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Functional Implications Resulting from Disruption of the Calcium-Binding Loop in Bovine α-Lactalbumin

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ABSTRACT

The strong calcium-binding site of α-lactalbumin comprises the carboxylate side chains of aspartic acid 82, 87, and 88 and the carbonyl oxygens of residues 79 and 84. A single methionine residue was selectively modified by controlled CNBr cleavage to yield homoserine at position 90. The CNBr-cleaved α-lactalbumin lost the ability to bind calcium strongly as monitored by intrinsic fluorescence, electrophoretic mobility, atomic absorption, and x-ray fluorescence. Remarkably, the modified protein was still competent in lactose biosynthesis, although activity was reduced to 1/40th that of the native form of the protein. Although the strong calcium-binding site was destroyed as a result of the cleavage of the calcium-binding loop, a secondary calcium site was retained that directly affects a rate enhancement of lactose biosynthesis when saturated, resulting in approximately a two- to threefold increase in rate at 1m*M* $CaCl_2$ with an activation equilibrium constant of 350 ± 40 μ*M*. (Key words: calcium-binding loop disruption, functionality, α-lactalbumin)

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Abbreviation key: α-LA = α-lactalbumin; Asp = aspartic acid; bis-ANS = 4,4′-bis[1-(phenylamino)-8-naphthalenesulfonate]; Ca(II)-α-LA = α-La bound strongly with a single calcium cation; CNBr = cyanogen bromide; CNBr-α-LA = cyanogen bromide-modified α-lactalbumin; K_m = Michaelis constant; Met = methionine; Tris = tris (hydroxymethyl)-aminomethane; Trp = tryprophan.

INTRODUCTION

α-Lactalbumin (α-LA) is the modifier protein in the lactose synthase (EC 2.4.1.22) complex. There has been intense interest in its three-dimensional structure because it is highly homologous to hen lysozyme in primary structure (16, 17) and also has the properties of a calcium-binding protein (11, 15). Recently the crystallographic structure of the calcium-binding loop elbow in baboon α-LA was reported

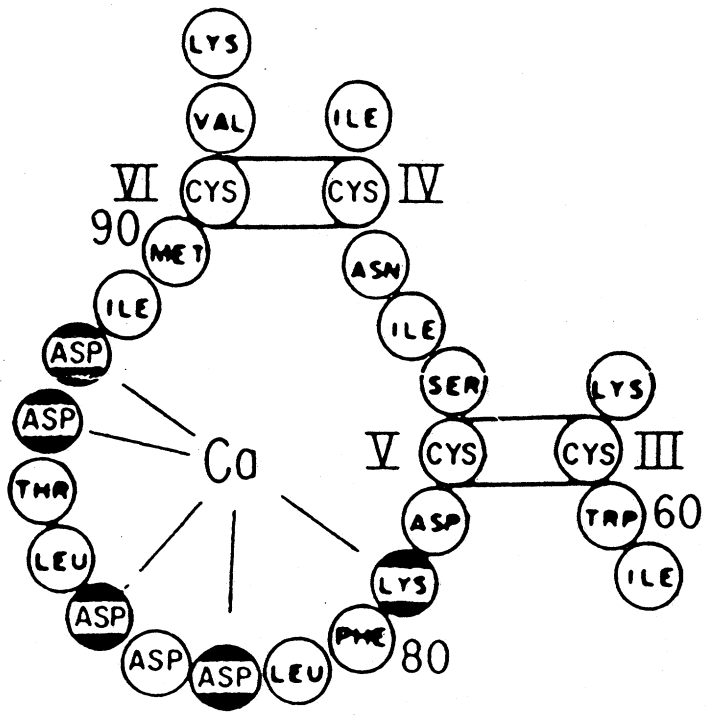

Figure 1. Depiction of the "calcium-binding loop" region of the primary structure of bovine α-lactalbumin (α-LA) after the corrected sequence of Shewale et al. (17).

that comprises the carboxylate side chains of aspartic acid (Asp) 82, 87, and 88 and the carbonyl oxygens of residues 79 and 84 (1, 18, 19). Although no crystallographic structure is available for bovine α-LA, the corrected sequence (17) predicts an essentially identical structure.

