Pathway of Disulfide-Coupled Unfolding and Refolding of Bovine α-Lactalbumin†

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ABSTRACT: α-Lactalbumin's four disulfide bonds have been used to probe the nature of its native, molten globule, and unfolded states. The Ca^{2+} concentration could be used to vary the stability of the native state because it binds a single calcium ion with high affinity. In the Ca^{2+}-bound native state, the Cys6–120 and Cys28–111 disulfides were reduced sequentially, reflecting their relative accessibilities. The native disulfides do not stabilize the native conformation as much as in other proteins, nor was the unfolding process as cooperative. After two disulfides were reduced, only part of the protein molecule appeared to remain folded. In the absence of Ca^{2+}, the native state was much less stable, and a molten globule-type conformation tended to be adopted. Formation of disulfide bonds in the reduced protein was initially noncooperative and nonspecific. The molten globule state increased the rate at which disulfides were formed, by tending to keep close in space cysteine residues that are distant in the sequence. A large number of nonnative disulfide pairings were preferred, and the disulfides were not stabilized to any great extent. The α-lactalbumin molten globule seems to be stabilized by nonspecific interactions and without a well-defined topology. The native protein was regenerated only when Ca^{2+} bound to and stabilized the three-disulfide intermediate lacking the Cys6–120 disulfide, which then rapidly formed the fourth disulfide bond. The rate-limiting intramolecular steps were rearrangement of disulfide bonds to native combinations in species with two and three disulfide bonds.

It is well-established, primarily from the work of Kuwajima (1989), that α-lactalbumin (αLA)[1] exhibits complex unfolding behavior. Three conformational states are populated under different conditions: the fully folded, native conformation; the fully unfolded state; and a poorly-defined compact intermediate state, generally known as the "molten globule". The nature of this third conformational state is important for understanding protein structure and folding and is currently the subject of much investigation [see Kuwajima (1989), Baldwin (1991), and Ptitsyn (1992) for reviews].

Bovine αLA is a very suitable subject for studying the nature of the molten globule state and of protein structure in general. The crystal structures of the baboon and human proteins have been solved to high resolution and are essentially identical (Acharya et al., 1989, 1991; Figure 1); the structure of bovine αLA is predicted to be very similar (Acharya et al., 1990). The native conformation binds a single Ca^{2+} ion, so the free Ca^{2+} concentration affects specifically the stability of the native conformation. The dissociation constant of αLA is dependent upon the ionic conditions and is estimated to be 2×10^{-8} M under the conditions used here (Hiraoka & Sugai, 1985; Ewbank, 1992); when the ion-binding site is fully saturated, the stability of the native conformation is proportional to the free Ca^{2+} concentration (Ewbank & Creighton, 1993).

αLA also has four disulfide bonds (Cys6–120, Cys28–111, Cys61–77, and Cys73–91; Vanaman et al., 1970) that can be used as probes of its conformation and stability. Disulfides are especially useful, as they are the only type of stabilizing interaction in folded proteins that can be trapped in a stable form (Creighton, 1978, 1986). The rates of reaction between protein and small-molecule thiols and disulfides can be interpreted in structural terms, because the thiol–disulfide interchange reaction is relatively simple and well-characterized (Szajewski & Whitesides, 1980; Gilbert, 1990). The kinetics of reduction of a protein disulfide, for example, reflect primarily its accessibility and conformational energy. The rate of formation of a protein disulfide bond reflects the conformation of the protein, its flexibility, and any conformational changes involved. Disulfides are made with characteristic rates and stabilities in unfolded proteins (Creighton, 1988). Folded conformations can cause them to be formed very rapidly and to be stabilized dramatically, but disulfide bonds that will be buried in a stable folded conformation are generally formed slowly, as well as being reduced slowly (Creighton, 1978; van Mierlo et al., 1991).

