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Physiological Roles of Zinc and Calcium Binding to α-Lactalbumin in Lactose Biosynthesis†

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ABSTRACT: Bovine apo-α-lactalbumin was shown to be severalfold more efficient than its calcium conformer as a cofactor in lactose biosynthesis. This rate enhancement was manifested in a 3.5-fold increase in V_{max}, with no differences in K_m(app) between the two α-lactalbumin forms. In the presence of zinc, which shifts Ca(II)–α-lactalbumin toward the "apo-like" conformation [Musci, G., & Berliner, L. J. (1985) *Biochemistry 24*, 3852–3856], the catalytic rate constant for lactose synthesis was identical for both the Ca(II) and apo conformers. Activity measurements at different temperatures, on the other hand, confirmed that calcium is important in stabilizing the protein (α-lactalbumin) against thermal denaturation. The stabilizing effect of calcium was independent of the presence of Zn(II), i.e., of the protein conformation. The physiological implications of these results are discussed.

The modifier protein α-lactalbumin (α-LA)[1] is the noncatalytic regulatory subunit of the "lactose" synthase complex (UDP-galactose:D-glucose 4-β-D-galactosyltransferase, EC 2.4.1.22). The association of α-LA with galactosyltransferase (GT) imparts a change in specificity of the latter enzyme from terminal *N*-acetylglucosaminyl acceptors to glucose. Previous work with several α-LA species has described the extremely strong binding of calcium and several lanthanides ($K_d \approx 10^{-9}$–10^{-12} M) to a specific site, which also bound Mn(II) (K_d

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[1] Abbreviations: α-LA, α-lactalbumin; GT, galactosyltransferase; UDP-Gal, uridine 5′-diphosphate galactose; GlcNAc, *N*-acetylglucosamine; bis-ANS, 4,4′-bis[1-(phenylamino)naphthalene-8-sulfonate]; UDP, uridine 5′-diphosphate; Glc, glucose; PK/LDH, pyruvate kinase/lactate dehydrogenase; T_m, thermal melting temperature; Tris, tris(hydroxymethyl)aminomethane.

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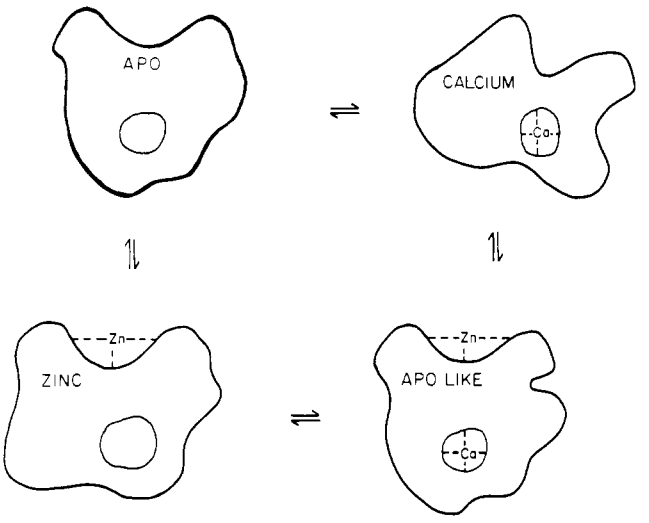

FIGURE 1: Conformational states of α-LA.

Table I: Summary of Cation Effects in Lactose Biosynthesis[a]

cation	K_d for α-LA	K_m(app) for GT	concn in milk
Ca(II)	0.2 nM (0.4 mM)[b]	(1 mM)[d]	1–5 mM[e]
Zn(II)	5.6 μM[c]	5.6 μM[c]	50 μM[f]
Mn(II)	31 μM (1.1 mM)[b]	15 μM (440 μM)[d]	0.1 μM[f]
α-LA			7–70 μM[g]

[a] Values in parentheses pertain to nonessential activation or secondary binding sites. [b] Murakami et al. (1982), pH 7.4, 20 mM Tris-HCl. [c] Murakami & Berliner (1983), pH 7.4, 20 mM Tris-HCl. [d] O'Keeffe et al. (1980), pH 7.4, 50 mM cacodylate. [e] Altman & Dittmer (1971). [f] Murthy (1974). [g] Hill & Brew (1975).