Permyakov et al. (15) and Murakami et al. (11) demonstrated the extreme sensitivity of intrinsic fluorescence spectra with several species of α-LA as measures of calcium binding. The fluorescence changes were consistent with a ligand-induced conformational change rather than direct contact quenching with the cation bound at the strong calcium site. Some functional implications of calcium binding to α-LA function in lactose biosynthesis were discussed by Musci and Berliner (13) as demonstrated by kinetic analyses.

Physiologically, α-LA has recently been shown to exhibit potent antitumor activity in human mammalian mammary carcinoma cell lines (M. Bano, S. Mohanam, S. Liu, M. P. Thompson, and W. R. Kidwell, unpublished results). Thus, the structural integrity of α-LA may have important implications in other physiological or pathological processes.

The calcium-binding loop sequence for bovine α-LA is shown in Figure 1. Note the position of the sole methionine (Met) 90 residue, which completes the loop to the cysteine (Cys) 89-Cys 72 disulfide bridge. To test the validity of this loop structure in solution, we examined and characterized the consequences of cyanogen bromide (CNBr) cleavage of the calcium-binding loop at Met 90.

MATERIALS AND METHODS

Proteins and Chemicals

Bovine α-LA (lot 52F-80751), purchased from Sigma Chemical Company, St. Louis, MO, typically contained .2 to .3 mol Ca(II)/mol protein. Bovine galactosyl transferase was isolated as described previously (7). 4′4-Bis [1-(phenylamino)-8-napthalenesulfonate] (Bis-Ans) was purchased from Molecular Probes,

Junction City, OR. Phenyl-sepharose CL-4B (lot 98F-0415) was purchased from Sigma.

Methods

Apo-bovine α-LA was prepared by passing the protein through a column of tris(carboxymethyl)ethylenediamine (Pierce Chemical Co.) after the procedure of Koga and Berliner (9). Protein concentration was estimated by optical absorption at 280 nm ($\varepsilon = 2.01$ ml/mg). Protein intrinsic fluorescence was measured on a Perkin-Elmer Model MPF-44A spectrofluorometer (Perkin-Elmer, Norwalk, CT) at 26.5 ± .5°C. All experiments were measured at pH 7.4, 40 m*M* tris(hydroxymethyl)-aminomethane (Tris) buffer in calcium-free H_2O unless noted otherwise.

Kinetic data were fit by nonlinear regression analysis. Lactose synthase activity was measured by the coupled spectrophotometric method of Fitzgerald et al. (6) on a Kontron Uvikon 860 spectrophotometer (Kontron Instruments, Everett, MA). Conditions were 310 μ*M* uridine 5′-diphosphate galactose, 1.8 m*M* $MnCl_2$, 16 m*M* glucose, 160 μ*M* NADH, .08 *M* KCl, 190 μ*M* phosphoenolpyruvate, pH 8.0, and 37 m*M* N-methylmorpholine.

Calcium Content

Buffered samples of α-LA (pH 7.4) were incubated with a slight stoichiometric excess of $CaCl_2$ followed by extensive dialysis versus the same buffer. For x-ray fluorescence, small aliquots (ca. 50 μl) of each sample of control were placed on separate nylon filter disks and allowed to evaporate at room temperature. The disks were then placed in a Kevex model 0700/7500 x-ray fluorescence analyzer and calibrated for calcium. A Perkin-Elmer model 5000 carbon oven atomic absorption instrument was also employed for calcium analysis. Again, α-LA samples were calibrated against $CaCl_2$ standards.

CNBr Cleavage

Following the methods of Doyen and Lapresle (4), a 15 mg/ml solution of α-LA in 70% formic acid was treated with 30 mg/ml CNBr at room temperature for 24 h. The protein was either dialyzed versus H_2O followed by 5 m*M* EDTA, 40 m*M* Tris, pH 7.4 or lyophilized, redissolved, and relyophilized to remove all unreacted CNBr.

