Structural Characterization of the Disulfide Folding Intermediates of Bovine α-Lactalbumin†

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ABSTRACT: Specific three- and two-disulfide intermediates that accumulate transiently during reduction of the disulfide bonds of Ca^{2+}-bound bovine α-lactalbumin have been trapped, isolated, and characterized. The three-disulfide intermediate was shown to lack the Cys6–120 disulfide bond, confirming the observations of others. The newly-recognized two-disulfide form has been shown to lack the Cys6–120 and Cys28–111 native disulfide bonds. The remaining native disulfide bonds in the two partially reduced derivatives of α-lactalbumin are stable only when the proteins are in a Ca^{2+}-bound state. Otherwise, they adopt an equilibrium between molten globule and unfolded conformations, and rapid thiol–disulfide interchange occurs, at a rate as high as when the proteins are fully unfolded in 8 M urea, to generate distinct mixtures of rearranged products. Urea gradient electrophoresis, circular dichroism, fluorescence, and ANS binding have been combined to give a detailed structural picture of α-lactalbumin, its derivatives with native and with nonnative disulfide bonds, and the fully reduced protein. The native structure of α-lactalbumin appears to be split by selective disulfide bond cleavage into at least one subdomain, which retains the Ca^{2+}-binding site. The α-lactalbumin molten globule state is shown largely to result from nonspecific hydrophobic collapse, to be devoid of cooperative or specific tertiary interactions, and not to be stabilized substantially by the native or rearranged disulfide bonds.

Single-domain proteins usually unfold reversibly by a cooperative process in which partially-folded intermediates between the fully folded, native state and the fully unfolded state are not stable at equilibrium. The small, but growing, number of proteins that are found to be exceptions to this rule appear under particular conditions to have conformational properties similar in some respects to those of the native state and others characteristic of the unfolded state. This has usually been interpreted in terms of a single conformational state, the so-called molten globule (MG),[1] intermediate between the native and unfolded states but otherwise poorly understood [see Kuwajima (1989), Baldwin (1991), and Ptitsyn (1992) for reviews]. This conformational state is especially important because a wide variety of globular proteins appear to pass through a transient MG-like state during folding (Ptitsyn et al., 1990). Detailed characterization of a stable molten globule state should therefore be relevant to the more general problem of how proteins fold.

One of the best-characterized MG states is that of bovine α-lactalbumin (Kuwajima, 1989). The native state of this protein is stabilized by binding of a single Ca^{2+} ion (Hiraoka et al., 1980; Acharya et al., 1989, 1991). When the Ca^{2+} is dissociated at acid pH, at elevated temperatures, or at intermediate concentrations of denaturant, αLA tends to adopt the MG conformation; it is compact (Dolgikh et al., 1981) and, as judged by NMR (Baum et al., 1989) and far-UV CD [e.g., Kronman et al. (1965); Kuwajima et al. (1981)], it contains some of the secondary structure present in the native protein. But the native tertiary interactions appear to be greatly diminished: the near-UV CD spectrum is greatly reduced, and there are few, if any, native tertiary interactions detectable by NMR (Baum et al., 1989). The α-lactalbumin MG state present at equilibrium appears to be similar to a kinetic intermediate state that is populated transiently during refolding (Kuwajima et al., 1985; Ikeguchi et al., 1986a).

Partially folded intermediates that are intrinsically unstable can be trapped in a stable form if their folding is coupled to disulfide bond formation. Native α-lactalbumin has four disulfide bonds, Cys6–120, Cys28–111, Cys61–77, and Cys73–91, when it is designated here as αLA. The results of an extensive kinetic analysis of disulfide bond formation and reduction in the bovine protein are reported in the preceding paper (Ewbank & Creighton, 1993). When αLA is folded, reductive cleavage of one of its four disulfide bonds, that between Cys6 and Cys120, occurs at a rate hundreds of times faster than that of any of the other three disulfides, and a unique three-disulfide intermediate (3SS) is produced (Shechter et al., 1973; Iyer & Klee, 1973). Ca^{2+}-bound 3SS maintains a folded conformation (Kuwajima et al., 1990) and is reduced specifically to generate a two-disulfide intermediate (2SS) that accumulates if Ca^{2+} is present at high concentrations (Ewbank & Creighton, 1993). This intermediate, which had not previously been observed, has been trapped

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[1] Abbreviations: αLA, α-lactalbumin with its four native disulfide bonds intact; apo-αLA, αLA without liganded Ca^{2+}; 3SS, bovine α-lactalbumin with the Cys6–120 disulfide bond reduced; 2SS, bovine α-lactalbumin with Cys6–120 and Cys28–111 disulfide bonds reduced; R, fully reduced bovine α-lactalbumin; [*n*SS] mixture of disulfide isomers other than *n*SS containing *n* disulfide bonds, derived from *n*SS by intramolecular disulfide rearrangements; $[nSS]^u$, mixture of isomers other than *n*SS containing *n* disulfide bonds, derived from *n*SS in 8 M urea by intramolecular disulfide rearrangements; ANS, 1-anilinonaphthalene-8-sulfonate; BPTI, bovine pancreatic trypsin inhibitor; CD, circular dichroism; DTT_{SH}^{SH}, reduced dithiothreitol; DTT_S^S, oxidized dithiothreitol; EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography; I-AEDANS, *N*-iodoacetyl-*N'*-(5-sulfo-1-naphthyl)ethylenediamine; λ_{max}, wavelength of maximum fluorescence emission; MG, molten globule; N, native, fully folded conformation; NMR, nuclear magnetic resonance; TFA, trifluoroacetic acid; U, fully unfolded conformation; UV, ultraviolet. Intermediates with all thiol groups trapped with iodoacetamide, iodoacetate, or I-AEDANS are designated by subscripts cam, cm, or AEDANS, respectively. For partially blocked intermediates, the blocked thiol groups are indicated, for example: $2SS^{6/120cam}$, 2SS with Cys6 and Cys120 carboxamidomethylated.

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and isolated, and the second cleaved disulfide bond is identified here as being that between Cys28 and Cys111.

The native disulfides of the homologous three-disulfide form of human α-lactalbumin have been shown previously to be stable when the protein is in its native conformation, with Ca^{2+} bound (Ewbank & Creighton, 1991). Otherwise, when human 3SS adopts a molten globule or unfolded conformation, its disulfides rearrange intramolecularly. The apo forms of both 3SS and 2SS of the bovine protein are also shown here to adopt molten globule and unfolded types of conformations; their remaining native disulfides are then unstable and spontaneously rearrange intramolecularly.

The structures of bovine αLA, the two intermediates 3SS and 2SS, together with their rearranged derivatives and the fully reduced protein (R), have been investigated directly by CD and intrinsic fluorescence, by transverse urea gradient gel electrophoresis, and by binding of the hydrophobic fluorescent dye ANS. The characterization of these forms has allowed a fuller understanding of the pathways of reduction and reformation of α-lactalbumin's disulfide bonds and of the stabilization of its different conformational states. In particular, it is shown that the native disulfides do not stabilize the MG state substantially, and vice versa, consistent with the tendency of the native disulfides of 3SS to rearrange when it is in this conformation. The partly folded conformation of Ca^{2+}-bound 2SS further illustrates the ability of selective disulfide bond cleavage to dissect folded proteins into stable subdomains.

MATERIALS AND METHODS

Materials. Trypsin (type XIII, TPCK-treated) and I-AEDANS (*N*-iodoacetyl-*N'*-(5-sulfo-1-naphthyl)ethylenediamine) were obtained from Sigma, and ANS was from Fluka. They were used without further purification. All other materials were as described elsewhere (Ewbank & Creighton, 1993).

Preparation of Trapped Fully Reduced and Partially Reduced Forms of α-Lactalbumin: (*a*) *Blocked Fully Reduced α-Lactalbumin* (R_{cam} *and* R_{cm}). DTT^{SH}_{SH} (100 mM) in 0.1 M Tris (pH 8.7), 0.2 M KCl, 1 mM EDTA was added to an equal volume of αLA (40 mg/mL in the same buffer). After 15 min of incubation at 25 °C, when the protein was fully reduced, all free thiols were blocked by the addition of $^1/_5$ volume of 0.6 M iodoacetamide or of 0.6 M iodoacetate (neutralized with KOH), in 0.5 M Tris (pH 8.0). After 2 min of further incubation at 25 °C, the protein was separated from the excess blocking reagent by rapid gel filtration into 20 mM ammonium acetate (Pharmacia Fast Desalting column) and then lyophilized.

(*b*) *Iodoacetamide-Trapped Two-Disulfide α-Lactalbumin* ($2SS_{cam}$). 2SS was purified by reversed-phase HPLC as described in the preceding paper (Ewbank & Creighton, 1993). After the addition of 1 M $CaCl_2$ to 20 mM, the free thiols were trapped with 0.6 M iodoacetamide as above. The protein was rechromatographed by reversed-phase HPLC (Dynamax-300A C_4 column 4.6 × 250 mm, with a linear gradient of 25% to 50% acetonitrile in 0.1% TFA) and lyophilized.

(*c*) *Iodoacetamide-Trapped Three-Disulfide α-Lactalbumin* ($3SS_{cam}$). One-tenth volume of 60 mM DTT^{SH}_{SH} in 0.1 M Tris (pH 7), 0.2 M KCl, 10 mM $CaCl_2$ was added to a 20 mg/mL solution of αLA in the same buffer. After 2 min of incubation at 25 °C, all free thiols were trapped with 0.6 M iodoacetamide as detailed above. The mixture was desalted, and then $3SS_{cam}$ was further purified by reversed-phase HPLC as above and lyophilized.

Preparation of Trapped Rearranged-Disulfide Forms of α-Lactalbumin. (*a*) *Iodoacetamide-Trapped Rearranged Two-Disulfide α-Lactalbumin* ($[2SS]_{cam}$ *and* $[2SS]^u_{cam}$). Lyophilized acid-trapped 2SS with free thiols, prepared as described in the preceding paper (Ewbank & Creighton, 1993), was dissolved to a concentration of approximately 120 μM in 10 mM HCl and then diluted 24-fold into either 0.1 M Tris (pH 8.7), 0.2 M KCl, 1 mM EDTA, or the same buffer containing 8.35 M urea. After 2 min at 25 °C, the free thiols were trapped with 0.6 M iodoacetamide, as above. One-fifth volume of acetonitrile (containing 0.1% TFA) was then added, and the mixture was applied to a C_4 reversed-phase column as above. The column was washed with 25% acetonitrile, 0.1% TFA to elute the excess blocking reagent, and then all the protein was eluted by a single step to 80% acetonitrile, 0.1% TFA. The protein-containing fraction was lyophilized. The mixtures obtained in the presence and absence of urea were designated $[2SS]^u_{cam}$ and $[2SS]_{cam}$, respectively.

(*b*) *Iodoacetamide-Trapped Rearranged Three-Disulfide α-Lactalbumin* ($[3SS]_{cam}$ *and* $[3SS]^u_{cam}$). 3SS was generated by partial reduction of Ca^{2+}-bound αLA and then diluted into an EDTA- or urea-containing buffer as described in the preceding paper (Ewbank & Creighton, 1993). After 2 min at 25 °C, the protein was trapped with 0.6 M iodoacetamide and purified in the same way as $[2SS]_{cam}$ as above. The mixtures obtained in the presence and absence of urea were designated $[3SS]^u_{cam}$ and $[3SS]_{cam}$, respectively.

