Minireview

α-Lactalbumin: structure and function

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Received 6 March 2000; received in revised form 14 April 2000

Edited by Vladimir Skulachev

Abstract Small milk protein α-lactalbumin (α-LA), a component of lactose synthase, is a simple model Ca^{2+} binding protein, which does not belong to the EF-hand proteins, and a classical example of molten globule state. It has a strong Ca^{2+} binding site, which binds Mg^{2+}, Mn^{2+}, Na^{+}, and K^{+}, and several distinct Zn^{2+} binding sites. The binding of cations to the Ca^{2+} site increases protein stability against action of heat and various denaturing agents, while the binding of Zn^{2+} to the Ca^{2+}-loaded protein decreases its stability. Functioning of α-LA requires its interactions with membranes, proteins, peptides and low molecular weight substrates and products. It was shown that these interactions are modulated by the binding of metal cations. Recently it was found that some folding variants of α-LA demonstrate bactericidal activity and some of them cause apoptosis of tumor cells.

Key words: α-Lactalbumin; Structure; Function; Metal cation binding

1. Introduction

α-Lactalbumin (α-LA) is a small (M_r 14 200), acidic (p*I* 4–5), Ca^{2+} binding milk protein, which is very important from several points of view. First of all, α-LA performs an important function in mammary secretory cells: it is one of the two components of lactose synthase, which catalyzes the final step in lactose biosynthesis in the lactating mammary gland [1]. The other component of this system is galactosyltransferase (GT), which is involved in the processing of proteins in various secretory cells by transferring galactosyl groups from UDP-galactose to glycoproteins containing *N*-acetylglucosamine. In the lactating mammary gland, the specificity of GT is modulated by interaction with α-LA, which increases its affinity and specificity for glucose:

$$\text{UDP-Gal} + \text{glucose} \xrightarrow{\text{GT}/\alpha\text{-LA}} \text{lactose} + \text{UDP}$$

The reaction takes place in the Golgi lumen and requires Mn^{2+} ions.

Second, α-LA possesses a single strong Ca^{2+} binding site [2,3] and for this reason it is frequently used as a simple, model Ca^{2+} binding protein. It is very convenient for studies of calcium binding effects on interactions of the protein with proteins, peptides, membranes and low molecular weight organic compounds, which frequently have physiological significance.

Third, α-LA has several partially folded intermediate states, which are being studied by many researchers interested in protein folding problems. It is very attractive for studies of the properties and structure of intermediate molten globule-like states since at acidic pH and in the apo-state at elevated temperatures α-LA is the classic 'molten globule' [3–5].

Fourth, it has been found recently that some forms of α-LA can induce apoptosis in tumor cells [6,7] which suggests that this protein can fulfill many important biological functions.

2. Primary, secondary and tertiary structure

Most of α-lactalbumins, including human, guinea pig, bovine, goat, camel, equine and rabbit proteins, consist of 123 amino acid residues (see for example [8]). Only rat α-LA contains 17 additional C-terminal residues. α-LA is homologous in sequence to the lysozyme family, but it exhibits cell lytic activity about 10^{-6} of the specific activity of hen egg white lysozyme [9]. X-ray crystallography has shown that the three-dimensional structure of α-LA is very similar to that of lysozyme [10,11]. Native α-LA consists of two domains: a large α-helical domain and a small β-sheet domain, which are connected by a calcium binding loop (Fig. 1). The α-helical domain is composed of three major α-helices (residues 5–11, 23–24, and 86–98) and two short 3_{10} helices (residues 18–20, and 115–118). The small domain is composed of a series of loops, a small three-stranded antiparallel β-pleated sheet (residues 41–44, 47–50, and 55–56) and a short 3_{10} helix (three residues per turn and an intrachain hydrogen bond loop containing 10 atoms; residues 77–80). The two domains are divided by a deep cleft between them. At the same time, the two domains are held together by the cysteine bridge between residues 73 and 91, forming the Ca^{2+} binding loop. A second important disulfide bridge 61–77 connects the two domains as well. Overall, the structure of α-LA is stabilized by four disulfide bridges (6–120, 61–77, 73–91, and 28–111).