Phenyl-Sepharose Purification

Utilizing a modification of the procedures of Lindahl and Vogel (10), 25 mg of the CNBr-reacted sample (after exhaustive dialysis) were applied in 40 m*M* Tris, 5 m*M* EDTA, pH 7.4, on a phenyl-Sepharose column (1.8 × 47 cm) and washed with the same buffer until the protein absorbance at 280 nm was zero. Subsequent elution with a buffer containing 1 m*M* $CaCl_2$ substituted for the EDTA resulted in elution of approximately 10 to 15% of the total applied protein as a sharp peak from the column, consistent with the release of intact α-LA strongly bound with a single calcium cation [Ca(II)-α-LA] (10). This latter "calcium peak" was fully active in the lactose synthase assay. Finally, the column was eluted with deionized water (pH 6.5 to 7.0), whereupon a major peak eluted approximately with the void volume. This latter "water peak" was reapplied in the EDTA buffer noted earlier to a smaller phenyl-Sepharose column (1.8 × 23 cm or .8 × 22 cm), which was chosen so that the column was overloaded by ca. two- to fivefold its capacity, which is .2 mg of protein/ml gel for the "water peak", whereupon a broad peak, almost equivalent to the amount overloaded, appears that reflects more highly purified CNBr-modified α-LA (CNBr-α-LA). Subsequent washing with H_2O elutes a mixture of CNBr-α-LA and an inactive protein impurity, the latter representing about 10% of the total protein sample. Finally, the first column (calcium elution) was repeated to ensure complete removal of trace calcium-binding material.

Amino Acid Analyses

Twenty-four-hour hydrolysates (6 *M* HCl) of protein were analyzed in triplicate using a Beckman 119 CL amino acid analyzer (Beckman Instruments, Fullerton, CA).

Polyacrylamide Gel Electrophoresis

Nondenaturing PAGE and SDS-PAGE were run according to the procedure of Thompson et al. (20) with 12% gels, pH 8.9.

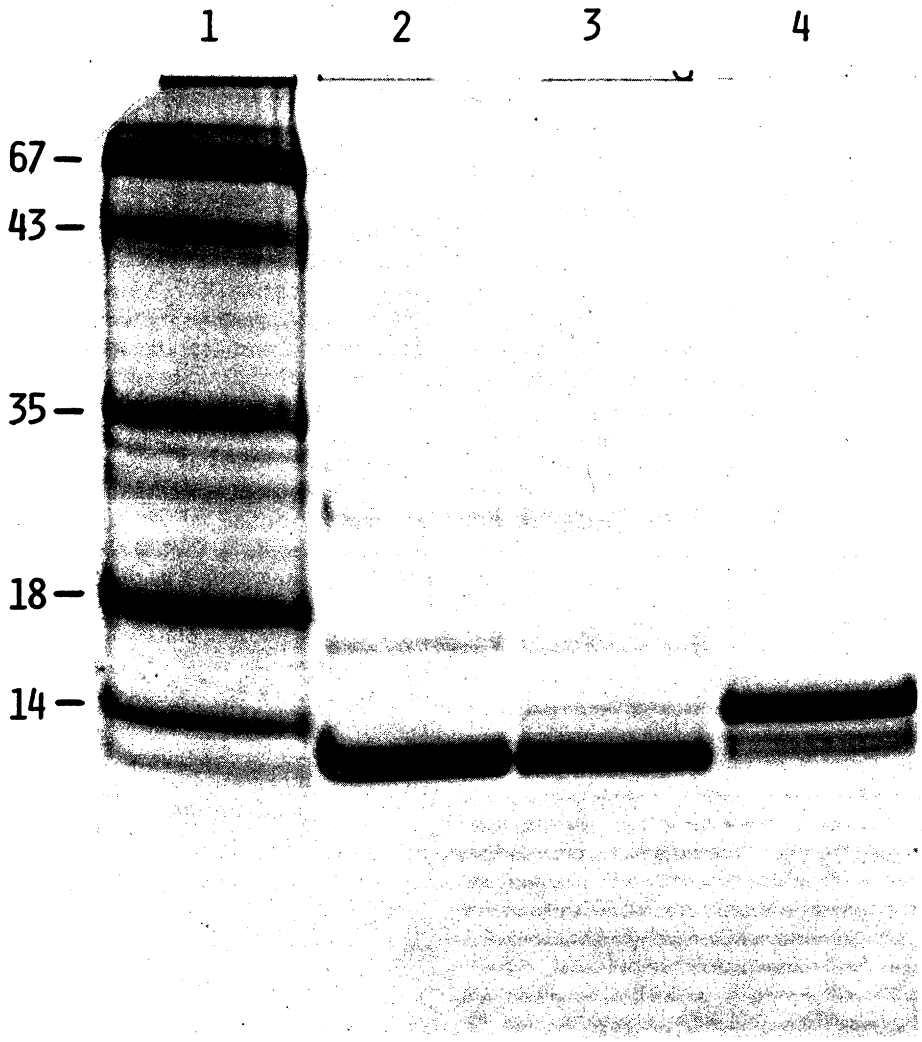

Figure 2. The SDS-PAGE patterns for native bovine α-lactalbumin (α-LA) (lane 2), formic acid treated native bovine α-LA (lane 3), and cyanogen bromide-modified α-LA (CNBr-α-LA) (lane 4). The standards (lane 1), which were reduced with mercaptoethanol before loading onto the gel, were 67 kDa, bovine serum albumin; 43 kDa, ovalbumin; 25 kDa, chymotrypsinogen; 18 kDa, β-lactoglobulin; 14 kDa, bovine α-LA. All other conditions are as noted in Materials and Methods.