The disulfide between Cys6 and Cys120 of αLA has long been known to be selectively reduced by DTT_{SH}^{SH} (Iyer & Klee, 1973; Shechter et al., 1973). The resultant three-disulfide form of α-lactalbumin (designated here as 3SS) has a near-native conformation in the presence of Ca^{2+}, and its three remaining disulfides are relatively stable (Kuwajima et al., 1990). When Ca^{2+} was removed from human 3SS, the protein adopted the molten globule conformational state (Ewbank & Creighton, 1991). Its disulfide bonds then rearranged spontaneously by intramolecular thiol–disulfide interchange, at

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[1] Abbreviations: αLA, α-lactalbumin with its four native disulfide bonds intact; apo-αLA, αLA without a liganded Ca^{2+}; 3SS, α-lactalbumin with the Cys6–120 disulfide bond reduced; 2SS, α-lactalbumin with Cys6–120 and Cys28–111 disulfide bonds reduced; R, fully reduced α-lactalbumin; [*n*SS], mixture of isomers other than *n*SS each containing *n* disulfide bonds, generated during reduction of αLA, oxidation of R, or intramolecular disulfide rearrangement of *n*SS in the absence of urea; $[nSS]^u$, mixture of isomers other than *n*SS with *n* disulfide bonds, generated during reduction of αLA, oxidation of R, or intramolecular disulfide rearrangement of *n*SS in 8 M urea; BPTI, bovine pancreatic trypsin inhibitor; CD, circular dichroism; DTT_{SH}^{SH}, reduced dithiothreitol; DTT_S^S, oxidized dithiothreitol; EDTA, ethylenediaminetetraacetic acid; GSH, reduced glutathione; GSSG, oxidized glutathione; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; nOe, nuclear Overhauser effect; RNase, ribonuclease; TFA, trifluoroacetic acid. Intermediates with all thiol groups blocked irreversibly by reaction with iodoacetamide or iodoacetate are designated by the subscripts cam and cm, respectively. For partially blocked intermediates, the thiol groups blocked are indicated; for example, $2SS^{6/120cam}$, 2SS with Cys6 and Cys120 blocked by reaction with iodoacetamide. Rate constants are indicated by lower case *k*, equilibrium constants by upper case *K*, with the process indicated by super- and subscripts as necessary. Where appropriate, the reagent used is indicated in the subscript.

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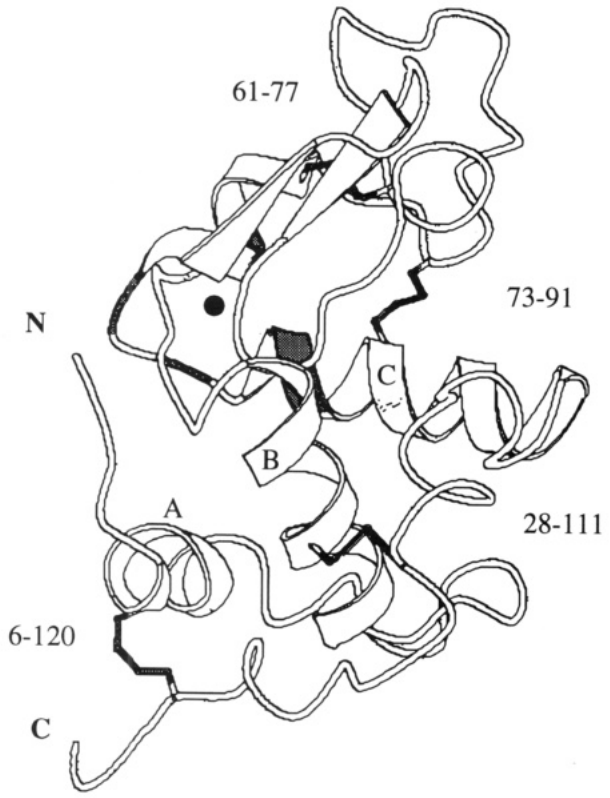


FIGURE 1: Schematic representation of the structure of baboon αLA, generated from the structure of Acharya et al. (1989) using the coordinates 1ALC of the Brookhaven Protein Structure Database and the program XRENDER (M. Noble, unpublished results). The structure of bovine αLA is predicted to be close to this structure (Acharya et al., 1990). The C-terminal residue is omitted from the deposited coordinates. Disulfide bonds are dark, with the cysteine residues listed beside each. Ca^{2+} is represented by a black circle, and the backbone of those residues that contribute to the Ca^{2+}-binding site is shaded. The major elements of secondary structure are shown as ribbons and include the three α-helices labeled A (residues 5–11), B (23–34), and C (86–99), a 3_{10} helix (76–82), and two strands of β-sheet (40–43 and 47–50).

the same rapid rate as when the protein was fully unfolded in 8 M urea. A large number of species with rearranged disulfides were generated that tend to maintain molten globule-like conformations. This indicated that the molten globule state is not stabilized to any significant extent by specific native tertiary interactions and that its overall topology need not be native-like.