3. Location of cation binding sites

One of the most interesting features of α-LA is its ability to bind metal cations. It does not belong to the EF-hand protein family. The protein has a single strong calcium binding site, which is formed by oxygen ligands from carboxylic groups of

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Abbreviations: α-LA, α-lactalbumin; GT, galactosyltransferase; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DSA, 5-doxylstearic acid; DSC, differential scanning calorimetry

PII: S0014-5793(00)01546-5

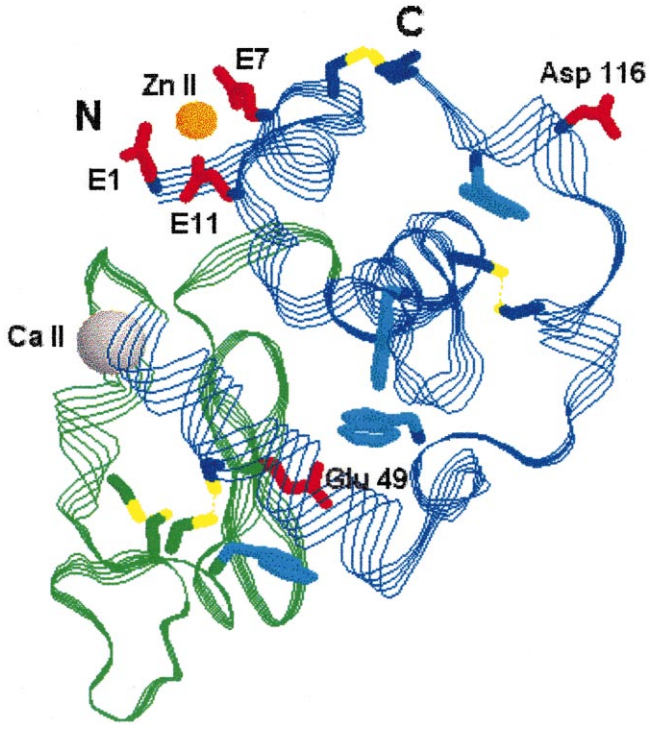

Fig. 1. X-ray α-LA structure derived from native buffalo (the structure was kindly presented by Dr. Acharya) and recombinant bovine protein (the structure was taken from Brookhaven Protein Data Bank) prepared in conjunction with Serge E. Permyakov from the Institute for Biological Instrumentation, Pushchino, Russia and Dr. Charles Brooks, Department of Veterinary Biosciences, Ohio State University, Columbus, OH, USA. α-Domain is shown in blue while β-domain is shown in green. Trp residues are shown in blue and S–S bridges are shown in yellow. The residues which take part in coordination of Zn^{2+} ions are shown in red.

three Asp residues (82, 87 and 88) and two carbonyl groups of the peptide backbone (79 and 84) in a loop between two helices. The loop contains two residues less than the typical EF-hand Ca^{2+} binding domain. In addition, one or two water molecules take part in direct coordinating Ca^{2+}. Overall the oxygen ligands form a distorted pentagonal bipyramidal structure. Recently a secondary calcium binding site was found by X-ray crystallography revealed in human α-LA 7.9 Å away from the primary strong calcium binding site [12]. Four residues are involved in Ca^{2+} coordination at this site in a tetrahedral arrangement (Thr-38, Gln-39, Asp-83 and the carbonyl oxygen of Leu-81). This secondary site is located near the surface of the α-LA molecule.

α-LA also has several zinc binding sites [13], one of which is located in the 'cleft' region (i.e. the region which forms the active site of lysozyme) [14]. In the X-ray structure of human α-LA the zinc is sandwiched between Glu-49 and Glu-116 (Asp in the bovine protein) of the symmetry-related subunit in the dimeric crystal unit cell (Fig. 1). This site was assigned to as the strong zinc site in human α-LA. The intramolecular distance between the strong zinc and calcium sites in bovine α-LA, as measured by Förster fluorescence energy transfer, was 14–18 Å utilizing Co(II) as acceptor and Tb(III) as donor, respectively [15]. This distance agreed well with the distance (17.5 Å) found in the human α-LA X-ray structure. Nevertheless, recent studies on α-LA mutants showed that the strong Zn^{2+} binding site in solution is not consistent with the site presumed from the human α-LA X-ray structure and, in fact, appears to be located near the N-terminus of the protein: site directed mutagenesis of Glu-1 to a Met residue results in the disappearance of the strong Zn^{2+} binding site in bovine α-LA [16]. A proposed site involves Glu-1, Glu-7, Glu-11 and Asp-37 (Fig. 1). The distance between the strong Ca^{2+} binding site and this putative N-terminus Zn^{2+} binding site is about 14 Å, which is in good agreement with the fluorescence energy transfer data [15]. In addition, there is evidence that some of the weak secondary Zn^{2+} sites in α-LA contain His residues [17,18].

