Functional Identification of Calcium Binding Residues in Bovine α-Lactalbumin†

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ABSTRACT: The functional role of previously identified calcium binding residues in α-lactalbumin (α-LA) was investigated by site-directed mutagenesis. Mutation of D82 to alanine did not effect the binding affinity for calcium, the protein structure, or its function in the lactose synthase assay, suggesting that this aspartate side chain is not essential for calcium binding or structural stabilization. In contrast, mutation of either D87 or D88 to alanine completely eliminated the strong calcium binding and altered α-LA as shown by several spectroscopically derived properties such as near- and far-UV CD and intrinsic fluorescence studies. These latter two mutants displayed significantly reduced abilities to stimulate lactose synthase activity (<3.5% of the maximal rate). Additionally, residues K79 and D84, which chelate calcium by backbone carbonyls, were mutated to alanine. K79A lost approximately 50% of its tertiary structure and stability (as determined by CD) but retained full calcium binding activity, indicating that at least the lysine side chain does not influence the carbonyl-mediated calcium coordination. In contrast, D84A lost approximately 25% of its tertiary structure and stability which was accompanied by a modest reduction in calcium affinity. Both mutants were able to stimulate normal lactose synthase activity. The triple mutant, D82A/D87A/D88A α-LA, lost its ability to bind calcium, similar to D87A and D88A. These studies clearly demonstrate the importance and variation of side chain interactions, which might be the seminal event in the establishment of the correct calcium binding loop conformation, possibly to stabilization and final folding of the overall protein structure.

α-Lactalbumin (α-LA)[1] is the mammary-specific modifier protein of the lactose synthase complex, comprised of galactosyltransferase (GT) (EC 2.4.1.90) and α-LA. α-LA is a model for protein folding because it forms a classic molten globule (MG) state during folding (Ptitsyn, 1987; Creighton, 1995; Kuwajima, 1996). The MG state is a compact structure which maintains secondary structure but lacks a stable tertiary conformation. The lack of a thermal unfolding transition, a characteristic of some native state proteins that fold via an MG state, is another characteristic of α-LA in the molten globule state.

The native structure of α-LA consists of two domains, an α-helical domain and a β-sheet domain, connected by a calcium binding loop. The structure is stabilized by four disulfide bridges (6−120, 61−77, 73−91, and 28−111). The two domains are held together by the cystine bridge between residues 73 and 91, forming a "loop" (Figure 1) which resides between these domains and binds calcium (Acharya et al., 1989, 1991). A second important disulfide bridge (61−77) connects the α-helical and β-sheet domains. Unlike other calcium-binding domains (such as EF-hands), the loop in this calcium binding domain is unique because it contains two fewer residues than the typical EF-hand (Kretsinger & Nockolds, 1973; Stuart et al., 1986; Acharya et al., 1989, 1991). The calcium binding loop contains two helices, a portion of the C-helix (residues 86−98) as well as a 3_{10}-helix (residues 77−80), which are analogous to, but smaller than, the helix−loop−helix motif of the classical EF-hand (Pike et al., 1996; Stuart et al., 1986; Acharya et al., 1989, 1991). These two helices articulate in a manner dissimilar to that of an EF-hand due to the β-turn-like loop region. The calcium ion is coordinated to three aspartic acid side chains (D82, D87, and D88), two backbone carbonyl oxygens (K79 and D84), and two water molecules which contribute

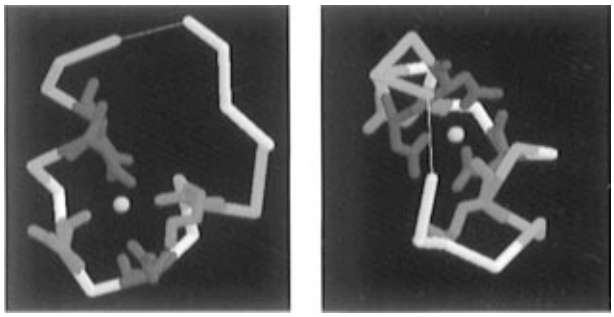

FIGURE 1: α-LA modeled from human α-lactalbumin (Bookhaven Protein Data Base entry 1HML) between residues 73 and 91 defined by the disulfide bond (yellow). Two views of the calcium binding loop containing portions of the C-helix (blue and red, residues 86−91) and the 3_{10}-helix (blue and green, residues 77−80). Calcium is shown in yellow. D87 and D88 are red and D84 and K79 green, and D82 is purple.

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[1] Abbreviations: α-LA, α-lactalbumin; GT, *N*-acetylglucosamine β-(1→4)galactosyltransferase; PCR, polymerase chain reaction; CD, circular dichroism; MG, molten globule; LB, Luria broth; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; BCA, bicinchoninic acid/copper sulfate protein assay.

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the last two oxygen ligands to a distorted pentagonal bipyramidal structure (Acharya et al., 1989, 1991).

