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THE CALCIUM-DEPENDENT ELECTROPHORETIC SHIFT OF α -LACTALBUMIN, THE MODIFIER
PROTEIN OF GALACTOSYL TRANSFERASE

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SUMMARY: α -Lactalbumin, the modifier protein of galactosyl transferase in the synthesis of lactose by the mammary gland, has been shown to undergo a Ca^{2+} -dependent electrophoretic shift. Such shifts, characteristic of most calcium modulated proteins, are related to gross conformational changes upon binding calcium when detected in the presence of detergent (SDS-PAGE). However, we detected the calcium shift for α -lactalbumin using non-denaturing PAGE (ND-PAGE) where electrical charge changes are observed upon binding calcium. In order for a shift to be observed between the apo and calcium bound protein, calcium ion binding to proteins must have minimal dissociation constants (K_{diss}) of 10^{-7} M; α -lactalbumin is reported to bind calcium at $K_{\text{diss}} = 10^{-10}$ to 10^{-12} M. The electrophoretic shift identifies α -lactalbumin in complex milk whey patterns of many species of mammals. © 1988 Academic Press, Inc.

α -Lactalbumin (α -LA) has been described as the modifier or modulator protein in lactose synthesis (1). It complexes with galactosyl transferase (UDP-galactose: α -D-glucose 4 β -D galactosyl transferase, EC 2.4.1.22), altering the substrate specificity of the enzyme to favor glucose as the acceptor molecule. The binding of Ca^{2+} , as well as Mn^{2+} , by α -LA has been established by Hiraoka et al. (2) and Murakami et al. (3). Stuart et al. (4), using X-ray diffraction, reported that a novel Ca^{2+} binding loop exists in α -LA. The amino acid residues, Lys-79, Asp-82, Asp-84, Asp-87, Asp-88, and two molecules of water are ligands for Ca^{2+} binding. This loop is formed by one disulfide bond between positions 73 and 91. Apparent further rigidity of the

loop is provided by a disulfide bond at position 77, which is in the loop, and residue number 61, which is adjacent to the loop.

MATERIALS AND METHODS

Isolation of whey proteins: Whey protein was collected from the milks of several species of mammals and α -LA purified from bovine whey by methods described by Quarfoth and Jenness (5).

Electrophoresis of proteins: Non-denaturing polyacrylamide gel electrophoresis (ND-PAGE) and SDS-PAGE were performed as described by Thompson et al (6). Electrophoresis was run at pH 8.3, tris-glycine buffer, 12.5% acrylamide.

RESULTS AND DISCUSSION

The SDS-PAGE method employed to detect the calcium binding of calmodulin depends on its unique conformational changes upon calcium binding. Although α -lactalbumin does have a high affinity calcium binding site, no calcium dependent changes were noted for this protein in SDS-PAGE. With knowledge of the strong binding constant for Ca^{2+} by α -LA ($K_{\text{diss}} = 10^{-10}$ - 10^{-12} M), yet prior to the report of Stuart et al. concerning crystallography of the calcium binding loop, we investigated the electrophoretic behavior of α -LA in ND-PAGE in the presence of calcium (30 mM) or in the presence of EGTA (30 mM) to chelate the bound Ca^{2+} (7). It was anticipated that the removal of Ca^{2+} would result in an increased migration rate of the protein toward the anode. Figure 1 is a demonstration of that phenomenon. Since glycosylated α -LA's may constitute as much as 25% of the α -LA found in bovine milk, the effect of Ca^{2+} removal on their migration rates was examined. Lanes 3 and 5 represent glycosylated α -LA, termed "fast" and "slow," respectively, in the presence of Ca^{2+} , whereas lanes 4 and 6 are the same proteins in the presence of EGTA. Clearly, like the non-glycosylated parent molecule in lanes 1 and 2, glycosylated α -LA undergoes the Ca^{2+} -dependent electrophoretic shift. S-Carboxymethylated α -LA (4 S-S bonds/14 kD) does not undergo the electrophoretic shift which supports the concept that the Ca^{2+} ligands must necessarily be in close proximity in the secondary structure of the molecule for strong Ca^{2+} binding to occur. Proteins

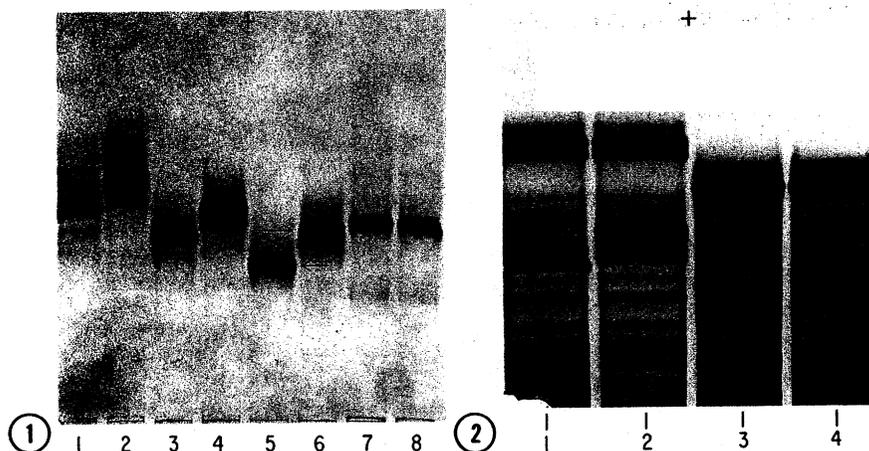


Figure 1. PAGE of bovine α -LA's. Non-glycosylated α -LA (lanes 1 and 2), glycosylated α -LA "fast" (lanes 3 and 4), glycosylated α -LA "slow" (lanes 5 and 6), S-carboxymethylated α -LA (lanes 7 and 8). Ca^{2+} had been added to proteins in lanes 1, 3, 5 and 7; EGTA was added to proteins in lanes 2, 4, 6, and 8.