Table 1
Apparent binding constants of metal ions for bovine α-LA

Cation	Association constants (M^{-1})	
	37°C	20°C
Ca^{2+}	2×10^{7}	3×10^{8}
Mn^{2+}		3×10^{8}
Mg^{2+}	211 ± 20; 46 ± 10	2000 ± 100; 200 ± 20
Na^{+}	36 ± 10	100 ± 10
K^{+}	6 ± 3	8 ± 3

4. Calcium-induced conformation changes

The binding of Ca^{2+} to α-LA causes pronounced changes in structure and function, mostly in tertiary, but not secondary, structure, which is clearly seen from both fluorescence [3,19], and CD [20] data. Calcium binding results in both a tryptophan fluorescence blue shift and a decrease in fluorescence quantum yield. Time-resolved fluorescence measurements demonstrated that the Ca^{2+}-induced effects are due to changes in the environment of all emitting tryptophan residues (four in bovine α-LA and three in human α-LA, Fig. 1) [21]. The pronounced fluorescence changes can be used as accurate monitors of the Ca^{2+} association constant, which is

very large and usually cannot be evaluated from direct Ca^{2+} titrations, but can be more accurately calculated from titrations of Ca^{2+}-loaded α-LA with a strong Ca^{2+} chelator [3].

5. Equilibrium and kinetic metal ions binding constants

Besides calcium, α-LA binds other physiologically significant cations such as Mg^{2+}, Mn^{2+}, Na^{+} and K^{+}, which can compete with Ca^{2+} for the same binding site [19,22]. They induce similar, albeit smaller, structural changes in α-LA. Table 1 lists comparative binding constants for these cations [19,23]. All these cations seem to bind to the calcium binding site.

In Table 1 we note that two binding constants are listed for magnesium since α-LA appears to possess secondary binding sites for this cation. The values of the binding constants for Mg^{2+}, Na^{+} and K^{+} ions are rather low but, taking into consideration their high concentrations in the cell, one might speculate that they could successfully compete with Ca^{2+} ions in vivo.

The synthetic peptide corresponding to the residues 72–100 of native α-LA contains the Ca^{2+} binding loop with a flanking helix comprising residues 86–98 of the α-domain and the 3_{10} helix of the β-domain. If Cys-73, Cys-77 and Cys-91 are replaced by alanines, it binds Ca^{2+} very weakly (10^2 M^{-1}) [24]. On the other hand, formation of the native disulfide bond between Cys-73 and Cys-91 does not increase its affinity to Ca^{2+} in water but increases it in 50% trifluoroethanol.

According to fluorescence stopped flow measurements, the values of dissociation rate constants for complexes of α-LA with Ca^{2+} and Mg^{2+} are practically the same and within 0.006 to 1 s^{-1} in the temperature region from 10 to 40°C [25]. The association rate constants for Ca^{2+} and Mg^{2+} in this temperature region are within 10^6–$10^7$and 10^1–10^2 M^{-1} s^{-1}, respectively. It is of interest that the association rate constant for the Ca^{2+}–α-LA complex is almost 1 to 2 orders of magnitude lower than the diffusion-controlled limit, which is rather unusual for calcium binding proteins. One of the possible reasons for this may be the existence of the S–S bridge connecting the ends of the Ca^{2+} binding loop in α-LA.

6. Protein stability

Cation binding to the strong calcium site increases the stability of α-LA. From differential scanning calorimetry (DSC) data, the binding of Ca^{2+} shifts the thermal transition to higher temperatures by more than 40°C [26,27]. The binding of Mg^{2+}, Na^{+} and K^{+} increases protein stability as well. The stronger an ion binds to the protein, the more pronounced is thermal transition shift.

Surprisingly the binding of Zn^{2+} ions to Ca^{2+}-loaded α-LA decreases thermal stability, causes aggregation and increases its susceptibility to protease digestion [13,28]. The thermal transition for calcium-loaded α-LA occurs at room temperatures at high zinc concentrations (Zn:protein molar ratio about 100). Overall the results also showed that α-LA is in a partially unfolded and partially aggregated state in the presence of high [Zn^{2+}].