Refolding of α-LA to its native structure was shown to be dependent upon the presence of calcium (Rao & Brew, 1989). However, in the *folded* protein, calcium is not absolutely required for lactose synthase activity; in fact, under certain *in vitro* conditions where the protein is not thermally unfolded, apo-α-LA is more active than Ca^{2+}−α-LA in the lactose synthase complex (Musci & Berliner, 1985). Selective CNBr treatment of the native protein cleaves the calcium binding loop at Met 90, completely disrupting its calcium binding ability, yet it maintains 2.5% of its biological activity (Berliner et al., 1991). These results also suggest that calcium is not essential for function, even though disruption of the calcium binding loop caused major changes in tertiary structure (D. M. Meinholtz and L. J. Berliner, unpublished results). Apo-α-LA is less stable than the calcium form, as observed, in thermal denaturation studies where apo-α-LA "melts" at 25 °C while holo-α-LA is stable up to 60 °C (Hiraoka et al., 1980, 1984; Permyakov et al., 1985; Permyakov & Burstein, 1986; Cawthern et al., 1996). Some data suggest that calcium remains associated with thermally denatured α-LA (Vanderheeren et al., 1996) which suggests that much of α-LA's structure can be altered before calcium binding is impaired. The data imply an important role for the calcium binding loop in folding.

α-LA is homologous to c-type lysozymes (Sugai & Ikeguchi, 1994; McKenzie & White, 1991). Human lysozyme has been genetically engineered to impart Ca^{2+} binding in the homologous loop region in bovine α-LA (Kuroki et al., 1989, 1992; Haezebrouck et al., 1993; Pardon et al., 1995). This resulted in a functional Ca^{2+}-binding form which was structurally more stable than the nonmutated lysozyme (Kuroki et al., 1989, 1992; Haezebrouck et al., 1993). A single mutation, A92D (human lysozyme numbering), homologous to D88 in bovine α-LA, bound Ca^{2+} with a dissociation constant of 2×10^{-7} M (Haezebrouck et al., 1993). However, as Linse and Forsen (1995) pointed out, the local coordination at the calcium ion is not the sole determinant of ligand binding as more distant changes in structure and dynamics can be significant.

In this study, we identify the relative thermodynamic contributions of functionally important side chains described by the X-ray structure (Stuart et al., 1986; Acharya et al., 1989, 1991) and their manifestations with respect to structural and functional integrity of α-LA. The functional roles played by each calcium binding residue were explored by substituting alanines and measuring Ca^{2+} binding as well as structural and functional properties.

MATERIALS AND METHODS

Materials. Bovine lactating mammary gland was provided by F. Schanbacher (Department of Dairy Science, The Ohio State University, Wooster, OH). Restriction enzymes, Taq polymerase, polynucleotide kinase, and T4 DNA ligase were from Boehringer Mannheim (Indianapolis, IN), New England Biolabs (Beverly, MA), or Gibco BRL (Gaithersburg, MD). R408 helper phage was from Promega, Inc. (Madison, WI). Primers were synthesized by Oligos, Etc. (Wilsonville, OR) or Integrated DNA Technologies (Coralville, IA) and used without further purification. Competent *Escherichia coli* strain DH5α was purchased from Gibco BRL and BL21-(DE3) from ATCC (Rockville, MD). Bovine α-LA (lot no. 128F-8140), UDP-galactose, NADH, pyruvate kinase, and lactate dehydrogenase were purchased from Sigma Chemical Co. (St. Louis, MO). DE-52 DEAE-cellulose was purchased from Whatman Lab Products (Hillsboro, OR). Chelex-100 was from Bio-Rad Laboratories (Richmond, CA). GT was purified as previously described (Grunwald & Berliner, 1978).

Preparation of PT7-7 Phagemid-Containing Methionyl-α-LA. Total RNA from lactating mammary tissue was extracted by the modified guanidinium isothiocyanate method of Goodman and Schanbacher (1991). cDNA was prepared by reverse transcription using the cDNA Cycle Kit (Invitrogen, Inc., San Diego, CA) with oligo [d(T)] as the primer. The DNA sequence coding α-LA was amplified from the cDNA by PCR. The 5′ primer coded for a N-terminal methionyl residue containing an *Nde*I restriction site in the mature protein and provided bacterially preferred codons in the first seven residues. The 3′ primer added a *Hind*III restriction site distal to the termination codon. The PCR product was cut and ligated into a pT7-7(+) phagemid (Maciejewski et al., 1995). Site-directed mutagenesis was performed as described by Kunkle et al. (1991). Mutants were selected by addition or deletion of translationally silent restriction sites. Two independent and complete DNA sequences for wild type (WT) and each α-LA mutant were obtained by the dideoxy-terminator method (Sanger et al., 1977) from both the 3′ and 5′ termini. The DNA coding for each mutant was confirmed by two independent methods: the presence of a unique restriction site and complete DNA sequencing.