Preparation of AEDANS-Trapped Partially Reduced Forms of α-Lactalbumin. One-fourth volume of 40 mM DTT^{SH}_{SH} in 0.1 M Tris (pH 8.7), 0.2 M KCl, 10 mM $CaCl_2$ was added to αLA at 12 mg/mL in the same buffer. After 2 min of incubation of 25 °C, the mixture of 3SS, 2SS, and R was trapped by the addition of $^1/_5$ volume of 0.2 M I-AEDANS in 0.5 M Tris (pH 8.0), to modify all free thiol groups covalently. After 10 min at room temperature, the AEDANS-trapped proteins were separated by reversed-phase HPLC as above, and the protein-containing fractions were lyophilized.

Peptide Mapping. The AEDANS-trapped proteins were resuspended in 0.2 M ammonium acetate, 100 μM $CaCl_2$, pH 7.8, at a concentration of approximately 0.5 mg/mL. One-third volume of a 1 mg/mL solution of trypsin in 0.2 M ammonium acetate, 10 mM EDTA, pH 7.8, was added, and the mixture was incubated at 25 °C for 2.5 h. The resulting peptides were separated by reversed-phase HPLC (Dynamax-300A C_4 column 4.6 × 250 mm, with a linear gradient from 0% to 56% acetonitrile in 0.1% TFA) and then subjected to amino acid analysis.

CD Measurements. αLA and its covalently-trapped disulfide derivatives were dissolved to a concentration of between 5 and 25 μM in 0.1 M Tris (pH 8.7), 0.2 M KCl that also contained either 10 mM $CaCl_2$ or 1 mM EDTA (neutralized with KOH). For spectra of the unfolded proteins, the buffer also contained 8 M urea. CD spectra were acquired at 25 °C on a Jobin Yvon CD6 spectrometer calibrated with (+)-10-camphorsulfonic acid, using cells of path length 10 mm and 1 mm, with subtraction of the buffer contribution. The results were expressed as mean residue ellipticity ($deg \cdot cm^2 \cdot dmol^{-1}$), calculated using mean residue weights for αLA, $3SS_{cam}$, $2SS_{cam}$, and R_{cam} of 115.4, 116.2, 117.3, and 119.2, respectively. Protein concentrations were calculated using an extinction coefficient at 280 nm of 28 500 M^{-1} cm^{-1} for αLA and 27 200 M^{-1} cm^{-1} for R_{cm} (Ikeguchi & Sugai, 1989). The value for R_{cam} was taken to be the same as for R_{cm} and those for $3SS_{cam}$ and $2SS_{cam}$ were assumed to be 28 000 and 27 500 M^{-1} cm^{-1}, respectively. The values for the rearranged

derivatives were taken to be the same as for the form with the corresponding number of native disulfide bonds.

CD spectra were also recorded for unblocked and partially blocked fully reduced α-lactalbumin. One-tenth volume of 100 mM DTT_{SH}^{SH} in 0.1 M Tris (pH 8.7), 0.2 M KCl, that also contained either 10 mM $CaCl_2$ or 1 mM EDTA (neutralized with KOH), was added to αLA at approximately 15 μM in the same buffer. After 15 min of incubation at 25 °C, when the protein was fully reduced, spectra were acquired as above. For spectra of the unfolded protein, the buffer also contained 8 M urea. Spectra of $R^{6/120cam}$ and $R_{28/111cam}^{6/120cam}$ were also acquired in an identical fashion, but for the substitution of $3SS_{cam}$ and $2SS_{cam}$ for αLA, respectively.

For all measurements in solution, acquisition of data was conducted as rapidly as possible with freshly dissolved protein solutions. An absence of time-dependent changes and lack of significant absorbance above 320 nm indicated the absence of aggregation.

ANS-Binding Studies. αLA and its covalently-trapped disulfide derivatives were dissolved to 25 μM in 0.1 M Tris (pH 8.7), 0.2 M KCl, 50 μM ANS, containing either a fixed concentration of $CaCl_2$ or 1 mM EDTA. The concentration of ANS was determined using an extinction coefficient of 5000 M^{-1} cm^{-1} at 350 nm (Weber & Young, 1964). The protein concentration was varied by serial dilution in the same buffer, thereby keeping the ANS concentration constant and so avoiding inner filter effects (Mulqueen & Kronman, 1982). The fluorescence emission at 490 nm was measured at 25 °C using an excitation wavelength of 350 nm, with correction for the contribution of the buffer.

Intrinsic Fluorescence. αLA and its covalently-trapped disulfide derivatives were dissolved to 1.8 μM in 0.1 M Tris (pH 8.7), 0.2 M KCl, containing either 10 mM $CaCl_2$ or 1 mM EDTA. The emission spectra were recorded at 25 °C between 300 and 450 nm with bandwidths of 8 nm using excitation at 280 nm, and with correction for the contribution of the buffer.

Urea Gradient Gel Electrophoresis. Transverse linear urea gradient polyacrylamide slab gels were prepared essentially as described by Goldenberg and Creighton (1984), with the use of a discontinuous high pH buffer system [gel buffer, Tris-HCl at pH 8.8; electrode buffer, Tris–glycine at pH 8.3; Davis (1964)], modified to include either 5 mM $CaCl_2$ or 1 mM EDTA in both the gel and the running buffer. The gradient of urea was 0–8 M and the compensating inverse acrylamide gradient was 14% to 11.5%. The electrode buffer was only used once (Ewbank & Creighton, 1993). Electrophoresis was at 25 °C at a constant current of 30 mA/gel.

RESULTS

Reduction of Ca^{2+}-bound αLA proceeds through specific three- and two-disulfide intermediates, 3SS and 2SS, respectively (Ewbank & Creighton, 1993). With Ca^{2+} bound, the disulfide bonds of 3SS and 2SS are relatively resistant to reduction and stable with regard to disulfide bond rearrangements. If, however, 3SS or 2SS were fully unfolded by the addition of 8 M urea, their disulfide bonds rapidly rearranged to give complex mixtures of disulfide bond isomers, $[3SS]^u$ and $[2SS]^u$, respectively. Removal of bound Ca^{2+} from 3SS or 2SS in the absence of denaturant also resulted in rapid disulfide bond rearrangements that generated, however, distinct mixtures of disulfide bond isomers, denoted as [3SS] and [2SS], respectively. As these intermediates have both disulfides and free thiols, they must be trapped in a stable form to be structurally characterized. There are two commonly used trapping methods: acid quench (which greatly reduces thiol reactivity by minimizing the reactive thiolate anion) and irreversible covalent modification of thiol groups, with iodoacetamide for example. Both methods were used for the kinetic analysis reported in the preceding paper and gave very similar results (Ewbank & Creighton, 1993). Acid-trapped intermediates can only be characterized at acid pH, however, where the single Ca^{2+} ion dissociates from αLA (Permyakov et al., 1981) and radically changes the protein's conformation (Kuwajima, 1989; Goto et al., 1990). Therefore, intermediates were trapped by covalent modification, which has the advantage of being irreversible and allowed characterization of the intermediates under the same conditions as used for the studies on disulfide formation and breakage. The blocking groups introduced may, however, influence the conformational properties of the protein, which is discussed further below.

αLA and all its derivatives were greater than 95% pure as judged by reversed-phase HPLC and native gel electrophoresis. The disulfide counting method described in the preceding paper (Ewbank & Creighton, 1993) was used to confirm that each species had the expected number of disulfide bonds and that no significant oxidation, reduction, or intermolecular association had occurred during the rearrangement of the two- and three-disulfide derivatives.

Identifying the Disulfide Bonds Broken in 2SS and 3SS. The intermediate 3SS is well-established and known to have the Cys6–120 disulfide broken (Shechter et al., 1973; Kuwajima et al., 1990). The intermediate 2SS in which two disulfide bonds had been cleaved had not previously been observed, so it was necessary to identify the second disulfide that had been reduced. This was accomplished by isolating the intermediate and performing peptide mapping.

Reduction by DTT_{SH}^{SH} of αLA in the presence of 10 mM Ca^{2+} was quenched, by the addition of I-AEDANS, when both 3SS and 2SS were present, and the two trapped proteins ($3SS_{AEDANS}$ and $2SS_{AEDANS}$) were purified to homogeneity. The two proteins were digested with trypsin to determine which cysteine residues were blocked by AEDANS. AEDANS has substantial absorbance at 340 nm, so those peptides that contained an AEDANS-modified cysteine residue could be readily identified. A comparison of the products of tryptic digestion of $3SS_{AEDANS}$ and $2SS_{AEDANS}$ is shown in Figure 1. The identity of the different peptides was established by amino acid analysis and reference to α-lactalbumin's primary structure (Shewale et al., 1984; Vilotte et al., 1987). Two AEDANS-containing peptides were obtained from $3SS_{AEDANS}$: tryptic fragments Cys6 to Arg10 and Leu115 to Lys122. These peptides each contained a single cysteine residue and confirmed that the disulfide linking Cys6 to Cys120 was cleaved in 3SS. The tryptic digest of $2SS_{AEDANS}$ had the same two AEDANS-containing peptides, plus two additional AEDANS-containing peptides identified as Gly17–Phe31 and Ala109–Lys114 (Figure 1). These peptides also each contain a single cysteine residue and indicate that the second disulfide bond cleaved was Cys28–111.

Urea Gradient Gel Electrophoresis. During transverse linear urea gradient electrophoresis, a protein migrates electrophoretically through a slab gel of polyacrylamide in which there is a linear gradient of urea perpendicular to the direction of migration. The rate of migration is sensitive to both the net charge on the protein and the degree of unfolding induced by the urea; compact species migrate more rapidly than their unfolded forms. The technique can therefore provide direct visualization of the dependence of protein conformation on denaturant concentration, and mixtures of species can be analyzed directly. The discontinuous buffer

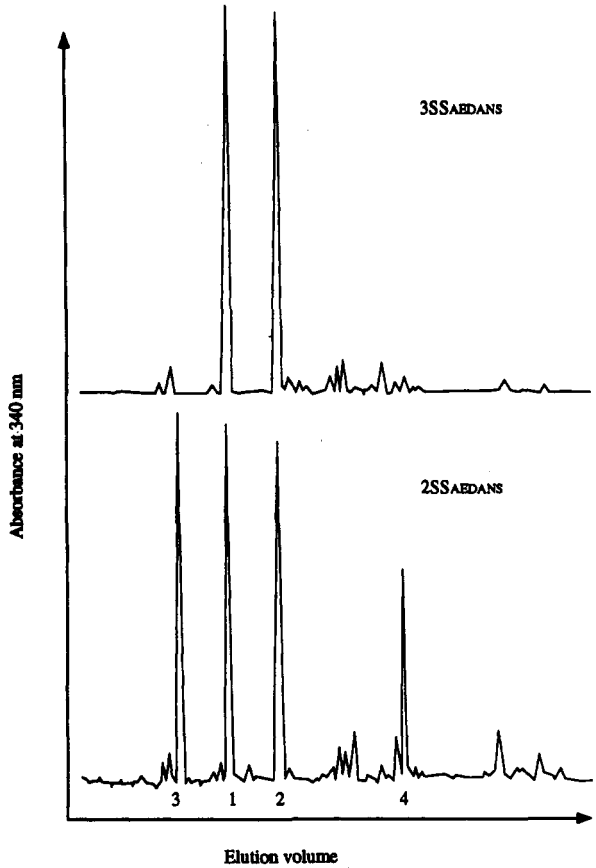

FIGURE 1: Comparison of the tryptic peptides from $3SS_{AEDANS}$ (top) and $2SS_{AEDANS}$ (bottom). Peptides were eluted from reversed-phase HPLC using the absorbance at 340 nm to identify those labeled with AEDANS. Amino acid analysis identified the peptides as being Cys6–Arg10 (1) and Leu115–Lys122 (2), Ala109–Lys114 (3) and Gly17–Phe31 (4). The peptide Gly17–Phe31 presumably resulted from cleavage at an atypical peptide bond and was not recovered in a stoichiometric yield.

system (Davis, 1964) used here runs at pH 9.5, close to that used here in the kinetic studies of disulfide bond reduction and re-formation (Ewbank & Creighton, 1993) and in the other physical studies reported here.