In the absence of calcium ions, but in the presence of physiological concentrations of magnesium, sodium and potassium ions, the thermal transition in α-LA occurs in the region from about 30 to 45°C [29]. This might be related to some temperature regulation of α-LA stability and function in the mammary gland.

The binding of metal cations also increases the stability of α-LA against the action of denaturing agents such as urea or guanidine hydrochloride [19]. Here, important features of the denaturation curves are distinct, intermediate molten globule-like states arising at intermediate denaturant concentrations.

Remarkably the binding of calcium stabilizes α-LA against pressure as monitored by a 200-Mpa increase in the pressure where denaturation occurs [30]. Interestingly, calcium binding increases the pressure stability of the calcium binding loop to a higher degree than the pressure stability of the overall α-LA secondary structure.

It is very important to point out that any denaturation transition in α-LA (temperature, pressure, denaturant concentration) depends upon metal ion concentration (especially that of calcium ion). Thus values such as denaturation temperature or urea or guanidine hydrochloride denaturing concentration are relatively meaningless for α-LA without specifying the metal ion content(s) and their solution concentration(s).

Kuwajima et al. [31,32] studied the kinetics of refolding of apo-α-LA by stopped flow pH jump experiments. The free refolding kinetics of the protein has a simple single exponential character. Chaperone GroEL was shown to retard the refolding of apo-α-LA by interacting with the molten globule state of the protein. The binding constant between GroEL and an early folding intermediate of α-LA is the order of 10^6 M^{-1}.

Lastly refolding of bovine α-LA from its 6 M GuHCl denatured state [33] indicates that folding of the protein with its four disulphide bonds intact corresponds to one of the limiting cases of protein folding in which rapid collapse to a globule with native-like fold is followed by a search for native-like side-chain contacts that enable efficient conversion to the close packed native structure.

7. Effects of N-terminus mutants on protein properties

Since we began studies of various mutant forms of α-LA, it was immediately apparent that wild-type recombinant bovine α-LA, which differs from the milk isolated native protein by the addition of an N-terminal Met, has more accessible tryptophan residues, lower thermostability and decreased calcium affinity compared to the native protein [34]. Enzymatic removal of the N-terminal Met restores the native properties of α-LA. Taken together, fluorescence, circular dichroism, and DSC results showed that recombinant wild-type α-LA in the absence of calcium ion is in a 'molten globule-like' state. The delta-E1 (or E1M) mutant, where the Glu-1 residue of the native sequence is genetically substituted, leaving an N-terminal methionine in its place after bacterial expression, shows almost one order of magnitude higher affinity for calcium and higher thermostability (both in the absence and presence of calcium) than the milk isolated native protein.

The effect of the single mutation in the N-terminus of α-LA is very interesting for many reasons. The charged Glu-1 in bovine α-LA is located on the protein surface and gives no evident contribution to the tertiary structure formation (e.g. salt bridges, hydrogen bonds with some proteins groups). At the same time, being charged group, it contributes to the balance of electrostatic interactions in the protein. Furthermore, as a hydrophilic residue, it strongly interacts with water

and therefore it probably tends to unfold the protein structure. The addition of Met to the N-terminus makes the situation even worse and it causes its transition to the molten globule-like conformation. The removal of Glu-1 abolishes such tendency, that is why, probably, the mutant protein becomes more stable. Perhaps, the Glu-1 residue in bovine α-LA is located in some critical region, which allows switch the protein structure from the highly rigid to the molten globule-like.

8. Acid transition

At low pH values protons can compete with Ca^{2+} for the same carboxylate oxygens and displace calcium ions at very acid pH values. One can describe the competition between calcium ions and protons for the same binding site by a simple scheme:

$$P + Ca^{2+} \Leftrightarrow PCa$$

$$P + nH^{+} \leftrightarrow PH_n$$

The fitting of the experimental fluorescence pH titration data by theoretical curves computed according to this scheme shows that the Ca^{2+} binding site in bovine α-LA contains three carboxylic groups with p*K*s about 5 [19], which agrees well with the structural data.