This study has been extended by measuring the rates of disulfide bond reduction, rearrangement and re-formation in bovine α-lactalbumin, under conditions that favor the native, molten globule, or fully unfolded states, and by trapping and characterizing the kinetic intermediates (Ewbank & Creighton, 1993). This provides useful information about the process of protein unfolding and refolding, the nature of the molten globule state, and the structures of partially-folded intermediates.

MATERIALS AND METHODS

Materials. Bovine αLA (type III, Ca^{2+}-depleted), GSSG and GSH, iodoacetamide, and iodoacetic acid (free acid) were obtained from Sigma. Iodoacetic acid was recrystallized from tetrachloromethane before use. DTT_{SH}^{SH} and DTT_{S}^{S} were obtained from Calbiochem. DTT_{S}^{S} was purified as described previously (Creighton, 1977a). Urea was ultrapure grade (BRL), and only freshly made urea-containing solutions were used. All other chemicals were reagent grade or better. Iodoacetamide- and iodoacetate-trapped reduced and partially reduced forms of α-lactalbumin were obtained as described in the following paper (Ewbank & Creighton, 1993). Protein concentrations were determined from the absorbance at 280 nm, using the extinction coefficients given in the following paper.

Rates of Disulfide Reduction of αLA. Reductions were initiated by the addition to a solution of αLA (15–30 μM) of one-third volume of solutions of DTT_{SH}^{SH} and DTT_{S}^{S} or of GSH and GSSG (neutralized with KOH) at given concentrations. Both were in 0.1 M Tris (pH 8.7), 0.2 M KCl, which contained either 1 mM EDTA (neutralized with KOH) or 10 mM $CaCl_2$. When the protein was to be unfolded, the buffer also contained 8 M urea. At various times, an aliquot was removed and the reaction was stopped by covalent modification (blocking) of all free thiol groups: the addition of $^1/_5$ volume of either 0.6 M iodoacetamide or 0.6 M iodoacetic acid (neutralized with KOH) in 0.5 M Tris (pH 8.0). After 2 min at room temperature, the samples were stored on ice prior to analysis by nondenaturing polyacryalamide gel electrophoresis. The discontinuous high pH electrophoresis system of Davis (1964) was modified to include either 1 mM EDTA or 5 mM $CaCl_2$ in both the gel and the running buffer. The running buffer in the cathode compartment was used only once; if used again, an undefined modification of the protein occurred during the electrophoresis. The gels were stained with 0.1% Coomassie Blue in 10% trichloroacetic acid, 10% sulfosalicylic acid and destained in 5% methanol, 7.5% acetic acid.

A second trapping method was also used: the addition of $^1/_4$ volume of 1 M HCl to aliquots of the reduction mixture. Such acid-quenched samples were stored on ice before analysis 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.

To determine the number of disulfide bonds present in iodoacetamide-trapped reduction intermediates, a disulfide-counting method was used. The mixture of trapped intermediates was separated from the excess iodoacetamide by adsorption to a reversed-phase HPLC column (Aquapore RP-300, 4.6 × 38 mm) and eluted with a single step from 25% to 80% acetonitrile in 0.1% TFA. The total mixture of species was lyophilized, resuspended in 0.1 M Tris (pH 8.7), 0.2 M KCl, 1 mM EDTA and fully reduced by the addition of $^1/_8$ volume of 100 mM DTT_{SH}^{SH} in the same buffer. After incubation at 25 °C for 15 min, the thiol groups thus generated were blocked with iodoacetate as above. The samples were then analyzed by denaturing polyacrylamide gel electrophoresis (the system described above modified to include 8 M urea in the separating gel). As a control, αLA was treated in the same way, to confirm that the removal of the iodoacetamide was complete and that no modification other than at free thiol groups occurred.