Binding of Ca^{2+} will affect the electrophoretic mobility because of the charge of the bound ion; there may also be effects caused by accompanying changes in the protein's conformation. Consequently, the urea-gradient gels give a wealth of information about the relative hydrodynamic volumes of the various species, both when folded and unfolded, about their binding of Ca^{2+}, and about the stabilities of their folded conformations and the relative cooperativity of their unfolding transitions. The patterns obtained with αLA, $3SS_{cam}$, $2SS_{cam}$, and R_{cam} in the presence and absence of Ca^{2+} are given in Figure 2. The results for αLA are consistent with previous results (Creighton, 1979), obtained before αLA's Ca^{2+}-binding was recognized (Hiraoka et al., 1980) and when the complex nature of its unfolding transition was not fully explicable.

Fully reduced α-lactalbumin does not have a measurable Ca^{2+} affinity, so the mobilities of the trapped species R_{cm} (Thompson et al., 1988) and R_{cam} are not affected by Ca^{2+}, and gels run under the two conditions could be compared (Table I). The presence of EDTA per se should not alter the mobility of α-lactalbumin, as the proposal that EDTA binds to the protein (Kronman & Bratcher, 1983) has been convincingly repudiated (Stemmer & Klee, 1990, and references cited therein).

R_{cm} had a constant mobility across the whole gradient, indicative of its being fully unfolded, even in the absence of urea. By contrast, the mobility of R_{cam} decreased gradually with increasing urea concentration, indicative of gradual unfolding, which was the same in the presence and absence of Ca^{2+}. In 8 M urea, where both R_{cm} and R_{cam} are fully unfolded, their electrophoretic mobilities are assumed to differ only due to the negative charge of the eight acidic cm groups of R_{cm}; in that case, their relative mobilities indicate that the net charge of the apo forms of αLA and of the other iodoacetamide-trapped forms was approximately −12 under the conditions of electrophoresis, which is consistent with their content of acidic and basic residues.

Various degrees of unfolding were observed for αLA and its three- and two-disulfide derivatives, as judged by their decreasing mobilities at high urea concentrations, both in the presence and absence of Ca^{2+}. In each case the curves leveled off before the end of the urea gradient, indicative of completion of the unfolding transition. A continuous electrophoretic band was always obtained, indicating that all the folding–unfolding transitions were rapid relative to the time of electrophoresis.

In the presence of EDTA, αLA, $3SS_{cam}$, $2SS_{cam}$, and R_{cam} each had distinct electrophoretic mobilities and underwent gradual decreases in mobility with increasing urea concentration (Figure 2); it was difficult to define start and end points for these broad unfolding transitions. Trapping with iodoacetamide does not introduce any charges, so the differences in mobility should reflect primarily differences in hydrodynamic volume, with αLA being the most compact.

The different mobilities of the unfolded proteins reflected the extent to which they were constrained against complete expansion by their disulfide bonds. The change in mobility appeared to be approximately proportional to the size of loop broken by each successive cleavage. Thus, cleavage of the Cys6–120 disulfide opened a 31-residue loop and produced a significant retardation of unfolded $3SS_{cam}$ relative to the unfolded αLA, and cleavage of Cys28–111 and of the last two disulfides (Cys61–77 and Cys73–91) opened 63- and 30-residue loops, respectively, with proportionate consequences for the mobilities of $2SS_{cam}$ and R_{cam}. Retention of disulfide bonds in the fully unfolded protein decreased the change in mobility upon unfolding of αLA and, to lesser extents, $3SS_{cam}$ and $2SS_{cam}$, and consequently decreased the apparent abruptness of their unfolding transitions.

αLA, $3SS_{cam}$, and $2SS_{cam}$ all bind Ca^{2+} with relatively high affinity in the absence of denaturant (Ewbank & Creighton, 1993). As expected, the electrophoretic mobilities of all three species were changed at low urea concentrations by the inclusion of Ca^{2+} during electrophoresis. Their mobilities were then indistinguishable, indicating that they all had very similar hydrodynamic volumes and adopted a native-like compactness.

No protons are released from αLA upon Ca^{2+} binding (Schaer et al., 1985), so Ca^{2+}-bound αLA, $3SS_{cam}$, and $2SS_{cam}$ should each have an overall charge less negative by two units relative to Ca^{2+}-free (apo) αLA and a decreased electrophoretic mobility (see Figure 3 for a schematic interpretation). The decrease in mobility of αLA upon binding Ca^{2+} was close to that expected if due only to a change in its net charge from −12 to −10. The decrease in mobility of $3SS_{cam}$ was not so great as for αLA, so there was probably a compensating compaction of $3SS_{cam}$ upon binding Ca^{2+}. In the case of $2SS_{cam}$, the protein's mobility was only slightly altered in the presence of Ca^{2+} (Figure 2), so compaction of the conformation on binding Ca^{2+} nearly compensated for the increase in charge.

The mobilities of αLA, $3SS_{cam}$, and $2SS_{cam}$ were not altered by Ca^{2+} in the presence of 8 M urea, so the unfolding of the proteins was accompanied by dissociation of bound Ca^{2+},

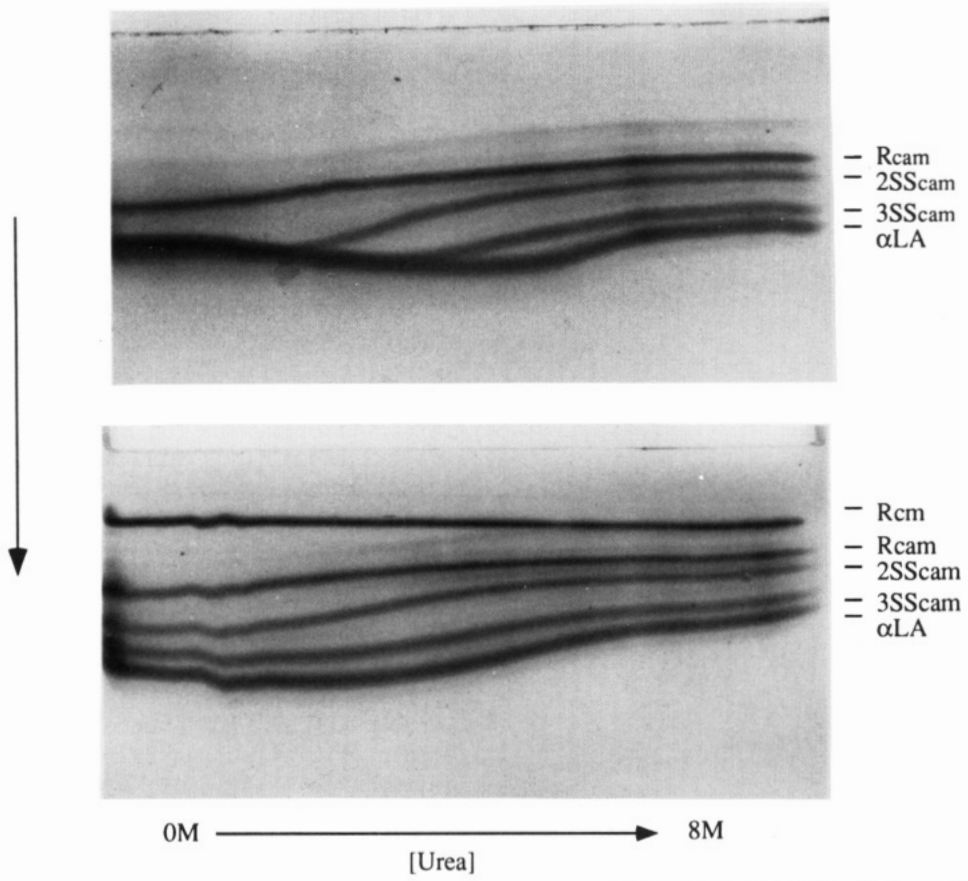

FIGURE 2: Urea gradient electrophoresis of αLA and its trapped reduction products in the presence (*top*) and absence (*bottom*) of Ca^{2+}. A 100-μL sample of a mixture containing each component (at a concentration of approximately 20 μM) was layered on the top of each gel. The upper gel contained 5 mM $CaCl_2$ and the lower one contained 1 mM EDTA. The gels were run at 25 °C at a constant 30 mA for 4 h and 2.5 h, respectively. The arrow indicates the direction of migration. For the lower gel, electrophoresis was stopped after 1.7 h, 100 μL of 20 μM R_{cm} was applied, and the electrophoresis continued. In this way, the band of R_{cm} did not overlap any of the other bands. The discontinuity at the 0 M urea end of the gradient of the lower gel was caused by a polymerization artefact.

Table I: Effects of Ca^{2+} and Urea on the Relative Electrophoretic Mobilities of αLA and Its Trapped Disulfide Derivatives during Discontinuous Polyacrylamide Gel Electrophoresis at pH 9.5 and 25 °C

	relative mobility		
species	1 mM EDTA	5 mM Ca^{2+} [a]	8 M urea[b]
αLA	100	80	78
$3SS_{cam}$	92	80	70
$2SS_{cam}$	81	80	57
R_{cam}	63	63	48
R_{cm}[c]	80	80	80

[a] The charge of the Ca^{2+} ion decreases the net negative charge of each species that binds it. [b] The mobility of each species was unaffected by the presence or absence of Ca^{2+} when unfolded by 8 M urea. [c] R_{cm} has a net negative charge 8 units greater than the apo forms of the other species.

consistent with previous results (Permyakov et al., 1985). Consequently, the unfolding transitions observed in the presence of Ca^{2+} were complicated by the twin effects of changes of conformation and of charge upon Ca^{2+} dissociation (Figure 3). This meant that the decrease in mobility caused by unfolding was compensated in part by an increase in mobility that resulted from the dissociation of the positively charged Ca^{2+} ligand from the unfolded protein. As a result of this effect and the constraints of the disulfide bonds on the hydrodynamic volume of the fully unfolded conformation, the unfolding transitions in the presence of Ca^{2+} did not appear particularly abrupt, although they were much more so than those of the apoproteins. The midpoint of the unfolding transitions for each species occurred at varying urea concentrations in the order αLA > $3SS_{cam}$ > $2SS_{cam}$.

The unfolding of αLA by denaturants is dependent upon Ca^{2+} concentration (Kuwajima et al., 1986; Ikeguchi et al., 1986b). The maximum concentration of Ca^{2+} that could be included during electrophoresis was 5 mM. In such Ca^{2+}-containing gels, a semicrystalline front was observed to run by the dye front presumably due to the migration of Ca^{2+} ions during the course of electrophoresis. Consequently, the Ca^{2+} concentration in the gels may have varied during the course of electrophoresis, and no rigorous analysis of the unfolding transitions was attempted. Under these conditions, the mobilities of αLA, $3SS_{cam}$, and $2SS_{cam}$ increased with increasing, but low, urea concentration, probably due to the affinity of the native state for Ca^{2+} being reduced by urea, shifting the equilibrium between Ca^{2+}-bound and apo forms of each.