= 31 μM) and Cd(II) (Murakami et al., 1982; Permyakov et al., 1981, 1985). Further studies confirmed the existence of a distinct zinc site (K_d = 5.6 μM) which also bound Co(II), Cu(II), and Al(III) (Murakami & Berliner, 1983). Most recently, Musci & Berliner (1985) were able to distinguish the four possible conformations of α-LA [apo, Ca(II), Zn(II), and Ca(II)–Zn(II) species] by the fluorescence emission characteristics of the bound dye bis-ANS. Of most note from these results was the existence of an "apo-like" conformation for the stoichiometric Ca(II)–Zn(II)-bound species. The interpretation of the data suggested that a hydrophobic surface on the protein was more exposed in all but the Ca(II) conformer. Figure 1 depicts the four α-LA conformers and the equilibria which connect them. Of particular interest was the physiological role of the Ca(II)–Zn(II)-bound α-LA conformer since the physiological concentrations of calcium (1–5 mM) and zinc (50 μM) are sufficient to shift the protein predominantly to the apo-like conformation (Altman & Dittmer, 1971; Murthy, 1974).

The goal of this work was to carefully assess the kinetic properties of apo-, Ca(II)–, and apo-like Ca(II)–Zn(II)–α-LA in the lactose synthase reaction under conditions which might approximate those in vivo. The protocols for these experiments had to take into consideration the facts that Mn(II) or Zn(II) was an *essential* cation cofactor for GT and that Ca(II) was an activator of GT catalytic rate in the presence of either of the former two metal ions (O'Keeffe et al., 1980; Powell & Brew, 1974). For reference, Table I summarizes kinetic and equilibrium parameters for Ca(II), Zn(II), and Mn(II) with bovine α-LA and GT, as well as their physiological levels in milk. For example, in order to maintain α-LA predominantly as the apo conformer, one must work with Mn(II) at concentrations (e.g., 10 μM) where GT is sufficiently activated while only a small fraction of α-LA is in the Mn(II) complex [i.e., in the "calcium" conformation (Murakami et al., 1982)].

Experimental Procedures

Proteins. Electrophoretically pure bovine α-lactalbumin (lot 52F-8075-1) from Sigma Chemical Co. typically contained between 0.3 and 0.4 mol of Ca(II)/mol of protein. Fully demetallized α-LA, which contained less than 2% bound calcium as determined by atomic absorption, fluorescence, and ^{1}H NMR (K. Koga and L. J. Berliner, unpublished results), was prepared as previously reported (Musci & Berliner, 1985). Galactosyltransferase (GT) was isolated as previously described (Grunwald & Berliner, 1978).

Chemicals. Ultrapure manganese chloride (99.999%, lot 0518) and zinc chloride (99.999%, lot 0208) were from Aldrich Chemical Co. UDP-Gal (lot 90F-7235), NADH (lot 33F-72001), phosphoenolpyruvate (lot 70F-3851), and PK/LDH (lot 44F-6074) were from Sigma Chemical Co. All other chemicals were reagent grade.

Methods. Lactose biosynthesis was measured spectrophotometrically by monitoring product UDP generation by the *split assay* method of Grunwald & Berliner (1978). This replaced the direct *coupled assay* of Fitzgerald et al. (1970) since we used Zn(II), which inhibits pyruvate kinase in the coupled assay (Kwan et al., 1975). The conditions were modified slightly from those previously reported by Murakami & Berliner (1983). A mixture containing GT (0.07 unit/mL), 504 μM UDP-Gal, 20 mM Glc, 15.2 μM apo-α-LA or Ca(II)–α-LA, and either 8.9 μM $MnCl_2$ or 53 μM $ZnCl_2$ in 10 mM Tris buffer, pH 7.4, was allowed to incubate at the desired temperature. Timed aliquots of this mixture were then analyzed for UDP by monitoring NADH consumption at 340 nm in a solution containing pyruvate kinase (50 units/mL), lactate dehydrogenase (35 units/mL), $MgCl_2$ (6.8 mM), NADH (248 μM), and phosphoenolpyruvate (458 μM) in the same buffer.[2]

α-LA concentration was measured spectrophotometrically by using ϵ = 2.01 mg^{-1} mL^{-1} at 280 nm. All glassware, plasticware, and pipets were carefully prewashed as noted earlier (Murakami et al., 1982); buffers and deionized double-distilled water were passed over a Chelex 100 column to remove trace metals. A Varian Model 635 spectrophotometer was used for all assays. Atomic absorption measurements were made on a Perkin-Elmer Model 360 instrument.