Analytical reductions were similarly carried out with the iodoacetamide-trapped two- and three-disulfide forms of α-lactalbumin, $2SS_{cam}$ and $3SS_{cam}$, prepared as described in the following paper (Ewbank & Creighton, 1993).

No rigorous efforts were made to exclude air from reduction mixtures, but the total thiol content was periodically assayed with Ellman's reagent (Riddles et al., 1979). The presence of 10 mM $CaCl_2$ markedly increased the rate of oxidation of thiol groups, but even in its presence, the total thiol content never decreased by more than 3% in 1 h. As GSSG is a potent reagent for promoting disulfide bond formation, even a very low level of oxidation of GSH could interfere with accurate

kinetic analysis. GSH and GSSG were separated and quantified (Chau & Nelson, 1991) by reversed-phase HPLC (Dynamax-300A C_{18} column, 4.6 × 250 mm) using a linear gradient of 0% to 2.5% acetonitrile in 0.1% TFA. Freshly dissolved GSH typically contained 0.25% GSSG, rising to 1.5% in 30 min in the presence of Ca^{2+}. This factor was included in simulations of the reduction kinetics.

Measurement of the Effect of Ca^{2+} Concentration on Reduction. To obtain fully Ca^{2+}-bound αLA, type III αLA was dissolved to a concentration of approximately 20 mg/mL in 0.1 M Tris (pH 8.7), 0.2 M KCl, 10 mM $CaCl_2$ and then desalted (G-25 fast desalting column, Pharmacia) into the same buffer without added Ca^{2+}. This protein was considered to be 100% Ca^{2+}-bound in light of αLA's very high Ca^{2+} affinity (Hiraoka & Sugai, 1985; Mitani et al., 1986). It was diluted in 0.1 M Tris (pH 8.7), 0.2 M KCl, and $CaCl_2$ at a given concentration, and reduction was initiated exactly as above by the addition of DTT_{SH}^{SH} in an identical buffer.

Preparation of Fully Reduced and Partially-Blocked Fully Reduced α-Lactalbumin (R, $R^{6/120cam}$, $R_{28/111cam}^{6/120cam}$). One-tenth volume of 100 mM DTT_{SH}^{SH} in 0.1 M Tris (pH 8.7), 0.2 M KCl, 1 mM EDTA was added to αLA at 5 mg/mL in the same buffer. After 15 min at 25 °C, the fully reduced protein, R, was separated from the excess DTT_{SH}^{SH} by rapid gel filtration into 10 mM HCl and lyophilized. The partially reduced and blocked forms $3SS_{cam}$ and $2SS_{cam}$ were treated in an identical fashion to generate $R^{6/120cam}$ and $R_{28/111cam}^{6/120cam}$, respectively.

Preparation of Two-Disulfide α-Lactalbumin (2SS). 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 at 25 °C, the reduction was stopped by the addition of $^1/_4$ volume of 1 M HCl. This resulted in the appearance of a slight precipitate that was removed by centrifugation. The 2SS was then isolated by reversed-phase HPLC on a C_4 column as above and lyophilized.

Preparation of $2SS^{6/120cam}$. One-third volume of 20 mM DTT_{SH}^{SH} in 0.1 M Tris (pH 8.7), 0.2 M KCl, 10 mM $CaCl_2$ was added to $3SS_{cam}$ at 3 mg/mL in the same buffer. After 5 min of incubation at 25 °C, the reduction was stopped by the addition of $^1/_4$ volume of 1 M HCl. The partially reduced $3SS_{cam}$ ($2SS^{6/120cam}$) was isolated by reversed-phase HPLC, as for 2SS above, and lyophilized.