The electrophoretic patterns, under nondenaturing conditions, for the trapped rearranged two- and three-disulfide derivatives of αLA are shown in Figure 4b. In each case there appeared to be many different rearranged species that were poorly resolved by electrophoresis or by HPLC (Figure 4a), as was observed previously for the rearranged three-disulfide forms of human α-lactalbumin (Ewbank & Creighton, 1991). The trapped products of rearrangement of 3SS generated in the absence of urea ($[3SS]_{cam}$) tended to have greater electrophoretic mobilities than those generated in the presence of urea ($[3SS]^{u}_{cam}$); similar observations were made of the products of rearrangement of 2SS. A proportion of $[3SS]_{cam}$ had a mobility greater than $3SS_{cam}$, with some species surprisingly having mobilities as great as apo-αLA. The majority of the $[2SS]_{cam}$ species were more compact than $2SS_{cam}$ as judged by their electrophoretic mobilities. All the

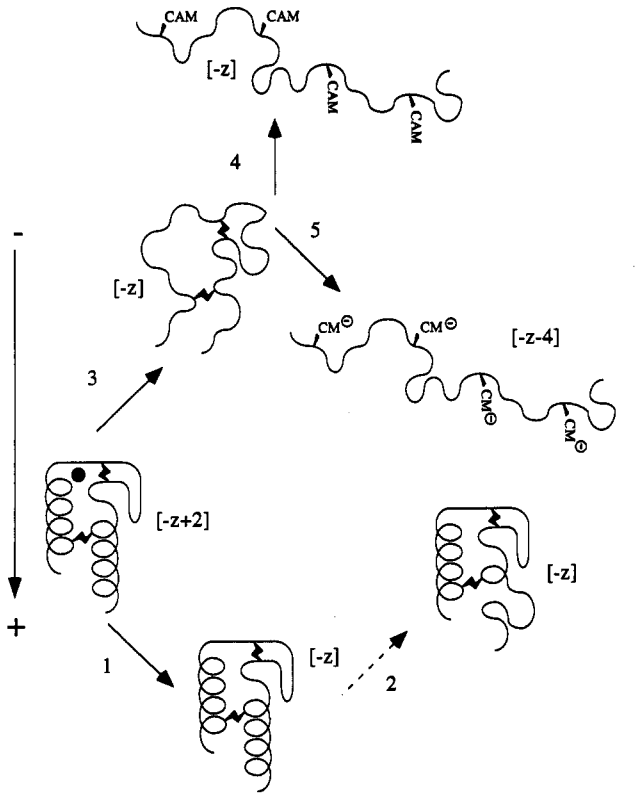

FIGURE 3: Schematic representation of the factors influencing a protein's electrophoretic mobility. The hypothetical protein with charge [–z] binds Ca^{2+} (solid circle) only when in a fully folded conformation. Removal of the ligand alters the protein's overall charge and hence its mobility (1), but may also alter the protein's conformation (2). Unfolding of the Ca^{2+}-bound protein is accompanied by a change in charge due to the dissociation of the ligand, which in this case compensates in part for the simultaneous change in conformation (3). The unfolded protein is constrained from complete unfolding by its two disulfide bonds (black zig-zags). When these are reduced and blocked with neutral iodoacetamide, there is no change in charge, but the protein's mobility is further decreased (4). Blocking the reduced thiols with iodoacetate increases the charge and hence the reduced protein's mobility (5). The large arrow indicates the direction of increasing mobility. For fully reduced α-lactalbumin, the nature of the blocking group influences the conformation of the fully reduced protein, but this added complexity has been omitted from this scheme.

species, including $[2SS]^{u}_{cam}$, had mobilities substantially greater than R_{cam}. Essentially identical patterns were obtained for the trapped rearranged proteins after electrophoresis in the presence of 5 mM $CaCl_2$, indicating that at least the majority of the rearranged species did not bind Ca^{2+} with even low affinity (Ewbank, 1992).

Each of the mixtures gave broad electrophoretic bands and gradual unfolding transitions upon urea gradient electrophoresis (Figure 5). The width of the band was not markedly greater in the transition region, indicating that the majority of species in each had similar unfolding transitions. The $[3SS]_{cam}$ species were slightly more stable and underwent a smaller relative decrease in mobility than did $[3SS]^{u}_{cam}$; the overlap between the two was less in the presence of 8 M urea. The distinction between the two forms was less pronounced in the case of the rearranged two-disulfide derivatives. Thus, all the rearranged species, even those that were generated in the presence of urea, possessed a degree of compactness in its absence.

Circular Dichroism. CD can give information both about a protein's secondary and tertiary structure. The near-UV CD spectrum contains contributions from each aromatic residue and disulfide bond in an asymmetric environment.

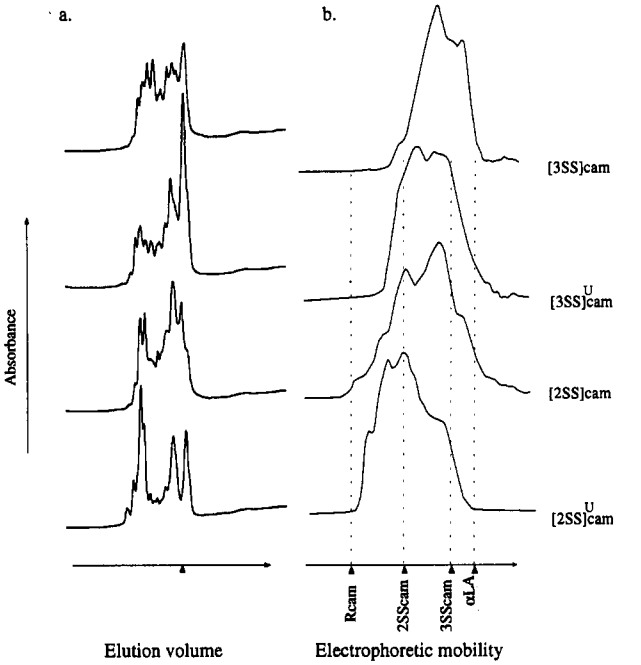

FIGURE 4: Patterns obtained after chromatographic (a) and electrophoretic (b) separation of blocked rearranged derivatives of α-lactalbumin. The mixtures are, from top to bottom, $[3SS]_{cam}$, $[3SS]^{u}_{cam}$, $[2SS]_{cam}$, and $[2SS]^{u}_{cam}$. (a) C_4 reversed-phase HPLC with a gradient of 25%–50% acetonitrile in 0.1% TFA. αLA, $3SS_{cam}$, and $2SS_{cam}$ are poorly resolved under these conditions and all elute close to the position given by the arrowhead. (b) High-pH discontinuous polyacrylamide gel electrophoresis at 25 °C in the presence of 1 mM EDTA. The arrowheads mark the positions of, from right to left, αLA, $3SS_{cam}$, $2SS_{cam}$, and R_{cam}.

The intensity and shape of the near-UV spectrum is therefore primarily a measure of the folded tertiary conformation. A protein's far-UV CD spectrum, on the other hand, reflects mainly the contribution of nonrandom conformations of the peptide backbone, possibly with an additional contribution from restrained aromatic residues and disulfide bonds (Manning & Woody, 1989; Bolotina, 1987).

The MG conformation is characterized by a near-native content of secondary structure and little or no tertiary structure [see Kuwajima (1989)]. Consequently, the near- and far-UV CD spectra can be interpreted semiquantitatively in terms of the relative populations of the N, MG, and U states, although detailed interpretation of the far-UV spectra in terms of secondary structure were not attempted (Kuwajima et al., 1985; Ikeguchi & Sugai, 1989). The CD spectra were obtained under the same conditions used for the kinetic studies of disulfide bond formation and breakage, although necessarily of disulfide intermediates that had been covalently trapped.

The far-UV spectra of αLA in the native conformation, with Ca^{2+} bound, and in the unfolded state in 8 M urea (Figure 6)closely resembled those previously reported [for example, Kuwajima et al. (1981), Gil'manshin et al. (1982),[2] and Ikeguchi and Sugai (1989)]. The near-UV spectrum of native αLA was very complex, reflecting the overlap of dichroic contributions from αLA's 12 aromatic residues and four disulfide bonds, which are spread throughout the protein molecule. The pronounced negative ellipticity at around 270 nm and the positive peak at 295 nm have been ascribed to Tyr and Trp residues, respectively (Cowburn et al., 1972). The far-UV spectrum of native αLA was typical of a largely α-helical protein with minima at around 208 nm and 222 nm. In 8 M urea, the spectrum was consistent with the protein

[2] The figure legends for Figures 1 and 2 in Gil'manshin et al. (1982) appear inverted.

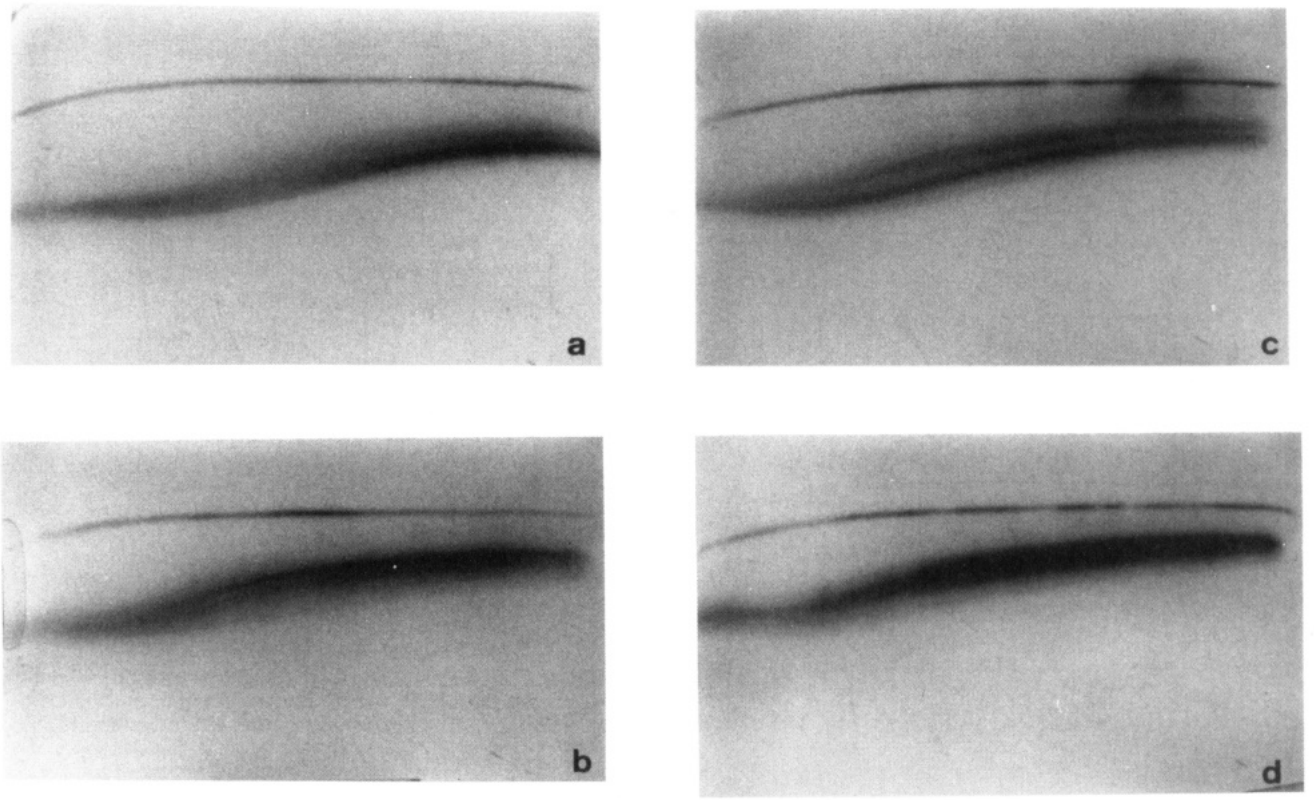

FIGURE 5: Urea gradient electrophoresis in the presence of 1 mM EDTA of iodoacetamide-trapped rearranged three- and two-disulfide derivatives of α-lactalbumin. The conditions were as given in the legend to Figure 2: (a) $[3SS]_{cam}$, (b) $[3SS]^{u}_{cam}$, (c) $[2SS]_{cam}$, (d) $[2SS]^{u}_{cam}$. The upper band in each is R_{cm}, applied two-thirds of the way through electrophoresis.

being fully unfolded (Kuwajima et al., 1991, and references cited therein). The near-UV spectrum was essentially featureless, and the intensity of the far-UV spectrum was greatly diminished.

