the desMetE49A mutant are

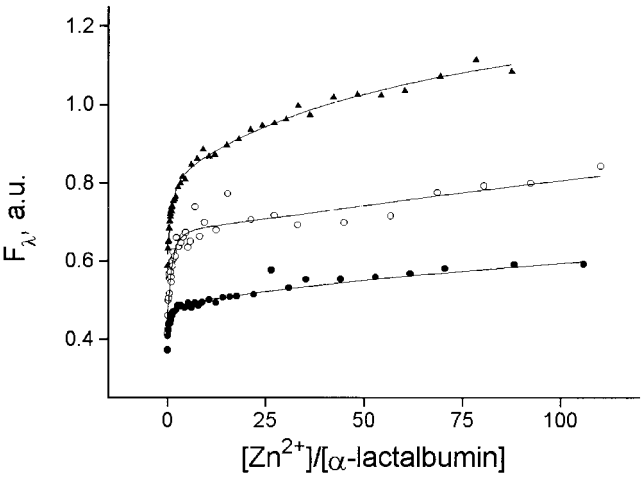

Fig. 1. Spectrofluorimetric Zn^{2+}-titration of desMetE49A (3.20 μM, ○), desMetE25A (2.15 μM, ●), and native bovine α-LA (6.06 μM, ▲) in 10 mM HEPES, 1 mM $CaCl_2$, [bis-ANS]/[α-LA] = 125, pH 7.8, at 21°C. Bis-ANS was excited at 385 nm, and the fluorescence signal was monitored at 508 nm for desMetE49A, 480 nm for desMetE25A, and 500 nm for native α-LA, respectively. Theoretical curves were obtained according to the sequential binding scheme (Scheme 1).

located in a slightly more mobile, polar environment and are (perhaps) more accessible to water than in the native protein. After the thermal denaturation transition the maximum positions of the fluorescence spectrum (λ_m) of the desMetE49A mutant and of the native protein are identical. Moreover, the desMetE49A mutant in both the apo- and calcium-loaded states is slightly less thermally stable than native α-LA, i.e., the thermal transition midpoint temperature (T_m) for the desMetE49A mutant is 2–4°C shifted to lower temperatures compared with the native protein. Table I summarizes the binding and fluorescence results for all proteins in this study. Consequently, the glutamic acid at position 49 is a contributor to the overall stability of the protein but is not a contributor to zinc chelation. This result led us to question the tentative identification by Ren et al.[8] of the zinc-binding site of human α-LA.

Additional Mutant Bovine α-Lactalbumins

Measurements of the fluorescence energy transfer showed that the distance between Tb^{3+} (strong calcium site) to Co^{2+} (strong zinc site of α-LA) is in the range of 14–18 Å.[9] Thus, as a criterion to identify other amino acid candidates as zinc chelation sites, we considered oxygen atoms of Asp and Glu side chains and imidazole nitrogens of His residues, which were located within these radii. Although zinc normally prefers sulfur and nitrogen ligands over carboxylates, α-LA contains no free cysteines and a single methionine in only one species. These criteria were applied to the three dimensional structures of buffalo (Acharya KR, unpublished data) and recombinant bovine α-LA (PDB 1HFZ). The advantage of using the structure of the native buffalo protein is that it differs from bovine α-LA at only two residues (positions 17 and 27), which appear to be conservative replacements. Although recombinant bovine α-LA is, in fact, derived from the native nucleic acid sequence, it contains the N-terminal methionine perturbation noted above and an additional M90V substitution.[18] Obviously, although neither structure is perfect, our choice of these structures was the best starting point to include the most likely candidates. The results of this search yielded the following carboxylate amino acid clusters as potential candidates: Glu 25, His 32, and His107, and Glu 1, Glu 7, Asp 11, Asp 37, and Asp 46, Glu 49, which we previously ruled out. We approached our identification with broad criteria allowing somewhat larger radii with cognizance that packing flexibility and conformational rearrangement in the solution state might result in overlooking a prime candidate.