RESULTS AND DISCUSSION

Met 90 Cleavage

The amino acid analyses of CNBr-α-LA versus the unmodified control or 70% formic acid-treated sample showed a substantial loss of Met. Figure 2 shows SDS-PAGE patterns for native versus unpurified CNBr-α-LA under nonreducing conditions: lane 1, molecular weight standards; lane 2, untreated α-LA; lane 3, formic acid-treated α-LA; lane 4, unfractionated CNBr-α-LA. Note the ca. 10% of the α-LA of noncleaved CNBr in lane 4, representing the ca. 10% contribution of uncleaved α-LA, which was originally reported by Doyen and Lapresle (4). When these α-LA species were examined by reducing SDS-PAGE (not

shown), the native protein and formic acid control migrated normally with an apparent molecular weight of 14 kDa. As expected from the sequence (Figure 1), however, reduced CNBr-α-LA migrated as two fragments: one peptide of about 33 residues and a larger peptide of about 90 residues. Another piece of evidence that bovine α-LA was specifically cleaved (at Met 90) into two peptide chains was confirmed by the following observations. Gel permeation chromatography in .18 *M* mercaptoethanol and 3 *M* urea (.1 *M* Tris Cl, pH 7.6) on Sephadex G-100 where two peaks were resolved at relative molecular weight (M_r) ranges, 3 to 4 and 9 to 10 kDa, respectively. (The identical separation was also observed when 50% acetic acid was used as an alternative eluant.) Upon SDS-PAGE of these two cleavage peptides, the 3- to 4-kDa peptide migrated with the dye front, and the 9- to 10-kDa peptide migrated almost identically with intact α-LA. Furthermore, the amino acid compositions of the two cleavage peptides were precisely as expected.

Somewhat unusual was the purification procedure for CNBr-α-LA, which was based on differential affinities of phenyl-Sepharose for the products of the CNBr reaction. The ca. 10 to 15% of uncleaved α-LA was retained to the column in the presence of EDTA and immediately released upon conversion to the calcium form (10). The CNBr-α-LA species and an inactive protein impurity (ca. 10%) were eluted only by distilled, deionized H_2O. However, because we subsequently found that the affinity for the impurity was stronger than that for CNBr-α-LA, the former protein was removed by overloading the column wherein CNBr-α-LA was not retained whereas the impurity was retarded. The impurity protein was not fully characterized but represented about 10% of the initially applied protein from the CNBr reaction mixture. Because it did not support lactose biosynthesis, we surmised that it was classified as either a denatured α-LA impurity or a hydrophobic milk protein impurity. Amino acid analysis of purified CNBr-α-LA (Table 1) showed the complete absence of Met.

[1]Human α-LA, which also contains a Met 90, showed similar electrophoretic behavior after CNBr treatment, but goat α-LA, which has a substitution at position 90, was unaffected by CNBr treatment.

Nondenaturing Electrophoretic Studies

An important physical diagnostic of calcium binding to α-LA is the associated change in electrophoretic mobility. The net protein charge, which is negative, becomes less negative upon binding Ca(II), resulting in a decreased mobility. Thompson et al. (20, 21) have shown that this electrophoretic shift of apo-α-LA versus Ca(II)-α-LA occurs for α-LA from several species. We have also observed this electrophoretic shift for the uncleaved, calcium-binding fraction of CNBr-α-LA and have found that this protein is indistinguishable from native α-LA, whereas purified CNBr-α-LA, however, does not exhibit the electrophoretic shift.[1]

Figure 3 depicts a nondenaturing, nonreducing PAGE study of native bovine α-LA (lane C), unfractionated CNBr-α-LA (lane A), the calcium-binding fraction from the CNBr-α-LA fractionation (lane D), and purified CNBr-α-LA (lane B), all in the presence of calcium. Comparison of lane A with lanes B and C shows that the band indicated by the upper arrow represents the uncleaved, calcium-binding fraction of CNBr-treated α-LA. [Note that

TABLE 1. Amino acid analyses of control and cyanogen bromide-modified α-lactalbumin (CNBr-α-LA).