Expression, Refolding, and Purification of Recombinant Bovine α-LAs. Phagemids containing DNA sequences for WT or mutant α-LAs were transformed into BL21(DE3) and grown in 1 L cultures of LB media containing ampicillin to an OD_{600} of 0.3. T7 RNA polymerase was induced with 0.4 mM isopropyl β-D-thiogalactopyranoside, and protein expression was continued for 3 h. Cells were collected by centrifugation at 6000 rpm for 15 min. Cell pellets were resuspended in 50 mL of 100 mM Tris (pH 7.5), 25 mM DTT, and 1 mM PMSF and lysed by two passes through a French pressure cell at 5000 psi. Inclusion bodies were collected by centrifugation at 20000*g* for 30 min. Pellets from 1 L fermentations were resuspended in 500 mL of 50 mM Tris (pH 7.5), 4 M urea, 5 mM β-mercaptoethanol, 0.5 mM 2-hydroxyethyl disulfide, 1 mM $CaCl_2$, and 0.01% (w/v) NaN_3, according to the method of Grobler et al. (1994). The pH was adjusted to 9.2, and the solution was allowed to mix at room temperature for 1 h. Solutions were clarified by centrifugation at 20000*g* for 30 min. The resultant supernatant was dialyzed against 2 L of 10 mM Tris (pH 7.5), 1 mM $CaCl_2$, 5 mM β-mercaptoethanol, and 0.5 mM 2-hydroxyethyl disulfide. The dialysate was changed three times with 2 L of buffer followed by two more 4 L changes.

The proteins were precipitated with 80% ammonium sulfate and centrifuged at 20000*g* for 30 min. The pellets were resuspended in 250 mL of 10 mM Tris (pH 7.5) and 1 mM $CaCl_2$, incubated at room temperature for 1 h, centrifuged at 20000*g* for 15 min, and dialyzed against 10 mM Tris (pH 7.5) and 1 mM $CaCl_2$. The dialyzed proteins were loaded into DEAE-cellulose columns (5 cm × 5 cm) previously equilibrated in the dialysate buffer and washed with 0.1 M NaCl in buffer until $OD_{280} < 0.010$. Proteins

were eluted with a 500 mL gradient from 0.1 to 0.5 M NaCl in the same buffer, monitored by UV absorbance, and dialyzed against 10 mM ammonium bicarbonate (pH 7.8). The products were lyophilized and stored (desiccated) at −30 °C. Each recombinant α-LA was expressed with yields between 7 and 16 mg per liter of broth. Proteins were greater than 95% pure as judged by SDS-containing gel electrophoresis under reducing conditions.

Verification of Homogeneity of Folded Proteins by HPLC. Proteins were chromatographed using the procedure of Wang et al. (1989). Briefly, proteins were resuspended in 0.1% TFA/water and applied to a C-18 reverse phase column (Waters Division, Millipore Corp., Bedford, MA, 2.8 × 300 mm, 5 μm particle size, 300 Å pore diameter) with a CH_3-CN/H_2O gradient (30 to 80%, 1 mL/min flow rate), monitored at 280 nm. All α-LA mutants eluted as a single peak and eluted within 1.5 standard deviations of the elution time of the WT protein.

Lactose Synthase Assay. Lactose synthase activity was monitored by the coupled assay of Fitzgerald et al. (1970). The assay medium contained 10 mM *N*-methylmorpholine (pH 8.0), 0.41 mM UDP-galactose, 2.4 mM $MnCl_2$, 0.21 mM NADH, 0.26 mM phosphoenolpyruvate, 20.1 mM glucose, 5.6 units of pyruvate kinase, 8 units of lactate dehydrogenase, and 0.26 μg/mL GT. α-LA concentrations varied between 0 and 25 μM and were determined both by absorbance using an extinction coefficient for native α-LA $E_{0.1\%,280nm}$ of 2.01 (Kronman & Andreotti, 1964) and by the BCA assay (Pierce, Rockford, IL). Initial rates of lactose synthesis were plotted as a function of α-LA concentration and fit to an apparent Michealis constant, $K_{m(app)}$, and V_{max} by nonlinear regression analysis.

Spectroscopic Methods. Fluorescence spectra were recorded with a temperature-controlled Perkin-Elmer LS50B fluorometer. Fluorescence thermal denaturations were performed in 10 mM HEPES (pH 8.0). Protein intrinsic fluorescence emission spectra (λ_{ex} = 280 nm) were recorded every 2 °C with a heating rate of 0.5−1 °C/minute. Calcium titrations of apo-α-LA and EGTA back-titrations of Ca^{2+}−α-LA were performed in 10 mM Chelex-100-treated ammonium bicarbonate at pH 7.8 and 25 °C.

CD spectra were recorded on a Jasco J-550A spectropolarimeter. A minimum of 1000 data points were averaged for each wavelength in the spectra.