9. Molten globule state

The acid state of α-LA at low pH values, which is the classical molten globule state, was studied extensively by many researchers beginning with Dolgikh et al. [4], who defined it as a compact state with fluctuating tertiary structure. The radius of gyration of native Ca^{2+}-loaded α-LA is 15.7 Å, but the acid molten globule has a radius of 17.2 Å [35]. Molten globule α-LA still retains a globular shape, but is simply 'swollen' from the native state. It is a highly hydrated state, containing about 270 bound water molecules; the intrinsic mass density of the swollen interior of the protein molecule is 5% less and the intrinsic compressibility coefficient 2 times higher than that of native molecule [36]. An analysis of a set of point mutations in the helical domain of α-LA allowed the identification of a stabilizing hydrophobic core, which likely contains some native-like packing interactions [37]. A subset of hydrophobic residues is most important for formation of the native-like topology [38,39]. The most persistent structure in the molten globule is localized in the helical domain and the helices most protected from hydrogen exchange in the molten globule are less protected in the native state than other regions of the protein [40]. It is interesting to note that an α-LA mutant in which all eight cysteines were mutated to alanine, was nearly as compact as wild-type α-LA at acidic pH [41]. Overall the architecture of the protein fold of α-LA is determined by the polypeptide sequence itself and not as a result of disulfide bond cross-linking.

10. Binding of low molecular weight organic compounds and peptides

α-LA interacts with various low molecular weight organic compounds and these interactions are modulated by cation binding. For example, α-LA binds UDP-galactose, the substrate of lactose synthase reaction, as well as UDP, and UTP [13,29]. The binding parameters depend upon the state of the protein, but the strongest binding constant for UDP-galactose falls in the range of 10^3 to 10^4 M^{-1}.

α-LA binds melittin, a short peptide from bee venom [42], which is frequently used as a model target protein for calmodulin. In contrast to the other calcium binding proteins, such as calmodulin or troponin C, α-LA binds melittin only in the *absence* of calcium ions. Apo-α-LA binds melittin with the binding constant 5×10^7 M^{-1}. Binding alters the melittin conformation from a random coil in solution to a helical structure in the binary complex with apo-α-LA.

α-LA possesses several classes of fatty acid binding sites. It binds 5-doxylstearic acid (DSA, spin-labeled fatty acid analog, $C_{22}H_{42}NO_4$), stearic acid and palmitic acid. The binding parameters depend upon the protein state. Apparent binding constants for DSA are in the range from 10^4 to 10^6 M^{-1} [43].

11. Interactions with membrane systems

α-LA also interacts with lipid membranes [44–49]. Sephadex G-200 chromatography of a mixture of α-LA and DMPC, DPPC or lecithin vesicles reveals that a significant portion of the protein binds to the vesicles where it is possible to subsequently study some physical properties of the protein in this state. The intrinsic fluorescence of vesicle bound α-LA is sensitive to two thermal transitions: The first is the gel-liquid crystal transition of the lipid vesicles; the second arises from the denaturation of the protein. The fluorescence maximum position suggests that, at low temperatures, tryptophan accessibility increases upon protein-vesicle association. Above the protein transition tryptophans appear to interact significantly with the apolar phase of the vesicles. Quenching experiments also suggest that tryptophan accessibility increases upon protein-vesicle association. Thermal denaturation of the liposome bound α-LA depends on the metal bound state of the protein [44,45].

At pH 2, where the protein rapidly inserts into the bilayer, the isolated vesicle–α-LA complex shows a distinct fluorescence thermal transition, consistent with a partially inserted protein possessing some degree of tertiary structure that unfolds cooperatively [44]. This is in contrast with the behavior of acid state α-LA in solution.

These results suggest a model where a limited expansion of conformation occurs upon membrane association at neutral pH, physiological temperature, with a concomitant increase in tryptophan exposure to solvent and external quenchers. The data may shed light on the in vivo function and mechanism of α-LA since it interacts with galactosyltransferase on membrane surfaces in the Golgi lumen.

12. Functions of α-LA

It has long been known that α-LA is a component of lactose synthase [1]: it complexes with galactosyl transferase only in the presence of substrates and modifies its specificity. Nevertheless, the role of metal cations in lactose synthase function is still far from clear. One of the substrates, which binds to galactosyltransferase, UDP-galactose, also binds to α-LA, but with rather low affinity [13,29]. It is unknown whether this binding is of any physiological significance or

not. Lactose synthase requires Mn^{2+} ions for optimal function and both galactosyl transferase and α-LA bind Mn^{2+} rather tightly.