Figure 2. PAGE of whey proteins of squirrel monkey (lanes 1 and 2) and opossum (lanes 3 and 4). Ca^{2+} had been added to proteins in lanes 1 and 3 and EGTA to proteins in lanes 2 and 4.

such as caseins, β -lactoglobulin and serum albumin, with K_{diss} ranging from 10^{-2} to 10^{-5} M (8), do not exhibit calcium dependent shifts in this gel system.

To date we have examined the whey proteins (milk proteins remaining after the removal of lipids and casein by conventional fractionation procedures and lactose and salts by dialysis) of over 40 species of mammals with respect to the Ca^{2+} -dependent electrophoretic shift of α -LA. Figure 2 shows the electrophoretic shift of squirrel monkey (*Saimiri sciureus*) α -LA. The relative mobility, R_m , for the shift is about 1.4 as compared with 1.16 for bovine α -LA. Notably, the whey protein PAGE pattern shows no α -LA for opossum (*Didelphis virginiana*). This is not to conclude that the whey is devoid of α -LA (this mammal produces lactose), but rather that either α -LA is too dilute to be observed in the pattern or that the K_{diss} for Ca^{2+} binding is lower for α -LA in that particular species of mammal. It is possible, moreover, that α -LA remains bound to the Golgi apparatus and is not secreted into the milk.

The R_m varies from 1.03 in house mouse (*Mus musculus*) to as great as 1.45 in gray squirrel (*Sciurus carolinensis*). Since the electrophoretic shift results from the exposure of negatively charged amino acids upon the removal of

Ca^{2+} , a shift of the magnitude of 1.40 or greater, as in gray squirrel, squirrel monkey, and mare α -LA, suggests that more than one strong Ca^{2+} binding site may exist in molecules of some species and that more negatively charged groups are released upon debinding of Ca^{2+} . Conversely, smaller or intermediate shifts suggest one binding site or weaker Ca^{2+} binding ligands due to amino acid substitutions within the loop. To date, however, all sequenced α -LA's contain essentially the same Ca^{2+} ligands in the binding loop, Table 1. Of particular interest in this table is that the substitution of glu for asp in position 84 does not prevent Ca^{2+} binding for rabbit or rat although R_m values for these two species, 1.08 and 1.04, respectively, are lower than those for the others listed.

Horse milk lysozyme (LZ) has been reported by Nitta et al. to bind Ca^{2+} . In view of the similarity of sequence with α -LA, this was predictable (4). We have observed that horse LZ undergoes the Ca^{2+} dependent electrophoretic shift (cathode migration) at pH 7.4 which clearly suggests strong Ca^{2+} binding.

It is tempting to term α -LA a calcium modulated protein. However, some uncertainty arises concerning the necessity of bound Ca^{2+} in the α -LA molecule for modifying galactosyl transferase. Calcium-free α -LA (apoprotein) is difficult to prepare. It is clear that Mn^{2+} is necessary for lactose synthesis.

Table 1: Amino acid sequence at the Ca^{2+} binding "elbow" of α -LA (residues 79-88) and the comparable sequence in lysozymes (LZ)

	79	80	81	82	83	84	85	86	87	88
Protein: α -LA										
Human (10)	Lys	Phe	Leu	Asp	Asp	Asp	Ile	Thr	Asp	Asp
Baboon (4)	Lys	Phe	Leu	Asp	Asp	Asp	Ile	Thr	Asp	Asp
Bovine (9)	Lys	Phe	Leu	Asp	Asp	Asp	Leu	Thr	Asp	Asp
Goat (11)	Lys	Phe	Leu	Asp	Asp	Asp	Leu	Thr	Asp	Asp
Guinea pig (18)	Lys	Leu	Leu	Asp	Asp	Asp	Leu	Thr	Asp	Asp
Rabbit (21)	Asn	Phe	Leu	Asp	Asp	Asp	Leu	Thr	Asp	Asp
Rat (17)	Lys	Phe	Leu	Asp	Asp	Glu	Leu	Ala	Asp	Asp
Wallaby (12)	Lys	Phe	Leu	Asp	Asp	Asp	Ile	Thr	Asp	Asp
Protein: LZ										
Horse (15)	Lys	Leu	Leu	Asp	Glu	Asn	Ile	Asp	Asp	Asp
Human (19)	Ala	Leu	Leu	Gln	Asp	Asn	Ile	Ala	Asp	Ala
Chicken (20)	Ala	Leu	Leu	Ser	Ser	Asp	Ile	Thr	Ala	Ser

With most proteins, such as calmodulin, the binding of Ca^{2+} results in the exposure of hydrophobic patches on the molecule's surface which provides for target protein interaction. With α -LA, however, Ca^{2+} binding results in a decreased hydrophobic surface as demonstrated by Lindahl and Vogel (13). It would appear that α -LA is totally saturated with Ca^{2+} in bovine mammary gland which it certainly is in the milk (30 mM Ca^{2+}). In the absence of Ca^{2+} at physiological temperatures, α -LA readily denatures (2). One would conclude, therefore, that Ca^{2+} is necessary for α -LA to modify or modulate lactose synthesis (14) and should be termed a calcium modulated protein.

The electrophoretic shift reported for α -LA was observed by ND-PAGE, not by SDS-PAGE where it is observed for most Ca^{2+} modulated proteins. Thus this method offers a quick screening device for the detection and purification of high affinity calcium binding proteins where charge changes occur in the absence of gross conformational changes.

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