To check the first site identified above, we prepared desMetE25A bovine α-LA, which had, in fact, a slightly increased zinc affinity compared with the native protein (Fig. 1). The fluorescence spectra of this mutant in both the native apo- or calcium-loaded states displayed a greater red shift than desMetE49A bovine α-LA, suggesting that it has a more relaxed (perhaps unfolded) structure, which is confirmed by its decreased thermal stability both in the presence and absence of calcium (Table I). Consequently, the glutamic acid at position 25 in bovine α-LA is not a contributor to zinc chelation. In addition, all of the mutant proteins studied here bound calcium with similar affinities, indicating a correctly folded, functional protein.

Previous investigators had not used the sensitive bis-ANS probe to monitor possible zinc binding in human α-LA. Thus, we performed bis-ANS zinc titrations, comparing the properties of native isolated α-LAs from human and bovine milk. As depicted in Figure 2 the titration curve shows human α-LA binds zinc with about an order of magnitude reduced affinity compared with native bovine α-LA. Overall, the results were somewhat dismaying because the distances between Glu 49 and Asp 46 were appropriate for zinc chelation on the same subunit in several species, despite the fact that this coordination did not appear in the human α-LA X-ray structure. These data revealed that the model for zinc binding in a homologous protein in a family (i.e., human α-LA) is not necessarily instructive for cation binding in other species.

The E1M bovine α-LA mutant was particularly of interest because it is known that human α-LA contains instead a Lys residue at position 1. Figure 2 shows the spectrofluorimetric Zn^{2+} titration curve for E1M bovine α-LA, which is compared with the two other mutants prepared in this study. The curve shows only some low affinity binding with a binding constant ($K \approx 10^3\ M^{-1}$, n = 1.7), which is several orders of magnitude lower than that observed for bovine α-LA. These numbers correspond to binding at secondary biologically nonrelevant sites. From these results we conclude that Glu 1 may be one of the critical residues for the strong Zn^{2+} binding site of bovine α-LA. Table I summarizes the binding and fluorescence results of each protein included in this study.

In conclusion, we propose Glu 1, Glu 7, Asp 11, and Asp 37 as a putative zinc-binding site on bovine α-LA. Because of the geometry and relevant distances between Glu 1 and

TABLE I. Comparison of the Metal-Binding and Thermal Stabilities of Native and Recombinant α-Lactalbumins[†]

Protein	K_{Ca} (M^{-1})	K_{Zn1} (M^{-1})	K_{Zn2} (M^{-1})	N	T_m (°C) +EGTA	T_m (°C) +Ca^{2+}	Fluorescence emission max., λ_m (nm) +EGTA 2°C	+EGTA 50°C	+Ca^{2+} 20°C	+Ca^{2+} 80°C
Bovine α-LA	$11.0 \cdot 10^7$	$4.3 \cdot 10^5$	$2.1 \cdot 10^3$	1.0	28	67.0	332.0	343.5	327.0	344.0
DesMetE49A	$3.9 \cdot 10^7$	$7.0 \cdot 10^5$	$1.9 \cdot 10^3$	1.5	23.5	65.5	335.5	343.5	329.0	342.0
DesMetE25A	$6.2 \cdot 10^7$	$24 \cdot 10^5$	$2.2 \cdot 10^3$	1.0	19.5	61.0	339.5	344.0	332.5	342.0
E1M	$7.5 \cdot 10^7$	n.d.[a]	$\approx 10^3$	n.d.	37.4	69.8	326.5	343.5	325.5	343.0
Human α-LA	$15.0 \cdot 10^7$	$0.7 \cdot 10^5$	$3.3 \cdot 10^3$	1.7	29	70.0	327.5	341.0	326.0	342.0

[†]Buffer was 10 mM HEPES-KOH, pH 7.8; 1 mM EGTA (+EGTA) or 1 mM $CaCl_2$ (+Ca^{2+}). Zinc binding was measured in the presence of 1 mM $CaCl_2$ at 21–24°C; calcium binding was measured at 40°C.
[a]n.d., not determinable.