Surprisingly, the role of calcium binding to α-LA in lactose synthesis is still unclear. On the other hand, we have learned that Zn^{2+} binding to α-LA can modulate lactose synthase function and this may be physiologically significant [48]. Zinc binding to α-LA changes both the apparent Michaelis constant K_m(app) and V_{max} of lactose synthase. These effects depend upon manganese concentration as well [48]: Zn^{2+} induces a decrease in both K_m(app) and V_{max} for Mn^{2+}, which results in an apparent increase, followed by a decrease, in lactose synthase activity at Mn^{2+} concentrations below saturation of the first Mn^{2+} binding site in GT. At high Mn^{2+} concentrations Zn^{2+} decreases lactose synthase activity.

Pelligrini et al. [49] found that proteolytic digestion of α-LA by trypsin and chymotrypsin yields three peptides with bactericidal properties. The polypeptides are mostly active against Gram-positive bacteria suggesting a possible antimicrobial function of α-LA after its partial digestion by endopeptidases. Hakansson et al. [50] revealed an α-LA folding variant with bactericidal activity against antibiotic-resistant and antibiotic-susceptible strains of *Streptococcus pneumoniae*. The active form of α-LA was purified from casein by a combination of anion exchange and gel chromatography. It is of interest that native α-LA could be converted to the active bactericidal form by ion exchange chromatography in the presence of a cofactor from human milk casein, characterized as a C18:1 fatty acid. As it was shown above, α-LA possesses several classes of fatty acid binding sites [43].

Recent data of Hakansson et al. [6] and Svensson et al. [7] address one more most intriguing possible function of α-LA. They found that some multimeric, yet not thoroughly characterized human α-LA derivative is a potent Ca^{2+}-elevating and apoptosis-inducing agent with broad, yet selective, cytotoxic activity, killing all transformed, embryonic, and lymphoid cells tested. The multimeric α-LA forms were isolated from the casein fraction of milk [7]. It was found that apoptosis-inducing fraction of α-LA contains oligomers of α-LA that have undergone a conformational change toward a molten globule-like state. Oligomerization appears to conserve α-LA in a state with molten globule-like properties in physiological conditions. As outlined above, it is now well known that aggregated forms of α-LA can be obtained in the presence of zinc ions [13] and it is interesting whether or not they possess cytotoxic activity as well. Multimeric α-LA was shown to bind to the cell surface, to enter the cytoplasm and accumulate in cell nuclei [7]. It is in line with our finding that apo- and Zn^{2+}-loaded α-LA (molten globule-like conformations) interact with model phospholipid membranes better than the Ca^{2+}-loaded protein [44]. Data of Köhler et al. [51] demonstrate that caspases (cysteine-containing aspartate-specific proteases) are activated and involved in apoptosis induced by aggregated α-LA and that direct interaction of α-LA with mitochondria leads to the release of cytochrome *c*, which may be an important step in the initiation of the caspase cascade in these cells.

Kit et al. [52] showed that oligonucleotides from human milk block both the cytostatic and cytotoxic effects of α-LA. They also found that both monomeric and multimeric α-LA binds oligonucleotides of various lengths [53]. They suggested that oligonucleotides are secreted from mammary cells and that α-LA and endogenic oligonucleotides can serve as factors of regulation of physiological state of mammary gland cells. Moreover, oligonucleotides could control the cytotoxic potential of milk.

13. Concluding remarks

It is intriguing that α-LA may potentially have a myriad of functions beyond its role in lactose biosynthesis. Yet practically the only cation which exists in vivo at high enough concentrations so that is likely to be bound to the (intact) protein is calcium (some population of the protein can be loaded by sodium and potassium). While we are at a disadvantage to date in being able to measure free unliganded concentrations of ions and metabolites in cellular compartments, most of the other cations are likely to be at too low level, including zinc. However, this does not address the binding of e.g. zinc to aggregated forms of α-LA since the binding is certainly cooperative at higher zinc:protein levels. Hence the relationship between zinc binding and protein aggregation, as well as the susceptibility to aggregation of many molten globule state proteins, points to a mechanism for zinc binding to promote immobilization and transport of the protein for nutritional purposes. Recall also that membrane/lipid association with α-LA compromises the protein as well, placing it in a molten globule-like state. Consequently consider the fact that zinc or other cation binding might induce α-LA aggregation to forms that have anticancer activity, perform various transport functions with apolar, lipophilic vitamins and metabolites as well as serving as a detergent to apoptotic events. The data presented show that some proteins, even such small proteins as α-LA, can perform several physiological functions depending on its location.

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