Ca^{2+}-bound $3SS_{cam}$ appeared to be vitrually fully folded as judged by CD. Its far-UV spectrum was very similar to that of αLA. The difference between the two in the near-UV was broad and featureless and could be attributed to the dichroic contribution of the broken disulfide bond (Kuwajima et al., 1990; Ikeguchi et al., 1992). Ca^{2+}-bound $2SS_{cam}$, on the otherhand, had a far-UV spectrum that was significantly diminished in comparison to that of Ca^{2+}-bound αLA. The difference between the two exceeded the expected contribution (theoretically and experimentally determined) of two broken disulfide bonds (Kuwajima et al., 1990, and references cited therein), indicative of a diminution in secondary structure content. There was also a significant change in the near-UV spectrum that could not be attributed solely to the loss of two disulfide bonds. The change was consistent with the removal of some of the chromophores from their asymmetric environments, and the spectrum still contained some fine structure (Figure 6), indicative of the contribution of more than one constrained chromophore [see for comparison, Craig et al. (1989)]. As all the $2SS_{cam}$ molecules had bound Ca^{2+} under these conditions, the CD spectra indicate that only part of the molecule was folded.

Removal of Ca^{2+} from αLA, by the addition of EDTA, resulted in approximately uniform $^1/_5$ reduction in the near-UV CD spectrum, reflecting a decrease in the amount of tertiary structure. In contrast, the ellipticity at 222 nm, indicative of helical content, was hardly changed (Figure 6). These observations are consistent with the results of others [e.g., Kuwajima et al. (1990) and Hiraoka and Sugai (1985)] and indicate that apo-αLA was in an equilibrium with 80% of the molecules in the native conformation and 20% in the MG.

The apo form of $3SS_{cam}$ had a near-UV CD spectrum that was much reduced compared to the Ca^{2+}-bound form, but its far-UV dichroism was substantially retained (Figure 6). Similar changes were observed upon removal of ligand from both bovine and human $3SS_{cm}$ (Kuwajima et al., 1990; Ewbank & Creighton, 1991; Ikeguchi et al., 1992). These results are consistent with apo-$3SS_{cam}$ being largely in a MG-like conformation.

Removal of Ca^{2+} from $2SS_{cam}$ resulted in a far greater diminution of CD signal in the near-UV than in the far-UV, but the change in the latter was also significant (Figure 6). Thus, as judged by CD, apo-$2SS_{cam}$ possessed no tertiary structure and less secondary structure than the Ca^{2+}-bound protein, consistent with partial population of the MG conformation.

The rearranged two- and three-disulfide forms all showed essentially featureless near-UV CD spectra (Ewbank, 1992). Their spectra in the far-UV were similar in shape to those of apo $3SS_{cam}$ and $2SS_{cam}$, and all were more negative than that of R_{cam} (Figure 7). The spectra were not significantly altered by 10 mM Ca^{2+}. The difference in the degree of unfolding measured by changes in the near- and far-UV CD spectra for these derivatives of α-lactalbumin is indicative of the existence of partly folded conformations intermediate between the native and unfolded states. A fuller interpretation of these spectra is given below.

The conformation of the fully reduced protein and the influence of blocking groups was investigated by comparing the CD spectra of R, $R^{6/120cam}$, $R^{6/120cam}_{28/111cam}$, R_{cam}, and R_{cm} (Figures 6 and 7). The minimal far-UV and featureless near-UV CD spectrum of R_{cm} (Figure 6) was typical of an unfolded protein and was identical to that previously reported for R_{cm} in 8 M urea (Ikeguchi & Sugai, 1989), indicating that R_{cm} was fully unfolded even in the absence of denaturant. By contrast, as judged by their similar ellipticities in the far-UV,

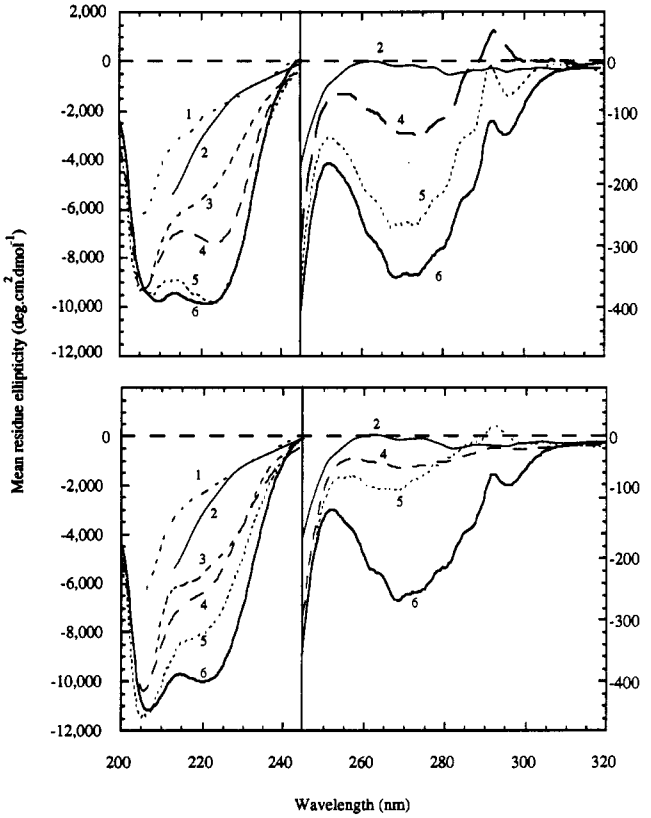

FIGURE 6: Far- and near-UV CD spectra of αLA and its trapped derivatives in the presence and absence of Ca^{2+} (upper and lower panel, respectively). The samples were αLA (thick line, 6), $3SS_{cam}$ (···, 5), $2SS_{cam}$ (– –, 4), R_{cam} (- - -, 3), R_{cm} (· · ·, 1), and αLA in 8 M urea (thin line, 2). The near-UV spectra of R_{cam} and R_{cm} were virtually identical to that of αLA in 8 M urea and have been omitted for the sake of clarity. In each case, the buffer was 0.1 M Tris (pH 8.7), 0.2 M KCl, and either 10 mM $CaCl_2$ or 1 mM EDTA, at 25 °C.

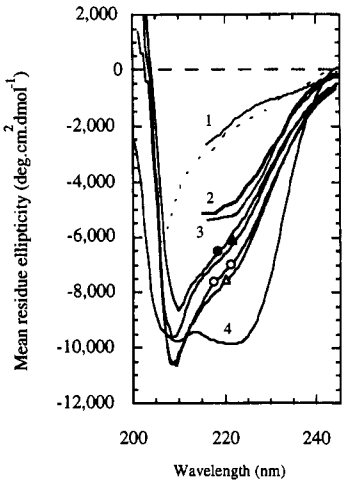

FIGURE 7: Far-UV CD spectra of rearranged derivatives of α-lactalbumin and fully reduced α-lactalbumin. The rearranged derivatives were in 0.1 M Tris (pH 8.7), 0.2 M KCl, 1 mM EDTA at 25 °C. Symbols: open triangle, $[3SS]_{cam}$; open circle, $[2SS]_{cam}$; closed triangle, $[3SS]^{u}_{cam}$; closed circle, $[2SS]^{u}_{cam}$. The spectra of reduced α-lactalbumin were recorded under the same conditions, but in the presence of 10 mM DTT^{SH}_{SH}: thin line, 1, R in 8 M urea; thick line, 2, R; thin line, 3, $R^{6/120cam}_{28/111cam}$. The spectrum of $R^{6/120cam}$ overlapped with that of R and has been omitted for the sake of clarity. The spectra of Ca^{2+}-bound αLA (thin line, 4) and R_{cm} (···) are included for comparison.

R, $R^{6/120cam}$, $R^{6/120cam}_{28/111cam}$, and R_{cam}, all possessed some secondary structure (Figures 6 and 7), but their near-UV spectra were virtually zero, indicative of an absence of tertiary structure (Ewbank, 1992). This negative ellipticity in the far-UV was virtually abolished by the addition of denaturant; in 8 M urea, their spectra were indistinguishable from that of R_{cm}, as illustrated for R in Figure 7. Thus, reduced α-lactalbumin with all eight free thiol groups, or with two, four, or all eight modified by reaction with iodoacetamide, adopted similar partially-populated MG conformations. While blocking the thiol groups with neutral cam groups did little to alter the conformation, complete carboxymethylation caused the protein to be fully unfolded. The spectra of none of the reduced forms, whether blocked or not, were altered by the presence of 10 mM Ca^{2+} (Figure 6; Ewbank, 1992), consistent with none of the reduced forms of α-lactalbumin binding Ca^{2+}.

Table II: Effect of Ca^{2+} on the Intrinsic Fluorescence Parameters of αLA and Its Trapped Disulfide Derivatives[a]

	λ_{max} (nm)		relative emission intensity	
species	10 mM Ca^{2+}	1 mM EDTA	10 mM Ca^{2+}	1 mM EDTA
αLA	323	332	32	42
$3SS_{cam}$	324	339	34	59
$2SS_{cam}$	335.5	338.5	59	68
$[3SS]_{cam}$	340	340	76	80
$[2SS]_{cam}$	340.5	340	93	93
$[3SS]^{u}_{cam}$	340.5	340.5	84	86
$[2SS]^{u}_{cam}$	341.5	341.5	113	113
R_{cam}	338.5	338.5	96	96
R_{cm}	345.5	345.5	99	100

[a] The buffer was 0.1 M Tris (pH 8.7), 0.2 M KCl at 25 °C, and the excitation wavelength was 280 nm.

Intrinsic Fluorescence. The intrinsic fluorescence of αLA reflects primarily the environments of its four tryptophan residues (residue numbers 26, 60, 104, and 118). The parameters λ_{max} and relative fluorescence emission were determined for αLA and its derivatives, in the presence and absence of Ca^{2+} (Table II). R_{cm} appeared the most unfolded species; its λ_{max} was at the longest wavelength, approaching that of free tryptophan (348 nm), while the spectrum of native Ca^{2+}-bound αLA was the most blue-shifted. The native conformation also quenched the fluorescence emission by a factor of 3, comparing Ca^{2+}-bound αLA to R_{cm}. The other spectra can be compared with these two extremes. Ca^{2+}-bound $3SS_{cam}$ appeared on this basis to be almost fully folded. The spectrum of Ca^{2+}-bound $2SS_{cam}$ was intermediate between the two extremes, in terms of both λ_{max} and emission intensity. The spectra of the other forms were red-shifted relative to Ca^{2+}-bound αLA, but only by around $^3/_4$ of the shift seen for R_{cm}, suggesting that none was as unfolded as R_{cm}. Similar trends were apparent in the fluorescence intensity but, curiously, the relative fluorescence intensity of $[2SS]^{u}_{cam}$ exceeded that of R_{cm}.