Preparation of Apo-α-LA. α-LA was decalcified by treatment in 0.1 mM EGTA of pH 8.0 for 15 min at 4 °C, followed by gel filtration chromatography on Sephadex G-25 (1 cm × 30 cm) in 10 mM ammonium bicarbonate (pH 7.8) to remove excess EGTA. The columns were thoroughly prewashed with EGTA and equilibrated with Chelex-100-treated ammonium bicarbonate buffer.

Data Analysis. Fluorescence thermal denaturation was analyzed as previously described (Permyakov & Burstein, 1985; Cawthern et al., 1996). Both emission intensity at 350 nm and λ_{max} were plotted as a function of temperature. Fractional conversion was calculated for each mutant's unfolding curve as previously described (Cawthern et al., 1996). The initial fluorescence intensity was used for normalization to compare the mutants. These procedures are also outlined in detail in Permyakov (1994).

Dissociation constants for calcium binding were obtained by titration of apo-α-LA with calcium followed by back-titration with EGTA. Typical protein concentrations were between 1 and 4 μM. The back-titration results were analyzed according to a simple competitive binding scheme:

$$\alpha\text{-LA} + Ca^{2+} \xrightarrow{K_p} \alpha\text{-LA}{-}Ca^{2+} \quad (1)$$

$$\text{EGTA} + Ca^{2+} \xrightarrow{K_e} \text{EGTA}{-}Ca^{2+} \quad (2)$$

where K_p and K_e are the dissociation constants of calcium for α-LA and EGTA, respectively. The K_e value used was 5.596 nM at pH 7.8. These procedures are outlined in detail in Permyakov (1994). The data were also analyzed by allowing the binding stoichiometry to vary with no apparent effect on the calculated binding constants (Bock & Shore, 1983; Lindahl et al., 1991).

Table 1: Calcium Dissociation Constants and Kinetic Parameters (Lactose Synthase Reaction) for Recombinant α-Lactalbumins[a]

protein	K_d (μM)	$K_{m(app)}$ (μM)	V_{max} (M/s)
WT	0.21 ± 0.06	8.8	1.8×10^{-7}
K79A	0.23 ± 0.06	8.8	1.9×10^{-7}
D82A	0.40 ± 0.10	8.7	2.0×10^{-7}
D84A	6.1 ± 1.0	9.2	1.6×10^{-7}
D87A	nd[a]	nd[b]	nd[b]
D88A	nd	nd	nd
D82/87/88A	nd	nd	nd
M90V	0.20 ± 0.06	9.5	1.7×10^{-7}

[a] Conditions were 10 mM ammonium bicarbonate at pH 7.8 and 25 °C. [b] Not detectable. Calcium binding was unable to be detected by titration using intrinsic fluorescence. [c] Not detectable. Detection of lactose synthesis was <3%.

RESULTS AND DISCUSSION

Previous X-ray studies identified a calcium binding loop conserved in α-LA and delineated those residues which bound calcium (Stuart et al., 1986; Acharya et al., 1989, 1991). One might expect an incremental diminution in the calcium binding constant with the sequential elimination of calcium binding ligands. In this work, we have identified residues that are functional for calcium binding by replacement with an alanine and examined the structural integrity of each protein as monitored by CD, fluorescence, thermal stability, and lactose synthase activity. The mutant α-LA's that were studied fell into three groups. The first group was composed of only D82A, a residue that contributes its β-carboxylate in calcium coordination; the second group consisted of D87A and D88A, which also bind calcium via the side chain β-carboxylate group, and D84A and K79A are residues that coordinate calcium via the backbone peptide carbonyl (Figure 1).

D82A α-LA. For each mutant, the calcium binding affinity was compared with that of the wild type α-LA by intrinsic fluorescence titration of the apo form as well as EGTA back-titration. Unexpectedly, the D82A mutant bound calcium with the same affinity as did the wild type (Table 1); i.e., removal of the coordinating oxygen ligand had no effect on the free energy of calcium binding. In addition, the calcium-induced λ_{em}^{max} shifts of the Ca^{2+}-bound (337 nm) and apo (348 nm) forms of both WT and D82A species were identical. The unimportance of this side chain to the structural integrity of Ca^{2+}−α-LA was confirmed by the near- and far-UV CD spectra at 10 °C shown in Figure 2A,B where no differences were observed in the near-UV CD of

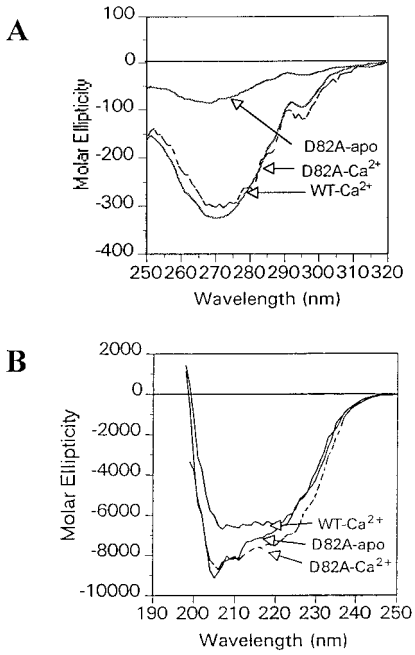

FIGURE 2: CD spectra of WT α-LA, D82A α-LA, and Ca^{2+}−D82A α-LA (---): (A) near-UV spectra and (B) far-UV spectra. The protein concentration was 1−1.5 mg/mL in 10 mM Tris and 1 mM $CaCl_2$ at pH 8.0 and 10 °C.