Results and Discussion

Lactose Biosynthesis in the Presence of Mn(II). Figure 2 depicts the effects of apo-α-LA (closed circles) or Ca(II)–α-LA (open circles) on the kinetics of lactose biosynthesis in the presence of Mn(II) as the metal ion cofactor for GT, pH 7.4, 26 °C. Both curves were fit to an *identical* K_m(app) = 8.5 × 10^{-8} M for either α-LA form, which is about 2 orders of magnitude below physiological concentrations (Table I); i.e., the (GT–α-LA) complex is most likely under saturation kinetics with respect to α-LA during lactose biosynthesis in vivo. However, a significant *difference* in catalytic rate was observed (ca. 2.5-fold) between apo-α-LA and Ca(II)–α-LA which was actually an underestimate. For example, choosing a concentration of 15.2 μM α-LA, which is within the physiological range (see Table I), we must account for the fact that at 8.9 μM Mn(II), only 77% of the apo-α-LA was actually in the apo conformation, the remaining 23% adopting the Mn(II)-bound (calcium) conformation. This consequently results in a reduction of the available free Mn(II) concentration to 6.9 μM, which reduces slightly the catalytic rate for GT compared to the rate in the presence of 8.9 μM free Mn(II).

[2] This coupling mix also contained 100 μM sodium azide to prevent bacterial growth.

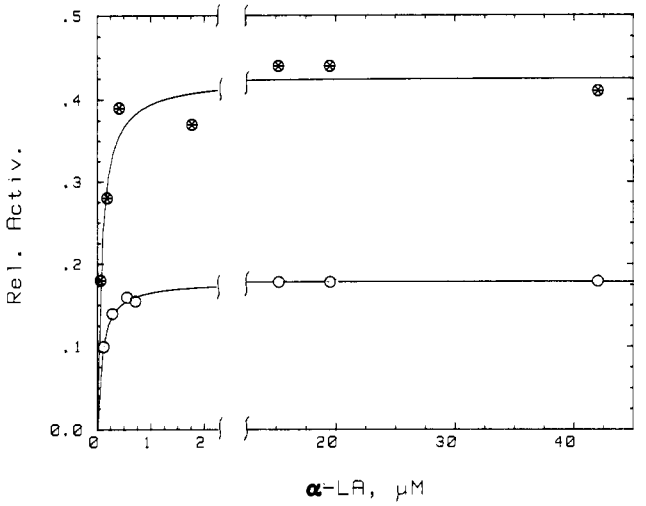

FIGURE 2: Kinetics of lactose biosynthesis in the presence of 8.9 μM Mn(II) and apo-α-LA (●) or Ca(II)–α-LA (○). Both curves were fit to K_m(app) = 8.5 × 10^{-8} M. Conditions were 10 mM Tris buffer, pH 7.4, 26 °C, GT (0.07 unit/mL), 504 μM UDP-Gal, and 20 mM Glc. Timed aliquots of the reaction mixture were analyzed for UDP generation by monitoring NADH consumption at 340 nm in a solution containing pyruvate kinase (50 units/mL), lactate dehydrogenase (35 units/mL), Mg(II) (6.8 mM), NADH (248 μM), and phosphoenolpyruvate (458 μM) in the same buffer. See Experimental Procedures for additional experimental details.