ANS Binding. The hydrophobic fluorescent dye ANS has been used as a diagnostic for the existence of molten globule states in equilibrium and kinetic studies (Ptitsyn et al., 1990; Semisotnov et al., 1991). Fully unfolded and fully folded states show relatively low affinity for ANS compared to partially-folded, molten globule states. The enhancement of ANS fluorescence by denaturant-induced molten globule states, relative to the low level of fluorescence seen with native or fully unfolded states, has been reported to be between 2- and 10-fold (Semisotnov et al., 1991).

The results of a titration of 50 μM ANS with αLA and its derivatives in the absence and presence of Ca^{2+} are shown in Figure 8. Linearity of fluorescence intensity as a function of protein concentration is a good indication that the dye is binding to the monomeric protein and that the protein is not aggregated (Mulqueen & Kronman, 1982). A linear correlation between protein concentration and ANS fluorescence was obtained in all cases, except for the rearranged derivatives. They all had

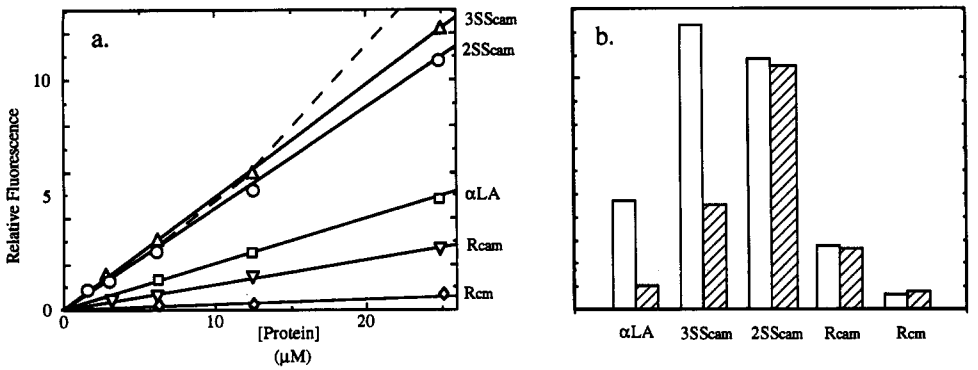

FIGURE 8: Binding of ANS by αLA and its blocked derivatives in 0.1 M Tris (pH 8.7), 0.2 M KCl at 25 °C. (a) Titration of 50 μM ANS with αLA and its blocked derivatives in the presence of 1 mM EDTA. The dashed line shows the nonlinear curve of $[3SS]^{u}_{cam}$. The other rearranged derivatives had a relative fluorescence of between 12 and 15 at 25 μM, but values less than those of $3SS_{cam}$ and $2SS_{cam}$ at lower protein concentrations, and so have been omitted for the sake of clarity. (b) Effect of Ca^{2+} on ANS fluorescence of αLA and its blocked derivatives. Bars: open, in the presence of 1 mM EDTA; hatched, 10 mM $CaCl_2$.

Table III: Contribution of α-Lactalbumin's Native Disulfide Bonds to the Stability of Its N, MG, and U States[a]

species	conformations present: Ca^{2+}-bound[b]	conformations present: apo[c]	K_d^{Ca} (M)	electrophoretic mobility of MG state[d]	folding equilibria in apo form: $\Delta G_{N\rightarrow U}$[e] (kJ·mol^{-1})	folding equilibria in apo form: $\Delta G_{MG\rightarrow U}$ (kJ·mol^{-1})
αLA	100% N	80% N, 20% MG	2×10^{-8} [f]		−9.7[g]	−6.7[g]
$3SS_{cam}$	≈100% N	80% MG, 20% U	2.5×10^{-6}	97.5	1.7	−3.4
$2SS_{cam}$	≈100% N_p[h]	60% MG, 40% U	2×10^{-5} [i]	97	6.9[h]	−1.0
R_{cam}	na	30% MG, 70% U	$>10^{-2}$	98	>21.1	2.1

[a] 0.1 M Tris (pH 8.7), 0.2 M KCl at 25 °C. [b] In the presence of 10 mM Ca^{2+}. [c] In the presence of 1 mM EDTA; values considered accurate to ±10%. [d] Calculated from the observed electrophoretic mobility of each apo form (Table I) by $Mobil_{apo} = f_{MG}Mobil_{MG} + f_{U}Mobil_{U}$; where f_U and f_{MG} are the fractional populations of U and MG states, respectively, and $Mobil_U$ is given by the observed mobility of the corresponding form in 8 M urea (Table I). [e] Calculated on the assumption that the observed affinity for Ca^{2+} reflects only the population of the N state; $\Delta G_{N\rightarrow U} = -9.7 - RT\ln(K_a/5\times10^7$ M^{-1}). [f] Estimated from values of Hiraoka and Sugai (1985) for the conditions used here. [g] Xie et al. (1991); 10 mM borate (pH 8.0), 1 mM EDTA, 0.2 M NaCl, 25 °C. These values give $\Delta G_{N\rightarrow MG} = -3.0$ kJ.mol^{-1}, which compares well with the value of −3.4 kJ.mol^{-1} obtained from the population of N and MG states of apo-αLA here. [h] N_p, native in part, remainder in collapsed unstructured state; see text for details. [i] Value for unblocked 2SS (Ewbank & Creighton, 1993).

substantial ANS fluorescence, which was increased in the aggregated state at protein concentrations greater than 10 μM.

Ca^{2+}-bound αLA and R_{cm}, which by the structural criteria detailed above were fully folded and fully unfolded, respectively, had the expected low affinity for ANS. The greater fluorescence of all the other forms of α-lactalbumin suggested that all had some molten globule character. The dramatic difference between the ANS fluorescence observed for apo- and Ca^{2+}-bound $3SS_{cam}$ enabled its dependence upon Ca^{2+} concentration to be measured (Ewbank, 1992). The apparent dissociation constant for Ca^{2+} (2.5×10^{-6} M) was 10-fold greater than that estimated from the effect of changing the Ca^{2+} concentration on the rate of reduction of 3SS measured in the preceding paper (Ewbank & Creighton, 1993).

DISCUSSION

αLA has been extensively studied recently in efforts to understand why it can exist under certain conditions in a stable partially-unfolded MG state, in addition to the fully folded, N, and fully unfolded, U, states (Kuwajima, 1989; Alonso et al., 1991; Ewbank & Creighton, 1991, 1993; Xie et al., 1991; Ikeguchi et al., 1992). Following the finding that a three-disulfide derivative of human α-lactalbumin could exist in the MG state at neutral pH (Ewbank & Creighton, 1991), the pathways and kinetics of disulfide bond reduction and reformation of bovine αLA were examined in far greater detail under conditions favoring the different conformational states, and various kinetic intermediates were identified (Ewbank & Creighton, 1993). To aid interpretation of these kinetic studies and to further understanding of α-lactalbumin's different conformational states, structural studies have been carried out on the trapped reduction intermediates under the same conditions as used for the disulfide folding studies. A variety of techniques that give complementary information have been used. Additional information for each species is given by its affinity for Ca^{2+}, by the disulfides it contains, by the rates at which these disulfides are rearranged and broken, and by the rates at which further disulfides are formed. In this way, a consistent description has been generated of the conformational properties of α-lactalbumin in terms of the N, MG, and U conformational states (Table III).

Disulfide Bonds of 3SS and 2SS. The native disulfide bond Cys6–120 has been confirmed to be that reduced rapidly in αLA and absent from 3SS (Shechter et al., 1973; Kuwajima et al., 1990). The free thiols in 2SS have been identified as those of Cys6, Cys120, Cys28, and Cys111, consistent with the Cys28–111 disulfide being the second to be reduced, directly in 3SS. The identity of the disulfides remaining in 3SS and 2SS could not be established directly because of the difficulty of obtaining proteolytic cleavage between the near neighbors Cys73 and Cys77. Indirect evidence, however, strongly supports the retention of the other native disulfide bonds. Re-formation of the Cys6–120 disulfide in 3SS regenerated native αLA. The reduction of Ca^{2+}-bound 3SS to 2SS occurred through the folded conformation and must have involved cleavage of one of the native disulfide bonds. Although it is theoretically posible that the partners in the two remaining disulfide bonds could have been swapped by a series of rapid thiol–disulfide rearrangement reactions (a minimum of three steps), Ca^{2+}-bound 2SS is relatively stable and there were no indications of disulfide bond rearrangements having occurred (Ewbank & Creighton, 1993). Additionally, the two remaining disulfide bonds of 2SS flank αLA's Ca^{2+} binding site, which would undoubtedly be disrupted by their rearrangement (see Figure 1 in the preceding paper; Ewbank

& Creighton, 1993). Both 3SS and 2SS bind Ca^{2+} with relatively high affinity and, in the absence of any evidence for disulfide bond rearrangements, it is very likely that 3SS differs from αLA only by the absence of the Cys6–120 disulfide, that 2SS lacks only this disulfide and Cys28–111, and that both retain the remaining native disulfide bonds.

Effect of the Blocking Groups. The kinetic intermediates had to be trapped covalently in order to study them under conditions like those used in the studies of disulfide bond reduction, rearrangement, and re-formation. Iodoacetamide was found to be efficient and specific in blocking the free thiols of 3SS, 2SS, and R. For [3SS] and [2SS], however, many different disulfide isomers are in rapid equilibrium, so there is the possibility that irreversible blocking might perturb the equilibrium mixture if the thiol groups of different species have different reactivities, but similar HPLC profiles were obtained of rearranged species trapped with various high concentrations of iodoacetamide (results not shown).

The introduced blocking groups might affect the conformation and stability of the protein due to their steric bulk and polar groups, as discussed recently by Ikeguchi et al. (1992). Ionization of cm or thiol groups would be expected by electrostatic repulsion to inhibit collapse of an acidic protein like αLA, which may be the reason that R_{cm} is apparently fully unfolded. At low pH, the cm groups will be neutralized, which may explain the observation of secondary structure by far-UV CD in R_{cm} at pH 2 (Ikeguchi & Sugai, 1989). The opposing factors of partial ionization of free thiols groups and steric bulk of cam groups appeared to balance or to be minimal with the reduced protein: R, $R^{6/120cam}$, $R^{6/120cam}_{28/111cam}$, and R_{cam} have 0, 2, 4, and 8 thiols blocked with cam groups, and the others as free thiols. All had similar conformations, with ~70% of the molecules in the U state and the remainder in the MG state, as judged by their CD spectra.

The effects of the blocking groups would be expected to be different in the fully folded state. The structure of trapped Ca^{2+}-bound 3SS was the same whether iodoacetamide or iodoacetic acid was used to block its two free thiol groups (Figure 4; Ikeguchi et al., 1992). For both 2SS and 3SS, urea gradient electrophoresis unfolding transitions of the cam and cm derivatives occurred over a similar denaturant range (results not shown). The rate of reduction of Ca^{2+}-bound $2SS_{cam}$ by DTT^{SH}_{SH} was, however, somewhat slower than that of the same protein with four free thiol groups [0.2 s^{-1} M^{-1} as against 1.4 s^{-1} M^{-1}], and a slight effect was observed with 3SS (Ewbank & Creighton, 1993). This presumably reflects a small net stabilizing effect of the cam group relative to the partially-ionized thiol. Apart from this small effect on stability, the cam derivatives used here appear to be close structurally to the unblocked reduction intermediates.