Disulfide Bond Formation in Partially and Fully Reduced α-Lactalbumin. Lyophilized R was dissolved to a concentration of approximately 50 μM in 10 mM HCl and then diluted 10-fold into 0.1 M Tris (pH 8.7), 0.2 M KCl containing DTT_S^S and DTT_{SH}^{SH} at given concentrations and either 1 mM EDTA or 10 mM $CaCl_2$. When the protein was to be unfolded, the buffer additionally contained 8.9 M urea. After incubation at 25 °C, aliquots were trapped at various times by covalent modification and analyzed either directly by electrophoresis or by subjecting the aliquots to the disulfide-counting method described above. Alternatively, the samples were trapped with acid and analyzed by reversed-phase HPLC as described above. To minimize air oxidation, all manipulations were performed under a positive N_2 pressure with solutions that had been thoroughly degassed and then saturated with N_2 prior to use. Control experiments, in which DTT_S^S was omitted, were also performed to monitor residual nonspecific oxidation. The presence of 10 mM Ca^{2+} greatly increased the background rate of oxidation, which invariably fitted first-order kinetics, with rates for different steps of between 0.0001 and 0.002 s^{-1}.

The rates of disulfide bond formation in $R^{6/120cam}$ and $R_{28/111cam}^{6/120cam}$, and in Ca^{2+}-bound 2SS and $2SS^{6/120cam}$, were followed in an exactly analogous fashion.

Disulfide Bond Rearrangement in 2SS and $2SS^{6/120cam}$. Lyophilized 2SS or $2SS^{6/120cam}$ was dissolved to a concentration of approximately 10 μM in 10 mM HCl and then diluted 4-fold into 0.1 M Tris (pH 8.7), 0.2 M KCl, 1 mM EDTA, or the same buffer at pH 8.7 containing 10.6 M urea. Aliquots were trapped after various times of incubation at 25 °C, either with acid or by covalent modification, and analyzed by reversed-phase HPLC or electrophoresis, respectively. To determine the rate of re-forming the native disulfide bonds by rearrangement, 0.5 M $CaCl_2$ in 0.1 M Tris (pH 8.7), 0.2 M KCl was added, to a final concentration of 10 mM, to samples that had been incubated in the presence of EDTA for 2 min. The samples were incubated further at 25 °C before trapping and analysis as above. Air oxidation was minimized as above.

Disulfide Bond Rearrangement in 3SS. The partially reduced intermediate 3SS was generated by the addition of $^1/_3$ volume of 400 μM DTT_{SH}^{SH}, in 0.1 M Tris (pH 8.7), 0.2 M KCl, 100 μM $CaCl_2$ to 25 μM αLA in the same buffer. After 2 min of incubation at 25 °C, >95% of the protein was in the 3SS form. The protein was diluted 5-fold into 0.1 M Tris, 0.2 M KCl, 1 mM EDTA, pH 8.7, or the same buffer at pH 8.7 containing 10 M urea; aliquots were trapped at various times with acid or with iodoacetamide and analyzed by reversed-phase HPLC or gel electrophoresis as above. The rate of re-forming the native disulfides was determined as for 2SS above.

Kinetic Analysis. The various species separated by gel electrophoresis were quantified by scanning laser densitometry, assuming that they all stained identically. HPLC analyses were quantified by integration of peak areas. All kinetic processes were modeled using a computer program (REDKINS; Ewbank, 1992) that simulated by numerical integration the time dependence of the concentrations of all reactants, given their starting concentrations and the values of the relevant rate constants. The results of disulfide bond breakage and formation obtained with different concentrations of DTT_{SH}^{SH} and DTT_S^S or of GSH and GSSG were interactively fitted by varying the rate constants until a single set for all the steps was obtained that adequately matched all the kinetic data under a given set of conditions.

Interpreting Thiol–Disulfide Kinetics. Protein disulfide bonds are reduced and formed by thiol/disulfide reagents in two sequential thiol–disulfide interchange reactions, proceeding through the mixed–disulfide intermediate. With a reduced protein with two accessible cysteine thiols (P_{SH}^{SH}) and a linear disulfide reagent like GSSG, the following steps occur:

$$P_{SH}^{SH} + GSSG \underset{k_{ex}/2}{\overset{2k_{ex}}{\rightleftharpoons}} P_{SSG}^{SH} + GSH \underset{k_r}{\overset{k_{intra}}{\rightleftharpoons}} P_S^S + 2GSH \quad (1)$$