	Residues per molecule		
Amino acid	Control (α-LA)	CNBr-α-LA	Sequence (17)
Lysine	12	12	12
Histidine	3	3	3
Arginine	1	1	1
Aspartic acid	21	20	21
Threonine	7	7	7
Serine	6	6	7
Glutamic acid	14	14	13
Proline	2	2	2
Glycine	6	6	6
Alanine	3	3	3
Half-cysteine	7	7	8
Valine	5	6	6
Methionine	1	0	1
Isoleucine	7	7	8
Leucine	13	13	13
Tyrosine	4	4	4
Phenylalanine	4	4	4
Tryptophan	ND[1]	ND	4
			123

[1]Not determined.

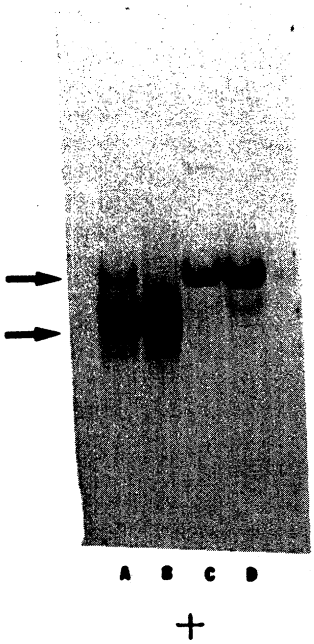

Figure 3. The PAGE of α-lactalbumin (α-LA) samples; conditions were 12% gels; samples were loaded with 10 m*M* $CaCl_2$. Unfractionated cyanogen bromide-modified α-LA (CNBr-α-LA) (A); phenyl-Sepharose purified CNBr-α-LA (B); native α-LA bound strongly with a single calcium cation [Ca(II)-α-LA] (C); purified "calcium peak" from phenyl-Sepharose chromatography (D).

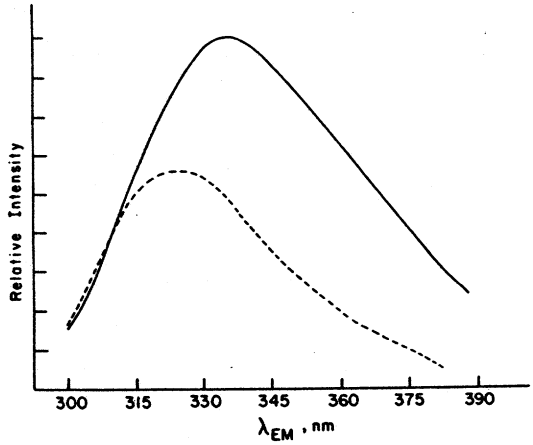

Figure 4. Intrinsic fluorescence spectra of various bovine α-lactalbumin (α-LA) samples at pH 7.4 (50 m*M* Tris) 25°C. An excitation wavelength of 280 nm was used to reduce light scattering although identical spectral emission lineshapes were observed over excitation (λ_{ex} = range 280 to 295 nm). The identical solid line emission spectrum (λ_{em} = 337 to 338 nm) was obtained in the absence of calcium for cyanogen bromide-modified α-lactalbumin (CNBr-α-LA), native apo-αLA, or a 70% formic acid-treated control. Upon addition of a 1- to 10-fold molar excess of $CaCl_2$, the dotted line blue shifted spectrum was obtained for the two controls, but CNBr-α-LA remained unchanged (solid line spectrum).

the amount of protein in lane D was overloaded relative to the amount of this material present in the upper band (arrow) of lane A.] This band is entirely absent in purified CNBr-α-LA (lane B).