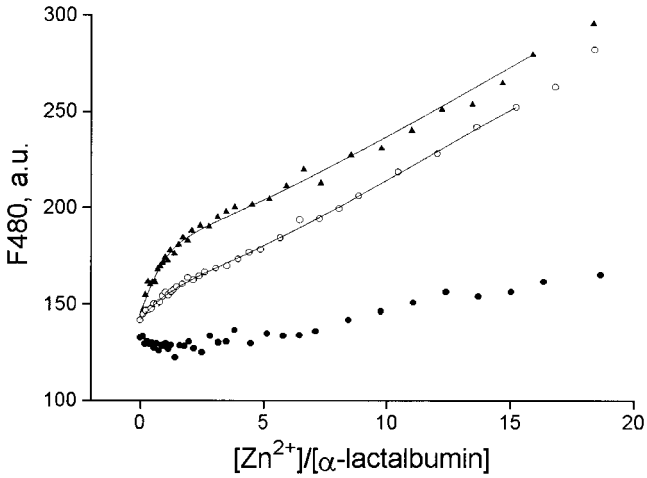

Fig. 2. Spectrofluorimetric Zn^{2+}-titration of E1M (4.55 μM, ●), native bovine (8.58 μM, ▲) and human (13.2 μM, ○) α-LA in 10 mM HEPES, 1 mM $CaCl_2$, [bis-ANS]/[α-LA] = 125, pH 7.9, at 24°C. Bis-ANS fluorescence was excited at 365 nm, and the fluorescence signal was monitored at 480 nm. Theoretical curves were obtained according to the sequential binding scheme (Scheme 1).

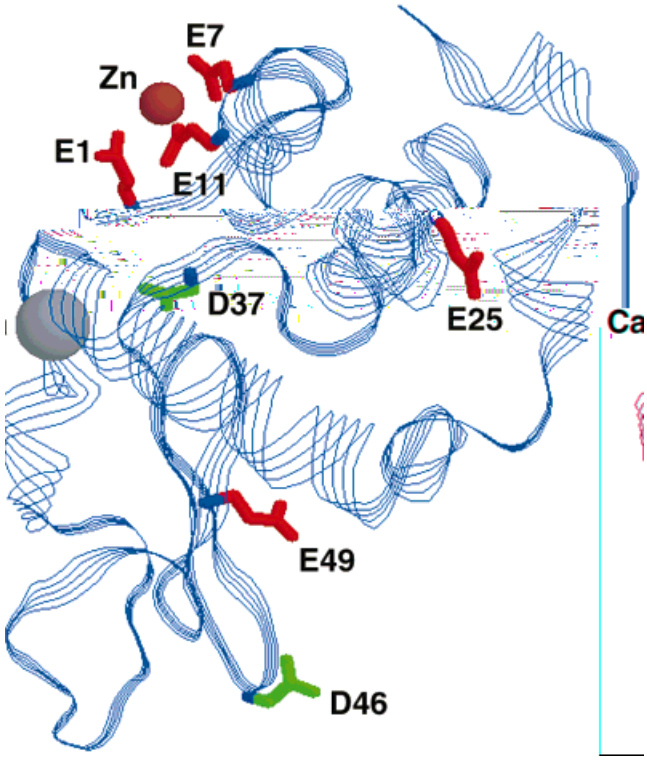

Fig. 3. Summary of the cation binding regions studied by site-specific mutation. The structure is adapted from the coordinates of buffalo α-lactalbumin (Acharya KR, personal communication) by using RasMOL molecular modeling software. The suggested putative zinc-binding cluster is shown near a zinc ion placed in the region of residues Glu 1, Glu 7, Asp 11, and Asp 37.

some or all of these three nearby carboxylate-containing residues, E1M bovine α-LA was an especially attractive residue to study by mutagenesis. Unfortunately, a native zinc-bound bovine α-LA X-ray structure has not been possible to date. Although the E1M mutant displayed significantly reduced (perhaps obliterated) coordination to the "strong" bovine α-LA zinc-binding site, it will, nonetheless, be prudent to sequentially mutate the other residues in this cluster to document their participation in zinc ligation. Figure 3 depicts a summary structural map based on the highly homologous buffalo α-LA structure that highlights the metal binding regions examined in this study. The putative site is highlighted by the placement of a Zn(II) cation near the residues suggested above. Whether the participation of Glu1 is by direct coordination or by a more complex, conformationally linked mechanism should be borne out by further mutational studies in this region. The contribution of peptide amide nitrogens may also be a factor that will require substantial NMR efforts in progress.