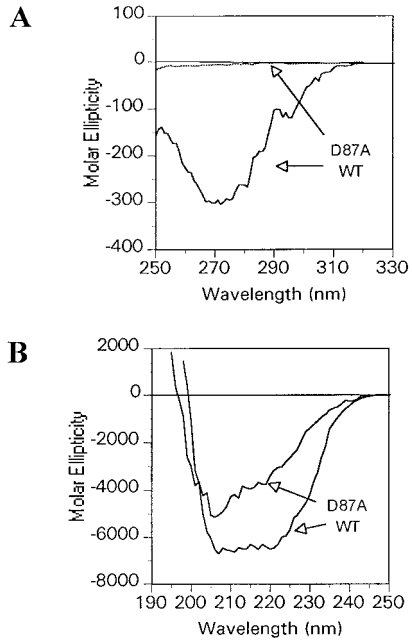

FIGURE 3: CD spectra of WT α-LA and D87A α-LA: (A) near-UV spectra and (B) far-UV spectra. All other conditions were as in Figure 2.

Ca^{2+}−D82A and Ca^{2+}−WT α-LA, respectively (Figure 2A). The far-UV CD spectra of the D82A mutant showed a slight decrease in ellipticity which compared well with that of the WT α-LA (Figure 2B). Furthermore, no additional differences were observed when the same studies were performed at room temperature, indicating the general stability of the calcium-bound species at elevated temperatures (data not shown). Comparing the near- and far-UV CD of the apo forms of both recombinant proteins, we observed a partial disruption of tertiary structure as shown by the typical loss of signal in the near-UV spectra (Figure 2A) indicating again that their relative stabilities (D82A *vs* WT α-LA) were similar. Thermal denaturation curves as monitored by changes in intrinsic fluorescence emission intensity showed no differences between Ca^{2+}−WT α-LA and D82A α-LA in melting temperature. Lastly, the functional integrity of D82A holo-α-LA, as measured by lactose synthase activity, was indistinguishable from that of WT α-LA [$K_{m(app)} = 8.7$ μM]. In conclusion, D82, identified by structural studies as a calcium binding side chain, appears to contribute no free energy to calcium binding and is not required for the proper calcium-induced folding to the mature structure (Rao & Brew, 1989).

D87A, D88A-α-LA. The removal of the β-carboxylate by mutation to alanine of either D87 or D88 totally obliterated strong calcium binding as monitored by intrinsic fluorescence titrations as described earlier (Table 1). This was in sharp contrast to the results with the D82A mutation described above. In addition, the $\lambda_{em}{}^{max}$ of the apo form was neither shifted nor quenched upon excess calcium addition [$\lambda_{em}{}^{max}{}_{(D87A)} = 348$ nm and $\lambda_{em}{}^{max}{}_{(D88A)} = 345$ nm]. The removal of the coordinating oxygen ligand at either position 87 or 88, eliminating calcium binding, demonstrates that the crucial functional determinants in these side chains are absolutely required. The consequence of this lethal modification to the maintenance of α-LA structure was obvious from the elimination of the near-UV CD ellipticity of D87A α-LA in the presence of excess calcium at 10 °C (Figure 3A) and also from the increased 205 nm/222 nm ratio in the far-UV CD (Figure 3B) which is highly indicative of molten globule α-LA. Identical results (data not shown) were obtained with D88A α-LA. It is noteworthy that, aside from the disulfide bridge deletion mutants of Peng et al. (1995), this represents the first example of a MG state α-LA under physiological pH conditions at room temperature. Thermal denaturation of either D87A or D88A α-LA in the presence of excess calcium as monitored by intrinsic fluorescence emission intensity showed absolutely no change up to 80 °C, which is indicative of the lack of a distinct thermal transition in molten globule α-LA (Ptitsyn, 1987). Lastly, both recombinant proteins showed less than 3.5% activity *vs* WT α-LA for the same galactosyltransferase concentration in the lactose synthase assay (up to 25 μM D87A or D88A α-LA). Since the altered $K_{m(app)}$ values were much higher than experimentally obtainable α-LA concentrations due to limited solubility and aggregation, we could only estimate a $K_{m(app)}$ (500−1000 μM) assuming an unchanged V_{max}. As discussed earlier, while calcium is required for the protein to adopt the native state (Rao & Brew, 1989), once this fully folded native state has formed, lactose synthase activity is not absolutely dependent upon the addition and/or removal of calcium. These results were very similar to the results observed by Berliner et al. (1991), where the calcium binding loop was cleaved by CNBr at M90. In contrast to the functional role of D82, both the side chains of residues D87 and D88, identified in structural studies as calcium binding determinants, have been shown to be critical for cation binding, structural stability, and function in solution. These results were surprising in that the absolute loss of calcium binding could be associated with the mutation of a single, structurally specific residue instead an incremental diminution in calcium affinity with the sequential withdrawal of calcium binding side chains.