Isolated purified CNBr-α-LA in lane B contains two minor bands and one major band (arrow), which run as a single band in SDS-PAGE (data not shown) comigrating with native α-LA, i.e., indicating that they consist of material of the same molecular weight as native α-LA (Figure 2). The differences in mobility between the adjacent bands in lane B are precisely half that observed for the electrophoretic shift of apo-α-LA upon Ca(II) binding, i.e., one charge different. Due to the conditions for CNBr cleavage (i.e., 70% formic acid for 24 h) where some deamidation of asparagine or glutamine residues may occur, a more negatively charged band relative to nondeamidated material will be expected. It is also known that CNBr cleavage precedes homoserine lactone hydrolysis, producing a species that will be one charge less negative than the hydrolyzed C-terminal homoserine species of CNBr-α-LA (5). Therefore, the presence of three bands in "purified CNBr-α-LA" might be expected on nondenaturing PAGE electrophoresis, which overemphasizes differences in net charge to mass ratio.[2]

Intrinsic Fluorescence

The intrinsic fluorescence emission spectra of CNBr-α-LA, native apo-α-LA, or a 70% formic acid control in the absence or presence of $CaCl_2$, are shown in Figure 4. In the ab-

[2]Elution of these species from DEAE-Sephadex with a KCl gradient (pH 7.4, 40 m*M* Tris buffer) (data not shown) produced only very poor separation, and the results were consistent with these three bands being material of the same weight but differing by one unit charge.

sence of Ca(II), all species yielded identical intrinsic fluorescence spectra (excitation = 280 nm, emission = 336.5 to 337.5 nm, 40 m*M* Tris HCl, pH 7.4). Upon exposure of up to 1 *M* $CaCl_2$, only native α-LA and the 70% formic acid control yielded the well-documented blue shifted spectrum (dotted line) reported earlier by Murakami et al. (11). Furthermore, the identity of the two spectra suggested that no tryptophan (Trp) damage occurred from CNBr exposure, because oxidized forms of Trp would be expected to alter the overall quantum yield.

The insensitivity of the intrinsic fluorescence emission to added calcium also suggests that the strong affinity for calcium has been substantially reduced or obliterated (12). As a direct physical check, we measured calcium content in CNBr-α-LA (compared with native α-LA samples), which had been initially exposed to stoichiometric concentrations of $CaCl_2$ before dialysis. The results from both atomic absorption and x-ray fluorescence for native α-LA showed evidence for a single bound calcium. However, an unpurified CNBr-α-LA sample (i.e., before passage through phenyl-Sepharose) contained only 11 to 12% of bound calcium per mole, which corresponded well with the ca. 10% of uncleaved protein noted earlier (4).

Lactose Synthase Kinetics

Although some of the structural features of α-LA appeared to be similar to intact apo-α-LA (as monitored by intrinsic fluorescence), a more sensitive test of a viable α-LA conformation is assessed from its ability to support lactose biosynthesis. Kinetic studies at 14.5°C showed that CNBr-α-LA did indeed support lactose synthesis but with an activity that was ca. 2.5% of intact 1:1 Ca(II)-α-LA in the absence of additional Ca(II) (Figure 5). Unfortunately, we were unable to deconvolute this activity into Michaelis constant (K_m) and maximum velocity contributions, because the K_m for CNBr-α-LA in the lactose synthase assay was estimated to be at least 500 μ*M*, which was experimentally impossible to measure accurately. Upon "titrating" with exogenous $CaCl_2$, a ca. 2.5-fold activation in activity was observed. The calcium activation curve (Figure 5) was fit to an apparent activation equilibrium

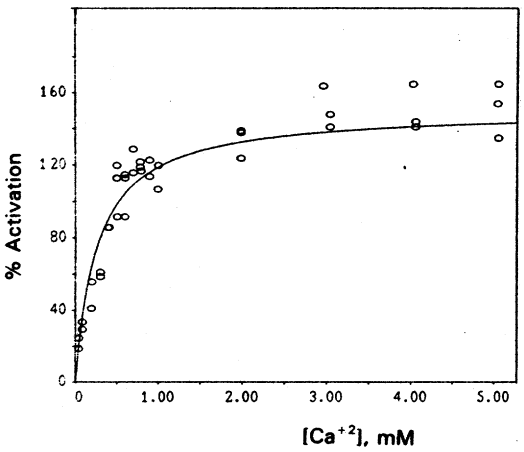

Figure 5. Kinetics of lactose biosynthesis in the presence of 1.8 m*M* $MnCl_2$ with increasing $CaCl_2$, 6 μ*M* CNBr-modified α-lactalbumin (CNBr-α-LA), (14.5 ± .5°C). The conditions are noted in Materials and Methods. The apparent Michaelis constant for Ca(II) under these conditions was 350 ± 40 μ*M*.

constant (K_{actn}) = 350 ± 40 μ*M*. It is interesting to note that secondary calcium binding to native α-LA has been reported previously by fluorescence (14) and nuclear magnetic resonance techniques with binding constants in the same range. Note, however, that native α-LA exhibits no calcium activation of lactose synthesis rate under identical conditions.