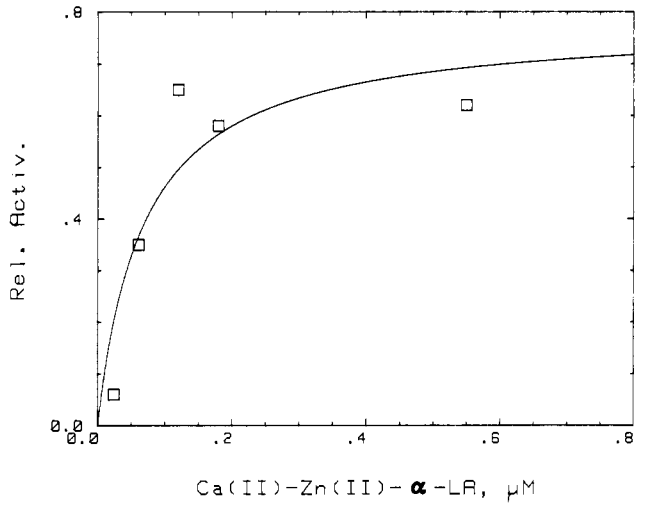

FIGURE 3: Kinetics of lactose biosynthesis in the presence of 53 μM Zn(II) and Ca(II)–α-LA, pH 7.4, 26 °C. The theoretical curve was fit to K_m(app) = 7 × 10^{-8} M which was also in agreement with the K_m(app) results of Murakami & Berliner (1983). All other conditions were identical with those in Figure 2. See Experimental Procedures for additional experimental details.

By first normalizing the observed rate for the apo conformer to 8.9 μM Mn(II) and subsequently solving for the true apo-α-LA rate from the expression

observed rate =
0.77(apo-α-LA rate) + 0.23[Ca(II)–α-LA rate]

we calculated a true apo-α-LA/Ca(II)–α-LA rate *ratio* of 3.5 under the conditions in Figure 2.

Lactose Biosynthesis in the Presence of Zn(II). Figure 3 depicts the effects of Ca(II)–α-LA on the kinetics of lactose biosynthesis in the presence of 53 μM Zn(II) as the metal ion activator for GT. This concentration of Zn(II) was sufficient to almost completely saturate GT as well as the zinc site on α-LA (see Table I), shifting it toward an apo-like conformation (Musci & Berliner, 1985). The K_m(app) = 7 × 10^{-8} M was identical with that for apo-α-LA or Ca(II)–α-LA in the presence of 8.9 μM Mn(II) (Figure 2). These data also agreed with the K_m(app) results of Murakami & Berliner (1983), who found identical K_m(app) and V_{max} values for *both* Zn(II)–(apo) and Ca(II)–Zn(II)– (apo-like) α-LA under the same conditions. Musci & Berliner (1985) showed that Zn(II) shifts α-LA toward an apo-like conformation whether or not the calcium site was metal bound. Consequently, no differences in either K_m(app) or V_{max} were observed at ca. 50 μM Zn(II) since apo-like conformations resulted in either case.

Table II: Initial Rates for Lactose Biosynthesis in the Presence of Apo-α-LA and Ca(II)–α-LA at Varying Temperatures

temp (°C)	relative rates[a] apo-α-LA	Ca(II)–α-LA	apo-α-LA/Ca(II)–α-LA rate ratio
26	1.00	0.95	1.05
45	2.03	2.12	0.96
50	1.05	3.19	0.33
55[b]	0	0	

[a] Rate of UDP generation vs. time in the presence of 53 μM Zn(II) and 15.2 μM α-LA at the temperature stated. All rates are normalized to apo-α-LA at 26 °C and were accurate to ±3%. See Methods for experimental details. [b] Thermal inactivation of GT occurs above 55 °C (Grunwald et al., 1982).

Temperature Effects on Lactose Biosynthesis in the Presence of Zn(II). From the data in Table I, it is clear that α-LA must be in the calcium form under physiological conditions. The question then remains as to the significance in vivo of Ca(II) binding to α-LA, since the results in Figure 2 above showed that the apo conformation was the most active. Calcium had been shown previously to play a physical role in stabilizing α-LA against thermal unfolding (Hiraoka et al., 1980; Hiraoka & Sugai, 1984). In order to find a physiological correlation to this phenomenon, we measured lactose synthase activity with temperature for apo-α-LA and Ca(II)–α-LA in the presence of Zn(II) as the metal ion cofactor for GT, and also to ensure that α-LA was always in an apo-like conformation. The results, which are summarized in Table II, showed that at each temperature between 26 and 45 °C, no differences were observed between the two α-LA forms. (The absolute rates increased in every case due to the temperature effect on the reaction rate.) Between 45 and 50 °C, the apo form lost substantial activity compared to the Ca(II) form. A total obliteration of activity was observed above 55 °C, which resulted from thermal inactivation of GT (Grunwald et al., 1982). The rate data in Table II compared well with the thermal denaturation measurements by CD of Hiraoka & Sugai (1984), who showed that apo-α-LA melted over the range 35–55 °C while Ca(II)–α-LA was stable up to 65 °C or higher (Hiraoka et al., 1980).[3]