K79A and D84A α-LA. These two residues fall in a special class in that they coordinate calcium through the

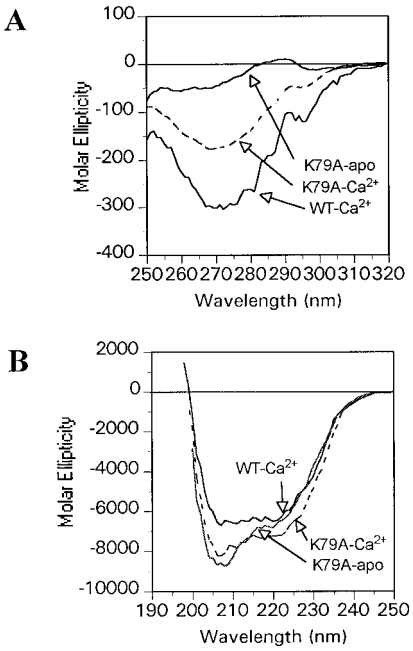

FIGURE 4: CD spectra of WT α-LA, K79A α-LA, and Ca^{2+}−K79A α-LA (---): (A) near-UV spectra and (B) far-UV spectra. All other conditions were as in Figure 2.

backbone peptide carbonyl. Not unexpectedly, removal of the cationic side chain of (conserved residue) K79 had no effect on calcium binding as monitored by intrinsic fluorescence (Table 1). On the other hand, D84A was unusual in that it displayed an incremental (≈30-fold) reduction in calcium binding affinity as would be expected for the removal of a single, coordinating oxygen ligand. The shifts in λ_{em}^{max} induced by calcium for both K79A and D84A α-LA (Ca^{2+}-bound, 337 nm; apo, 348 nm) were nearly identical to that of WT α-LA. The near-UV CD of K79A α-LA in the presence of excess calcium at 10 °C (Figure 4A) lost almost 50% of its ellipticity, and from the slightly altered 205 nm/222 nm ratio in the far-UV CD (Figure 4B), there was some indication of loss of a structural stability. This was more evident at room temperature where the near-UV CD of Ca^{2+}−K79A α-LA showed an ≈67% loss in tertiary structural features (Figure 5A). Remarkably, the near-UV CD of Ca^{2+}−D84A α-LA lost only ≈25% of its ellipticity at 10 °C but lost ≈50% at room temperature (Figure 5B); however, in the far-UV CD, changes similar to those of Ca^{2+}−K79A α-LA were observed (data not shown). Comparing the apo forms of both recombinant proteins in this group, we observed more disruption of tertiary structure in the near-UV spectra (Figure 4A) than was found for D82A or WT (Figure 2A). The fluorescence thermal denaturation curves did not provide as convincing evidence with respect to the stability of the holo forms of K79A and D84A *vs* WT α-LA in melting temperature. Resolution of these data awaits further studies by differential scanning calorimetry. Lastly, the functional integrity of the holo forms of K79A and D84A α-LA, as measured by lactose synthase activity, was unchanged *vs* that of WT α-LA (Table 1). The side chain of K79, which projects into the solvent, had no obvious effect on the chelation of calcium, yet the structure and the stability of this mutant have been subtly altered. In contrast, removal of the carboxylate side chain of D84A α-LA showed a reduction in calcium binding affinity, accompanied by more pronounced changes in structure and stability. It is clear

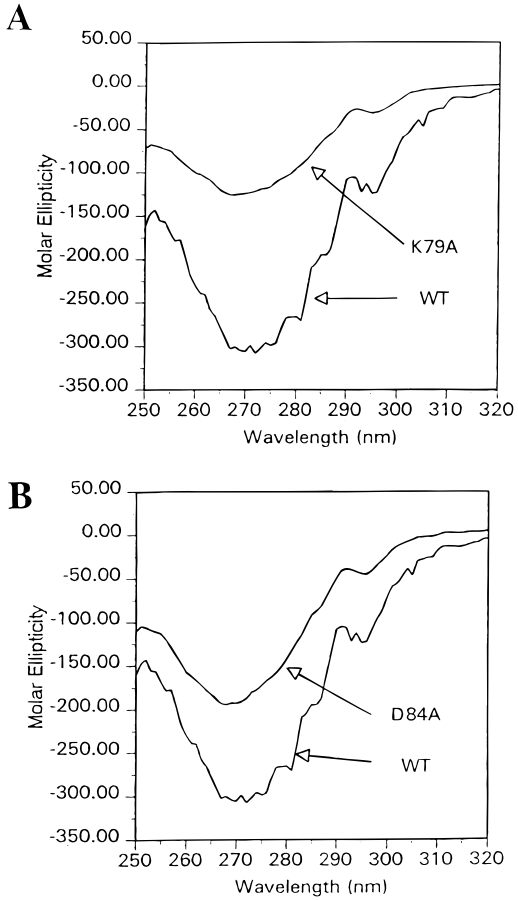

FIGURE 5: Room-temperature (22 ± 2 °C) near-UV CD spectra of (A) K79A α-LA and (B) D84A α-LA. All other conditions were as in Figure 2.