Aggregation of Partially Folded Proteins. Partially-folded proteins, especially molten globules that expose an increased hydrophobic surface relative to their folded forms, are prone to aggregation, which affects their physical properties and makes difficult their characterization. Aggregation is known to occur with α-lactalbumin (Kronman et al., 1964; Ikeguchi & Sugai, 1989), but its occurrence is often not apparent with many techniques. Aggregation in the studies described here usually involved formation of intermolecular disulfide bonds and was then very obvious by gel electrophoresis, as even dimeric complexes have greatly reduced electrophoretic mobilities (Ewbank & Creighton, 1993). At protein concentrations greater than 100 μM, there was also spectroscopic indications of time-dependent aggregation of all the derivatives, with the exception of the fully folded Ca^{2+}-bound αLA and $3SS_{cam}$. Even at submicromolar concentrations, ANS promotes the aggregation of αLA at acid pH (Mulqueen & Kronman, 1982; Semisotnov et al., 1991), and here at 50 μM promoted the aggregation of the rearranged derivatives (Figure 8); in the absence of ANS, however, they behaved as monomeric species at low concentrations. Aggregation was found not to be a significant problem in these studies when the protein concentration was kept below 25 μM and is very unlikely to have affected the results presented here.

Ca^{2+} Binding. A single tightly-bound Ca^{2+} ion specifically stabilizes the native conformation of α-lactalbumin (Acharya et al., 1989, 1991). The native conformation is far less temperature stable in apo-αLA than when Ca^{2+} is bound (Kuwajima et al., 1986), and the disulfides are much less stable and are reduced rapidly (Ewbank & Creighton, 1993). This effect of Ca^{2+} makes it possible to stabilize the native conformation specifically by varying the Ca^{2+} concentration. Fully reduced α-lactalbumin and the derivatives with some of the native disulfide bonds have the potential, however energetically unfavorable, to adopt an N-state conformation that would differ from αLA's only in the number of native disulfide bonds. None of the disulfide bonds are directly involved in the Ca^{2+}-binding site, so each N-state conformation would be expected to bind Ca^{2+} with the same affinity, K_d^{Ca}, that of apo-αLA where the N conformation predominates. So long as only specific binding by the N conformation is measured, the stabilizing effect is directly proportional to the free Ca^{2+} concentration when the protein is fully saturated. This can be illustrated with the following equilibria for any species i able to adopt the N conformation:

$$U_i \overset{K_{f,i}}{\leftrightarrow} N_i \underset{Ca^{2+}}{\overset{K_d^{Ca}}{\leftrightarrow}} N{\cdot}Ca^{2+} \qquad (1)$$

where $K_{f,i}$ is the equilibrium constant for folding of the apo form of species i and K_d^{Ca} is the dissociation constant of the native conformation for Ca^{2+}, which is assumed to be independent of the species. The apparent equilibrium constant for folding of species i in the presence of Ca^{2+}, $K_{f,app,i}$, will be given by

$$K_{f,app,i} = \frac{[N_i] + [N{\cdot}Ca^{2+}]}{[U_i]} = K_{f,i}\left(1 + \frac{[Ca^{2+}]}{K_d^{Ca}}\right) \qquad (2)$$

When the protein is saturated, at high Ca^{2+} concentrations, the stability is directly proportional to the free Ca^{2+} concentration:

$$K_{f,app,i} = \frac{K_{f,i}}{K_d^{Ca}}[Ca^{2+}] \qquad [Ca^{2+}] \gg K_d^{Ca} \qquad (3)$$

The N state of α-lactalbumin is stabilized not only by Ca^{2+}, however, but also by a variety of monovalent and divalent metal ions and by Tris (Permyakov et al., 1985; Hiraoka & Sugai, 1985), so quantitative comparisons of the Ca^{2+}-free form under varying conditions in different studies are uncertain. The data of Hiraoka and Sugai (1985) indicate that the value of K_d^{Ca} for αLA is approximately 2×10^{-8} M under the conditions used here (Ewbank, 1992). In this case, the N state should be stabilized by 32 kJ/mol by the presence of 10 mM free Ca^{2+} and should become populated to a significant amount in any species if it is present in the apoprotein to an extent greater than 10^{-6}.

When the native conformation of any species is not populated sufficiently in the absence of Ca^{2+} to be detectable directly, its stability can be estimated from the observed affinity of the species for Ca^{2+}. The apparent dissociation constant for Ca^{2+}

of a species i obeying eq 1 will be given by

$$K_{d,app,i} = \frac{[U_i] + [N_i]}{[N{\cdot}Ca^{2+}]}[Ca^{2+}] = \left(\frac{1}{K_{f,i}} + 1\right)K_d^{Ca} \quad (4)$$

Where the N state is not substantially populated in the apo form, this simplifies to

$$K_{f,i} = \frac{K_d^{Ca}}{K_{d,app,i}} \quad (5)$$

With $K_d^{Ca} = 2 \times 10^{-8}$ M and concentrations of Ca^{2+} of up to 0.01 M, the presence of the N state can be quantified over 6 orders of magnitude. If a species i does not detectably bind Ca^{2+} at a free concentration of 10 mM, the native conformation is populated in the apo form of i to an extent no greater than 10^{-6}.

The above discussion treats the N state as a cooperative structure, as is usually the case, but α-lactalbumin is somewhat unusual in that only part of the native conformation can be present, as in Ca^{2+}-bound 2SS (see below). Indeed, the Ca^{2+} binding site of αLA consists of only residues 79–88, in a short segment of polypeptide chain, so the above discussion can be modified to include the possibility of just a local Ca^{2+} binding site existing.

Structure of Apo-αLA. The results presented here suggest that dissociation of Ca^{2+} from αLA causes the MG state to be populated in a fraction of the molecules, approximately 20% as judged by the near-UV CD, rather than causing a local perturbation of the N conformation. The presence of the MG state would account for the change in the physical properties of αLA (Permyakov et al., 1985; Hiraoka & Sugai, 1985) and would be reflected in the 5-fold enhancement of ANS binding and the alteration of intrinsic fluorescence properties. The change in intrinsic fluorescence has been attributed to a change in the environment of all emitting tryptophans (Ostrovsky et al., 1988), with a major contribution from Trp118 (Murakami et al., 1982), which is distant from the Ca^{2+}-binding loop. This conclusion is also consistent with the greatly increased susceptibility of the apoprotein to proteolytic digestion (Hirai et al., 1992) and with a recent calorimetric study of apo-bovine αLA (Xie et al., 1991), which concluded that the MG state is some 15% populated in apo-αLA in the absence of denaturant under conditions reasonably similar to those used here.

Structure of 3SS$_{cam}$. With Ca^{2+} bound, $3SS_{cam}$ adopts a near-fully folded conformation: its electrophoretic mobility was indistinguishable from that of αLA, it had native-like CD and fluorescence spectra, and its remaining disulfide bonds were reduced relatively slowly (Ewbank & Creighton, 1993). Ikeguchi et al. (1992) have reached the same conclusion. The relative ANS fluorescence of Ca^{2+}-bound $3SS_{cam}$ was, however, found here to be comparable to that of apo-αLA, which as described above appears to exist in a partially populated MG state. This ANS binding may be the consequence of a local relaxation in the region of the cleaved disulfide bond. The thiol groups of Cys6 and Cys120 in Ca^{2+}-bound 3SS tended to interchange slowly with the Cys28–111 disulfide, reflecting a substantial dynamic flexibility in the molecule. For the analogous three-disulfide derivative of lysozyme, minor conformational rearrangements occurred in the C-terminal region (Radford et al., 1991). The absence of a disulfide bond may also influence the quantum yield of the bound dye or the number of available ANS binding sites.

Apo-$3SS_{cam}$ had all the characteristics of a molten globule (Kuwajima, 1989) under the conditions used here. It was comparatively compact and possessed considerable secondary structure as judged by CD. It displayed a dramatic enhancement of ANS fluorescence, had an intrinsic fluorescence spectrum distinct from that of αLA in either the N or U state, and did not have significant tertiary interactions as judged by its near-UV CD spectrum and the rapid rate at which the disulfide bonds of the unblocked form rearranged (Ewbank & Creighton, 1993). The N state of apo-3SS predominates at low temperature, however; reduction of apo-αLA essentially stopped at the 3SS stage at 1 °C (Iyer & Klee, 1973), and apo-$3SS_{cam}$ at 4 °C had a CD spectrum like that of the native protein (results not shown).

Partly-Folded Structure of 2SS$_{cam}$. The results presented here indicate that part of Ca^{2+}-bound $2SS_{cam}$ was in a folded conformation under the standard conditions: it retained αLA's Ca^{2+} binding site, it had a far-UV CD spectrum indicative of some helical structure, its two remaining disulfide bonds were somewhat resistant to reduction (Ewbank & Creighton, 1993), it underwent a moderately cooperative urea-induced unfolding transition, and it was as compact as αLA (Table I). It exhibited significant ANS binding, however, and had substantially altered intrinsic fluorescence and CD spectra relative to folded αLA. The corresponding unblocked form, Ca^{2+}-bound 2SS, preferentially formed nonnative disulfide bonds, while the rate of formation of the Cys28–111 disulfide was of a similar order of magnitude to that of a disulfide in a fully unfolded protein (Ewbank & Creighton, 1993). Such observations could be consistent with a three-state equilibrium between molecules in which some of the molecules are fully N, fully MG, or fully U, as has been assumed with the other species described here, but this is unlikely in the case of Ca^{2+}-bound 2SS: all of the molecules were saturated with Ca^{2+}, so an equilibrium was unlikely.

An explanation for these findings is that the region of the molecule around the Ca^{2+} binding site, encompassing at least residues 61–91 (see Figure 1 in the preceding paper; Ewbank and Creighton, 1993), exists in a folded conformation when Ca^{2+} is bound, while the remainder is in a collapsed but unstructured conformation. That the disordered part of 2SS is collapsed, but does not appear to have secondary structure, suggests that the two phenomena may not need to occur concomitantly. The absence of secondary structure in the unfolded part of 2SS also suggests that there may need to be a critical amount of disordered polypeptide chain for the collapsed MG state with secondary structure to be stable.

This partly-folded structure of Ca^{2+}-bound $2SS_{cam}$ contrasts with the usual cooperativity of protein unfolding transitions and demonstrates that part of the protein can unfold independently. There is supporting evidence from X-ray crystallography for local, partial unfolding in native human α-lactalbumin (Harata & Muraki, 1992). This change, a helix–loop transition that was suggested to be caused by the protonation of His107, occurred just in the region that would be predicted to be affected by reduction of the Cys28–111 disulfide. Thus, αLA appears to consist of two structural subdomains, which correspond only partly to those defined crystallographically (domain 1, residues 1–37 and residues 86–123; domain 2, residues 38–85; Acharya et al., 1991). It should be noted, however, that the two halves are not totally independent, for reducing the Cys28–111 disulfide bond to produce 2SS increased greatly the rate of reduction of the remaining two disulfides in the other part of the molecule (Ewbank & Creighton, 1993).

The closest counterpart of Ca^{2+}-bound 2SS may be the one-disulfide folding intermediate of BPTI containing only the Cys30–51 disulfide bond (van Mierlo et al., 1992, 1993; see below). The structure of Ca^{2+}-bound 2SS is reminiscent

of the critical substructure model of Kuwajima et al. (1989), which was proposed as a high-energy transition state between the MG and N states. In contrast, Ca^{2+}-bound $2SS_{cam}$ has a stable equilibrium structure, although trapped as a kinetic intermediate. The ability to generate stable partly-folded structures when only some of the native disulfides are present demonstrates the power of the disulfide approach to studying protein structure and folding.