CONCLUSIONS

Physiological Roles of Ca(II) and Zn(II). The results presented above suggest distinct roles for calcium and zinc binding to α-LA. While calcium stabilizes the protein against thermal denaturation, zinc is crucial in shifting the protein toward the optimally active apo-like conformation. Furthermore, Musci & Berliner (1985) showed that calcium (or manganese) in the low millimolar range binds to secondary

[3] The approximate "T_m" observed from these activity measurements was somewhat higher than physiological temperature; however, it should be noted that T_m values are strongly influenced by ionic strength (Hiraoka & Sugai, 1984). While ionic strength effects can only partially explain the difference here, no significant changes were observed in the K_m(app) for apo-α-LA in our lactose synthase assay, which rules out stabilization contributions from GT–apo-α-LA complex formation (G. Musci and L. J. Berliner, unpublished experiments).

site(s) on α-LA which also shifts the protein toward the apo-like conformation. This suggests that if calcium concentrations in the Golgi lumen (where lactose synthesis takes place) are similar to those in milk, a mixture of Ca(II)–Zn(II)– and Ca(II)–Ca(II)–α-LA exists, where all of the protein is in an "active, apo-like" conformation. It is also interesting to note that Lindahl & Vogel (1984) showed that apo-α-LA binding to phenyl-Sepharose was destabilized in the presence of low concentrations of calcium. They suggested that Ca(II) binding to α-LA may weaken its association with GT in the lactose synthase complex. However, they also showed that Zn(II) or Mn(II) and various lanthanides [but not Ca(II) in the low millimolar range] induced *binding* rather than release of the α-LA from the hydrophobic support. Musci & Berliner (1985) showed that calcium binding to site I of α-LA reduced the exposure of a hydrophobic surface on the protein. They also showed that Zn(II), as well as relatively low (millimolar) concentrations of *Ca(II)* or *Mn(II)*, "reversed" the effects of calcium bound to site I by shifting the protein toward the apo-like conformer. Lactose synthase activity measurements also confirmed these findings, as no difference was found between Ca(II)–α-LA and apo-α-LA in the presence of Zn(II) (Murakami & Berliner, 1983).

Under physiological conditions, α-LA is certainly in the calcium form since the K_d is in the nanomolar range (Murakami et al., 1982; Permyakov et al., 1985). Assuming that free Zn(II) concentrations in the Golgi lumen are similar to those in milk, ca. 50 μM, α-LA should be predominantly as the apo-like conformer in vivo. On the other hand, free calcium concentrations in the low millimolar range could also shift α-LA partially toward an apo-like conformation (Musci & Berliner, 1985). Kuhn et al. (1982) have measured membrane permeabilities in the Golgi apparatus, which suggests the presence of pores of about 1 nm in diameter. These would easily pass divalent cations, implying that Ca(II) and Zn(II) concentrations in the Golgi lumen should be similar to those in the cytosol. Whether they are similar to those in other compartments of the Golgi, such as the secretory vesicles (which transport out milk products), is not yet known.

It is also important to remember that both cations also regulate GT function. Assuming that their concentrations in the Golgi lumen are similar to those in milk, zinc can saturate the enzyme while Ca(II) further activates the Zn(II)-catalyzed rate (O'Keeffe et al., 1980).

Lastly, it may be interesting to consider whether Ca(II) is instrumental in effecting release and transport of biosynthesized α-LA from the endoplasmic reticulum to the Golgi lumen (Hill & Brew, 1975). If α-lactalbumin binding to the membranes of the endoplasmic reticulum is analogous to α-LA binding to phenyl-Sepharose (Lindahl & Vogel, 1984) or bis-ANS (Musci & Berliner, 1985), only stoichiometric amounts of Ca(II) would be necessary to effect release. It is likely that a balance between calcium and zinc concentrations "fine tunes" the protein conformation, modulating both α-LA release and its modifier activity in lactose biosynthesis.

ADDED IN PROOF

Further confirmation of the relevance of the bis-ANS binding site to the GT–α-LA interaction was exemplified by the potent bis-ANS inhibition of the lactose synthase reaction.

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Registry No. GT, 9054-94-8; lactose, 63-42-3; lactose synthase, 9030-11-9; manganese, 7439-96-5.

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