from all of these results that each residue plays a distinct role in the chelation of calcium and that there are additional contributions from the side chains as well to both the structure and stabilization of both this unique calcium binding loop and the global structure of the protein.

Other Mutants. Since we had anticipated incremental changes in calcium affinity with stepwise elimination of each residue contributing side chain oxygen binding ligands, we constructed a triple mutant D82A/D87A/D88A which, not surprisingly, behaved identically with respect to either D87A or D88A α-LA in calcium affinity and functional integrity (Figure 3 and Table 1). The triple mutant was more similar to D88A α-LA where the λ_{em}^{max} of the apo or Ca^{2+} form was unique (λ_{em}^{max} = 345 nm). The far-UV CD 205 nm/222 nm ratio of D82A/D87A/D88A α-LA at 10 °C was much larger than that observed for either D87A or D88A α-LA, indicating a cumulative loss of structure with each additional mutation. At room temperature the spectral features more closely approached that found for MG α-LA than those of any of the other mutants.

Thermal denaturation in the presence of excess calcium showed absolutely no change up to 80 °C, which was identical to that found for D87A and D88A α-LA, i.e., the lack of a distinct thermal transition for molten globule α-LAs (Ptitsyn, 1987). Lastly, the recombinant triple mutant showed less than 3.5% of the activity of WT α-LA in the lactose synthase assay, similar to that found with D87A and D88A α-LA above (Table 1). In summary, the results found with D82A/D87A/D88A α-LA, while not totally unexpected, contained features found in each of the individual mutants.

For example, the distinct 3 nm blue-shifted fluorescence emission maximum of D88A α-LA is also observed with the triple mutant. The thermal stability of the triple mutant is much less; i.e., each successive mutation produces a cumulative loss of structure.

The mutant M90V was prepared because of the suggestion of Wang et al. (1989), who found that this replacement was necessary for efficient expression. While this substitution was not obligatory for expression in our vector system, we nevertheless studied some of the physical and functional properties of this mutant since this residue coincidentally falls in the calcium binding loop. The data showed that, while the calcium affinity was not altered, both the near- and far-UV CD of Ca^{2+}-M90V α-LA were quite similar to that shown for D84A α-LA shown above. The fluorescence emission maximum of the calcium form was less blue-shifted ($\lambda_{em}^{max} = 340$ nm), while the apo form was normal ($\lambda_{em}^{max} = 348$ nm). The fluorescence quenching upon calcium binding was unusual, approaching 50% of that observed for all α-LAs capable of binding calcium. The apo form was also less stable *vs* WT α-LA as deduced from fluorescence thermal denaturation studies. Overall, it appeared that this mutation imparted a reduction of structural stability compared to that of the wild type species.

Speculations on the Structural Basis of These Substitutions. While we cannot describe the complete folding process in detail, it is enticing to speculate from these results that the D87/D88 carboxylate pair, which appears to contribute the majority of the free energy during calcium binding, initially docks or coordinates the calcium ion into the cation binding region, while secondary steps involve the backbone interactions with K79 and D84, followed by capping with D82 and the two solvent water molecules. Some justification for this model comes from the observation that D82A and WT α-LA were equally competent in their calcium binding abilities. The backbone residues, particularly D84, were found to have an important contribution to calcium binding. Hence, side chain interactions might be the seminal event in the establishment of the correct calcium binding loop conformation and possibly in stabilization and final folding of the overall protein structure.

Both D87 and D88 exist within the first turn of the C-helix of the α-helical domain of this protein. Their juxtaposition(s) in space appears to be highly dictated by the holoprotein structure as judged from the X-ray coordinates. Furthermore, we note that D87 and D88 are involved in unique internal hydrogen bonding contacts. Specifically, D88, which is not accessible to the solvent, besides acting as a ligand to calcium ion, makes contacts to internal water molecules (via OD1) and is also hydrogen bonded to OD1 of D87. Acharya et al. (1989) noted that "such interactions are usually observed in protein structures involving bound water molecules, which are partly responsible for the stabilization of the structure." Consequently, when the carboxylate side chains of either D87 or D88 are eliminated and replaced with alanine, where only the β-carbon remains, a key element in structural stabilization has been lost. The importance of these residues is even more dramatic than previously thought (Acharya et al., 1989). While replacement of strategic residues in the "loop region" of human lysozyme provided calcium binding affinity (Kuroki et al., 1989, 1992; Haezebrouck, 1993), the ability of α-LA to bind calcium does not depend solely upon the local coordination at the calcium ion. Linse and Forsen (1995) studied several calcium binding proteins and typically found that the surface within 5−15 Å of the cation has a net negative charge and that the hydrophobic core is more favorably packed in the presence of calcium. Removal of the side chains from D87 and D88 in the Ca^{2+}-binding loop of bovine α-LA disrupts the Ca^{2+} coordination sphere by changing the overall net negative charge of this region; hence, the protein does not adopt a native conformation.