The structure of Ca^{2+}-bound $2SS_{cam}$ may also be compared with the suggested transient folding intermediate of the homologous lysozyme, which was proposed to resemble the equilibrium MG state of αLA and which is split into two subdomains, one structured and the other not (Miranker et al., 1991; Radford et al., 1992). The presumed transient folded subdomain in lysozyme overlaps with, but is distinct from, the proposed native-like part of Ca^{2+}-bound $2SS_{cam}$. Thus, the major C-helix, which includes Cys91 that participates in a disulfide bond resistant to reduction in Ca^{2+}-bound $2SS_{cam}$, is also present in lysozyme's early folding intermediate. In contrast, the preceding 3_{10} helix (residues 76–82 in αLA), which is presumed also to be structured in Ca^{2+}-bound $2SS_{cam}$, on the basis of the resistance of the Cys61–77 disulfide bond to reduction, lies in the unstructured subdomain of lysozyme's early folding intermediate (Miranker et al., 1991; Radford et al., 1992). This difference may be a consequence of the absence of a Ca^{2+}-binding site in lysozyme, since the bound Ca^{2+} greatly stabilized the folded conformation of 2SS and it was stable only at high Ca^{2+} concentrations.

Removal of Ca^{2+} from $2SS_{cam}$ resulted in comparatively small changes in the protein's intrinsic fluorescence and ANS binding properties. There was, however, a substantial reduction in the protein's compactness and stability. Its remaining disulfide bonds were reduced 40 times more rapidly than in the Ca^{2+}-bound protein (Ewbank & Creighton, 1993), and its near-UV CD spectrum was absent. Hence no part of apo-$2SS_{cam}$ appeared to be in a folded conformation. Apo-$2SS_{cam}$ did have a marked far-UV spectrum, however, and underwent a gradual unfolding transition. The overall helical content of $2SS_{cam}$, as judged by the intensity of its CD signal at 220 nm changed little upon removal of ligand, but there was a substantial change in the shape of the spectrum, similar to that seen for $3SS_{cam}$. These observed properties of apo-$2SS_{cam}$ are consistent with it being in an equilibrium between approximately 60% of molecules in the MG state and 40% in U. Cleavage of the Cys28–111 disulfide therefore seems to shift the conformational equilibrium from the MG toward the U state. This would also be consistent with the greater overlap, relative to 3SS, between the products of disulfide bond rearrangement of apo-2SS in the absence and presence of urea, [2SS] and $[2SS]^u$, respectively (Figure 4).

Comparison with the BPTI Intermediates. Parallels can be drawn between the structures of α-lactalbumin's trapped intermediates and the disulfide bond intermediates that populate the folding pathway of BPTI [reviewed by Creighton (1978), Goldenberg (1992), and van Mierlo et al. (1992)]. Thus, intermediates (30–51, 5–55), (30–51, 14–38), and (5–55, 14–38), which each lack one native disulfide bond, and the one-disulfide species (5–55) all adopt conformations of varying stability that are extremely native-like, probably due to the high stability of the N state of BPTI; they resemble Ca^{2+}-bound 3SS of α-lactalbumin. Intermediates in which the only native disulfide is Cys30–51, i.e., (30–51), (30–51, 5–14), and (30–51, 5–38), all have similar structures in which the main elements of native secondary structure are intact, but the N-terminal fourth of the molecule is disordered (Darby et al., 1992). Consequently, these species are analogous to Ca^{2+}-bound 2SS. In the case of α-lactalbumin, a substantial part of the stabilization of the folded part of the molecule comes from Ca^{2+} binding, whereas in the BPTI derivatives it is provided primarily by nonpolar interactions between elements of secondary structure and by a disulfide bond. Both cases, however, illustrate that the contribution of an interaction can be local, even in a protein whose structure exhibits cooperativity when fully folded (Creighton, 1978; Goldenberg et al., 1992). In contrast to the disordered part of Ca^{2+}-bound 2SS, the disordered part of the analogous BPTI intermediates is not collapsed (van Mierlo et al., 1992, 1993). Neither BPTI nor its derivatives lacking native disulfide bonds appear to adopt a stable MG state, unlike α-lactalbumin. The precise physical basis for such a distinction is an outstanding question in the study of MG states.

Structures of the Rearranged Species. When disulfide bond partners may exchange, the most prevalent pattern of disulfide pairing will reflect the most stable conformation, as was first demonstrated for insulin and ribonuclease A (Givol et al., 1965). If 3SS or 2SS are fully unfolded by the addition of 8 M urea, their remaining disulfide bonds are not stable, but interchange rapidly with the free cysteine thiol groups to give many rearranged forms, with no detectable nonrearranged species remaining. Even in the absence of any denaturant, the folded conformations of 2SS and 3SS are destabilized upon removal of Ca^{2+}, they adopt the MG and U conformations, and their disulfide bonds rearrange, just as rapidly as when they are fully unfolded in 8 M urea. Less than 4% and 2% of 3SS and 2SS, respectively, would be expected to remain at equilibrium, on the basis of the ratios of the forward and reverse rate constants for their rearrangement (Table II in the preceding paper; Ewbank & Creighton, 1993), and none were detected. As they were generated only upon removal of Ca^{2+}, the rearranged species cannot bind Ca^{2+} as tightly as the species 3SS and 2SS, and there was no effect of Ca^{2+} on the rearranged products' conformational properties.

Disulfide rearrangements of apo-3SS and apo-2SS in the presence and absence of urea produced different mixtures of intermediates. The average properties of the mixtures $[3SS]_{cam}$, $[3SS]^u_{cam}$, $[2SS]_{cam}$, and $[2SS]^u_{cam}$ in the absence of urea were distinct from those expected for a mixture of completely unfolded proteins; all have some secondary structure as judged by CD, have an intrinsic fluorescence spectrum blue-shifted relative to the fully unfolded R_{cm}, have a substantial affinity for ANS (which promotes their aggregation), are partially collapsed, and undergo denaturant-induced unfolding transitions of low cooperativity. They thus possess some MG-like character in the absence of denaturant which, on average, appears to decrease in the order $[3SS]_{cam} > [2SS]_{cam} \approx [3SS]^u_{cam} > [2SS]^u_{cam}$ as the amount of unfolded character increases. The previous conclusion that the iodoacetate-trapped three-disulfide species obtained after rearrangement of human 3SS in the presence of 8 M urea remained unfolded when the denaturant was removed (Ewbank & Creighton, 1991) has been reassessed in light of the current findings on bovine $[3SS]^u_{cam}$. The former species exhibit marked ANS binding (Ewbank, 1992) and are concluded also to have some MG-like character in the absence of urea.

The rearranged species exhibited a range of electrophoretic mobilities (Figures 4 and 5). In the absence of denaturant, a small proportion of $[3SS]_{cam}$ surprisingly had a mobility greater than that of $3SS_{cam}$ and comparable to that of apo-αLA. Unless disulfide rearrangement causes very substantial alterations in the pK_a values of ionized groups, some of the 3SS molecules must upon rearrangement either become even more compact or undergo a radical change in overall shape.

Stabilization of the N State by Disulfide Bonds. By quantifying the extents to which the N and U states are populated in the apo forms of the various species with different disulfide bonds, the contribution of the disulfides to the stability of the N state can be estimated (Table III). The Cys6–120 disulfide stabilizes αLA's state by 11.4 kJ/mol, as calculated from the difference in the free energy between their respective U and N states for αLA and $3SS_{cam}$; this is in satisfactory agreement with the difference of 10.1 kJ/mol between αLA and $3SS_{cm}$ measured by Kuwajima et al. (1990). This stabilization is presumably decreased by about 10 kJ/mol by the apparent strain in the Cys6–120 disulfide bond (Kuwajima et al., 1990; Ewbank & Creighton, 1993). The energetic contribution of the Cys28–111 disulfide bond to the stability of the N state is considerably less (≈5 kJ/mol), but this is a minimum value for the entire native conformation, for only part of the Ca^{2+}-bound 2SS appeared to be folded. Values of ≥7 kJ/mol are obtained for the other two native disulfides, assuming that they are equivalent.

Reducing the disulfide bonds of α-lactalbumin therefore destabilizes substantially the N state, so that in the absence of Ca^{2+} it is no longer populated substantially after more than one disulfide bond is cleaved and occurs to $<10^{-6}$ in the fully reduced protein, R. The lowered population of the N state causes the apparent affinity of the protein for Ca^{2+} to be decreased accordingly (eq 5).

Molten Globule States. The observed properties of αLA and its disulfide derivatives were consistent with a similar MG state for each, at least in terms of secondary structure content and compactness (Table III). The relative electrophoretic mobilities of the MG states of $3SS_{cam}$, $2SS_{cam}$, and R_{cam} could be calculated to estimate their hydrodynamic volumes (Table III) and were found to be very similar, with an average value of 97.5. This is only slightly less than the mobility of the N state (100.6) and considerably greater than that of αLA in the U state with the four native disulfide bonds intact (78) and without any disulfides (48). These values are consistent with the similar compactness of the N and MG states of αLA reported previously (Kuwajima et al., 1989). Consequently, the unfolding transition monitored by urea-gradient electrophoresis, in the absence of Ca^{2+}, is primarily that between the MG and U states. The urea-induced unfolding transitions of apo-αLA, apo-$3SS_{cam}$, and apo-$2SS_{cam}$ and of R_{cam} were very gradual (Figure 2), indicative of limited structural cooperativity of their MG states.

The native disulfide bonds contribute much less to the stability of the MG state than they do to that of the N state (Table III). The contribution of each was ≈2–3 kJ/mol; consequently, the MG state was still populated in R, in the absence of any disulfide bonds. A similar conclusion was reached by Ikeguchi et al. (1992) for the Cys6–120 disulfide bond. For the MG state, the stabilization is nonspecific: when either 3SS or 2SS is in a MG conformation, their native disulfides are not stable; they undergo rapid thiol–disulfide interchange to give a large number of rearranged species with more stable disulfide bonds, which can contain little or no common specific structure, but appear to maintain a MG-like conformation. The products of the rearrangement of apo-3SS appear less unfolded than the corresponding two-disulfide forms. Thus, the small general contribution of disulfide bonds to the increased occupancy of the MG state may result simply from the additional conformational constraint contributing to the maintenance of a compact molecule (Dill & Shortle, 1991). The conformational entropy of the MG state is presumably reduced by disulfide bonds only slightly less than is that of the U state.

Certain elements of native secondary structure, for example part of the major C-helix, are retained at acid pH in the MG state of disulfide bond-intact guinea pig α-lactalbumin, and there is evidence for the persistence of a native-like clustering of aromatic residues (Baum et al., 1989). At least some of these specific features of αLA's MG state may be the result of the retention of the four native disulfide bonds. The energetically preferred rearrangements of the disulfides in the MG state are extensive and involve all the cysteine residues (Ewbank & Creighton, 1993), which reduces the likelihood that any specific super-secondary contacts are retained in all the rearranged species. With fewer of the fixed constraints provided by native disulfide bonds to maintain the overall native fold, the MG state is likely to be a more dynamically flexible conformational state.

The results presented here support the increasingly accepted view that MG states are stabilized largely by nonspecific hydrophobic interactions (Kuwajima, 1989; Ewbank & Creighton, 1991; Mitaku et al., 1991; Hughson & Baldwin, 1991). In this case, the higher order interactions, above the level of secondary structure, that stabilize the N state would not be expected to be important for the stabilization of transient MG intermediates during protein folding; certainly as judged by the rearranged species, collapse and formation of secondary structure appear not to require an overall native fold. The MG state is, however, nearly as compact as the N state and must consequently serve to limit severely the conformational space that may be sampled by a polypeptide chain during folding; indeed, it substantially increases the rate of initial disulfide bond formation in reduced α-lactalbumin (Ewbank & Creighton, 1993).

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