DISCUSSION

There are several lessons from this study about homologous families of proteins. The most apparent is that, although high sequence homology may dictate protein folding, it does not predict many subtle features about function. Certainly, the structural similarities with lysozyme have been well known despite their (evolutionarily divergent) functional differences. At a more subtle level, however, the unique binding of zinc and its modulation of bovine α-LA structure and lactose synthase activity[3,5] might have been presumed to exist in other species, such as human α-LA. A second lesson about extrapolating X-ray structural information to solution properties is particularly relevant to human α-LA where a consensus zinc-binding site was identified from a single X-ray structure

but never verified in solution. These results had been freely extrapolated to all species of α-LA. It is relevant to note that the X-ray article of Ren et al.[8] pointed out that zinc coordination might very well be species dependent.

Another aspect relates to the conditions required for ligand binding for crystal studies. The concentration of Zn^{2+} used in the human α-LA structure (5 mM) corresponded to multiple sites leading to aggregation in the solution studies with bovine α-LA. Ren et al.[8] also noted that any higher concentrations than 5 mM promoted aggregation of human α-LA. Considering that the "effective" α-LA concentration in a crystal approaches 100 mM, one needs little more than a stoichiometric amount of Zn^{2+} if the binding constant is in the 0.1–1 μM range. In summary, the careful application of site-specific mutational studies has led to the potential discovery of a strong zinc site candidate in bovine α-LA.

Physiological Significance of Zinc Binding to α-Lactalbumin

The in vitro "activity" of Zn(II) with various α-LAs has been well documented: a threefold stimulation of V_{max} in lactose biosynthesis for Ca(II) loaded α-LA[19]; altering the Michaelis-Menten kinetic parameters for other substrates of the galactosyl transferase lactose synthesis reaction[20]; induction of aggregated complexes of both bovine and human α-LA at higher [Zn(II)]/[α-LA] ratios[5]; alterations in α-LA conformation that shift it toward apo- or partially unfolded states[3] and decreases thermal stability and susceptibility to protease digestion.[21,22] As noted above, the strongest zinc-binding site lies within 14–18 Å of the calcium binding loop of bovine α-LA,[9] although the presence of an identical site in human α-LA is doubtful. The physiological importance of such binding would require biological concentrations of free, uncomplexed Zn(II) of the order of μM or higher. The data for zinc levels in milk report values around 50 μM; however, these were most likely total, not free Zn(II) concentrations.[23] The only carefully measured value for unliganded zinc was reported in horse serum at levels around 10^{-10} M, which would certainly rule out the significance of most or all of the function extrapolated to breast fluid or milk.[24] Although lactose biosynthesis and secretion occur in the Golgi lumen of the lactating mammary gland cell, detailed metal ion measurements in this organelle have yet to be reported.

On the other hand, the zinc-induced aggregation of α-LA is a cooperative, partially irreversible process.[5] Because the levels of α-LA in milk substantially exceed those concentrations required for lactose biosynthesis, there may be several physiologically relevant functions for (zinc) aggregated α-LA. One may simply be nutritional, both as a source of bioavailable zinc and digestible protein. Preliminary studies in our laboratory appear to support increased zinc affinity with increasing α-LA concentration (S. Permyakov, L. Wasserman, L.J. Berliner, unpublished results). A more specific physiological function may relate to the ability of α-LA to both inhibit mammary tumor cell growth and to trigger tumor cell death via apoptotic mechanisms. Although the inhibition of MCF-7 human neoplastic mammary epithelial cells in culture required intact α-LA molecules (vs. a derivative containing a chemically cleaved calcium binding loop), the participation of aggregated α-LA forms is not known.[25] On the other hand, two reports from the group of Hakannson showed that some, as of yet not fully characterized, aggregated forms of human α-LA displayed unique apoptotic behavior with mammary tumors.[26,27] Collectively, the suggestion of aggregated (and other) α-LA forms and a growing body of anecdotal medical reports on breast-feeding and decreased incidence of mammary cancer may have some linkage to zinc associated α-LA forms as well.[28] Although the purpose of this article was to point out cautions in extrapolating sequence homologies to subtleties of function, the single strong zinc site in bovine α-LA is probably only one of several involved in α-LA aggregation.

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