Furthermore, in human α-LA, an additional water molecule hydrogen bonds between the side chain carboxylate oxygens of D87 and D84 (Acharya et al., 1991). We showed the functional importance of D84 (which binds calcium only through the backbone carbonyl oxygen) where the D84 mutation reduced the calcium affinity by greater than 1 order of magnitude and imparted changes in the secondary and tertiary structure from CD and thermal measurements. Note that calcium binding lysozymes contain an Asn at the position corresponding to D84 in the α-LA sequence, resulting in calcium binding affinities that are 100−1000-fold lower (Nitta et al., 1987, 1988). Hence, it appears that loss of the water bridging this side chain with D87 appears to have functional consequences.

K79 binds calcium via the backbone carbonyl oxygen, and replacement of the side chain by mutation to alanine has no apparent effect on calcium binding yet modestly affects protein structure and stability. The contribution of this basic side chain may be a partial charge stabilization of the apoprotein as noted by Acharya et al. (1991). This was certainly borne out here in both the temperature-dependent CD (Figures 4 and 5) and fluorescence thermal unfolding studies where K79A apo-α-LA was significantly less stable than WT α-LA yet showed similar stabilities in the presence of calcium (data not shown).

We have shown the relative effects of elimination of the side chains of residues involved in calcium binding, some of which were quite varied and dramatic in their manifestation of protein structure, stability and function. Yet, when the absolute calcium affinity of WT α-LA was compared with that of α-LA isolated from milk, there was an almost 100-fold increase in the dissociation constant (as measured under our conditions and those of others; Murakami et al., 1982; Permyakov et al., 1981). Considering, however, that the functional properties of the recombinant WT protein were equivalent to those of the biological isolate, the principal difference between recombinant and biological isolates of α-LA was the presence of a methionyl residue at the N terminus of the recombinant protein. The specificity of the methionine aminopeptidase in some bacterial expression systems depends heavily on the side chain of the N-penultimate residue. Bovine α-LA, as well as most of the other mammalian species, does not contain short, hydrophobic residues at this position and is unlikely to be cleaved. To our knowledge, we are the only investigators who have reported the calcium affinity constants for these recombinant (methionyl) α-LAs. Examination of the three-dimensional structures of those α-LAs available in the Brookhaven Protein Data Base to date (i.e., baboon or human α-LA) demonstrates that addition of an N-terminal methionyl can spatially perturb the calcium binding loop. Preliminary studies cleaving methionyl residues from WT α-LA result in an increased calcium binding affinity (M. Narayan, C. L. Brooks, P. J. Anderson, and L. J. Berliner, unpublished results). The N terminus is directed toward the side of the

calcium binding loop and constrained in that position by the disulfide bridge between residues 6 and 120, as well as critical intermolecular hydrogen bonds between the ϵ-amino group of (conserved) Lys 5, the side chain oxygen of (highly conserved) Gln 117, plus the carbonyl oxygen of Leu 119 and hydrogen bonds between Phe 3 and Thr 38. The N terminus of human α-LA is only 4 Å from the carboxylate of Asp 83. As the side chain of Met may extend 5.4 Å from the α-carbon, it appears likely that the addition of an N-terminal Met could approach the calcium binding loop. X-ray studies have shown that the most rigid part of the molecule in baboon, human, guinea pig, and goat α-LAs was consistently the calcium binding loop (Acharya et al., 1991; Pike et al., 1996). Interestingly, in the recombinant bovine α-LA crystal structure, it was found that the temperature factors of the calcium binding loop (residues K79, D82, D84, D87, and D88) were 3−6-fold larger than that of the isolated human form (Pike et al., 1996), suggesting to us a perturbed calcium binding loop in a *N*-methionyl protein.

In conclusion, our current studies demonstrate different functional and energetic contributions of the residues shown by structural studies to coordinate calcium in α-LA. Recombinant proteins able to bind calcium retained most or all of the structural and thermal stability properties of α-LA. α-LAs with severely impaired abilities to bind calcium were shown to be in the MG form. These observations are certainly consistent with those of Rao and Brew (1989), who demonstrated the requirement of calcium for correct folding. Further studies will investigate the function of the N terminus in the calcium binding loop.

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