The purification of CNBr-α-LA was essential. The uncleaved calcium-binding peak (i.e., uncleaved α-LA) was fully active in the lactose synthase assay, thus obscuring the small, but real, lactose synthase activity of isolated CNBr-α-LA. Although it might be argued that this small activity of isolated CNBr-α-LA was due to the presence of trace amounts of uncleaved α-LA, the fact that this activity is enhanced by secondary calcium binding proves that this cannot be the case, because uncleaved α-LA is not activated by secondary calcium binding.

The results reported in this communication emphasize the importance of the calcium-binding loop in α-LA function in lactose biosynthesis. Although it is well established that Ca(II) is important for maintaining the thermal stability of α-LA (8, 13) as well as modulating its binding affinity to artificial membranes (2), the current results clearly show that selective

cleavage of Met 90 yields an α-LA form that is no longer capable of strong calcium binding. Because the Trp residue or residues responsible for the blue shift found for native Ca(II)-α-LA are not in the calcium loop and are structurally removed from this locus, CNBr-α-LA has lost the ability to promote this conformational change (12). Furthermore, activity decreased significantly (1/40) for CNBr-α-LA in the lactose synthase reaction. On the one hand, the substantial loss in calcium-binding affinity was expected, considering that the unique polypeptide loop that defines this calcium coordination site was cleaved at its midpoint. On the other hand, the extent to which this alteration affected other properties of α-LA was not obvious and might have eluded x-ray analysis.

It is also interesting to speculate on the factors behind the small but significant activity of CNBr-α-LA compared with native α-LA. This substantial difference in activity suggests that CNBr-α-LA must be predominantly in an unfolded state. That is, the ca. 1/40 relative rate is reconciled by the small fraction of folded, kinetically competent CNBr-α-LA in solution. Specifically, the results suggest that CNBr-α-LA is subject to thermal denaturation (because the activity at 14°C is higher than at 25°C), and, although the "folded" form of CNBr-α-LA is competent as a modifier protein, its structure and conformation are probably different. This is similar to the kinetic studies of Musci and Berliner (13) with (partially thermally unfolded) native apo-α-LA, in which the rate changed with increasing temperature consistent with a diminishing concentration of folded α-LA. Nevertheless, the (secondary) weaker calcium binding, as expressed by the activation of lactose synthase activity of CNBr-α-LA, suggests that this aspect of cation binding is not lost as a result of Met 90 cleavage. With native α-LA, additional Ca(II) did not activate lactose biosynthesis (12), yet a rate activation occurs when Zn(II) binds to Ca(II)-α-LA, shifting the protein toward an "apo-like conformation" and raising the maximum velocity for Ca(II)-α-LA to a value equal to that of apo-α-LA. Musci and Berliner (14) monitored secondary calcium binding to α-LA by fluorescence with bis-ANS, yet preliminary bis-ANS experiments with CNBr-α-LA were insensitive to calcium (Meinholtz and Berliner, unpublished results). Thus, from the data available at this juncture, we may conclude that the primary calcium-binding loop imparts an important structural integrity to native α-LA that affects the overall structure, at least in the conformation of residues that interact with galactosyl transferase. The Trp residue(s) that are affected by the calcium-induced structural change of intact apo-α-LA displays an intrinsic fluorescence emission spectrum in CNBr-α-LA, which is identical to that in unmodified apo-α-LA. Furthermore, the unique secondary calcium-binding properties of α-LA are retained in CNBr-α-LA, although the "linkage" between the apo- and "apo-like"-Ca(II) conformers in native α-LA is obviously lost because the strong calcium-binding loop is lost.

At this juncture, therefore, the results constitute a qualitative confirmation in solution of the importance of the calcium-binding loop for α-LA structure and function. We also note that the results with human CNBr-α-LA demonstrated a total loss of tumor growth inhibition activity (M. Bano, S. Mohanam, S. Liu, M. P. Thompson, and W. R. Kidwell, unpublished results). Thus, the structural integrity of the calcium-binding loop and its influence on overall α-LA structure go well beyond its modifier activity in lactose biosynthesis. Further studies are in progress on the nature of the folding characteristics of CNBr-α-LA (Meinholtz and Berliner, unpublished).

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