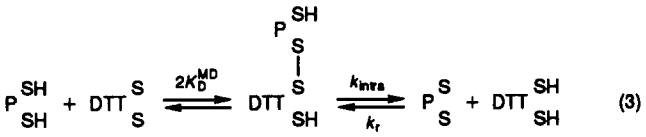
Although thiol–disulfide reactions proceed through the thiolate anion (Creighton, 1986), the nonionized forms are shown here, and subsequently, for simplicity. The mixed disulfide can either form an intramolecular protein disulfide bond or be reduced by GSH. For exposed, normally reactive thiol groups and disulfides, thiol–disulfide interchange occurs with an intrinsic rate constant k_{ex}, which under the conditions used here is ≈10 s^{-1} M^{-1} (Creighton & Goldenberg, 1984; Darby & Creighton, 1993); the value of k_{ex} and of the other thiol–disulfide rate constants will vary if any of the thiol reactants or products have atypical ionization properties (Szajewski & Whitesides, 1980).

The magnitude of k_{intra} reflects the energetic changes associated with formation of an intramolecular disulfide bond and is most pertinent to protein folding. If the value of k_{intra} is large, the observed rate of disulfide bond formation with GSSG is governed solely by the initial reaction of the disulfide reagent with a protein thiol group to form the mixed disulfide and is first-order in the concentration of GSSG. During reduction, the rate constant for the initial step, k_r, generally has the value k_{ex}, unless decreased by inaccessibility of the disulfide bond or increased by conformational strain. When the mixed-disulfide does not accumulate to substantial levels, but is present at steady-state levels, the rate of disulfide reduction is second-order in the concentration of GSH and inversely proportional to k_{intra}. The value of k_{intra} can be estimated from the observed rate of reduction of a protein disulfide with GSH, $k_{r,G}$, if the mixed-disulfide is normally reactive and accessible:

$$k_{r,G} = (k_r/k_{intra})k_{ex}/2 \quad (2)$$

The value of k_r can be obtained from the observed rate of reduction of the same disulfide by DTT^{SH}_{SH} (see below), which is found to be 2 times the value measured with GSH (Darby & Creighton, 1993). With more than two cysteine residues, the calculated value of k_{intra} should be corrected by the number involved (Creighton & Goldenberg, 1984).

The rate of disulfide bond formation is most relevant to protein folding with DTT^{S}_{S}. Its mixed-disulfide intermediate is intrinsically unstable, as it rapidly re-forms the stable intramolecular disulfide bond of DTT^{S}_{S}. Formation of the mixed disulfide with either of two cysteine residues is therefore an unfavorable preequilibrium, with the equilibrium constant $2K^{MD}_{D}$:



(3)

Consequently, the observed rate constant for disulfide bond formation with DTT^{S}_{S} is given by

$$k_{f,D} = 2K^{MD}_{D}\, k_{intra} \quad (4)$$

The value of K^{MD}_{D} should be double the equilibrium constant for thiol–disulfide interchange between GSH and DTT^{S}_{S}:

$$2GSH + DTT^{S}_{S} \rightleftarrows GSSG + DTT^{SH}_{SH} \quad (5)$$

The value of this equilibrium constant is pH-dependent above pH 8 (Chau & Nelson, 1991), and the appropriate value of K^{MD}_{D} will depend upon the pH and the pK value of the thiol group (Darby & Creighton, 1993). Analysis according to the results of Szajewski and Whitesides (1980) indicates that the appropriate value for pH 8.7 and typical protein thiol groups is $K^{MD}_{D} = (100\ M)^{-1}$. Since the rate constant for thiol–disulfide interchange between P^{SH}_{SH} and DTT^{S}_{S} should be $2k_{ex}$ (compare eqs 1 and 3), the rate constant for forming DTT^{S}_{S} from the mixed-disulfide can be estimated from the value of K^{MD}_{D} to be $\approx 1.2 \times 10^3\ s^{-1}$. Therefore, during reduction of protein disulfides with DTT^{SH}_{SH} at the usual rates, the observed kinetics will be first-order in the concentration of DTT^{SH}_{SH}, and the initial step, k_r, will be rate-limiting.

RESULTS

Direct Analysis of Disulfide Bond Breakage and Reformation. The kinetics of disulfide bond reduction and reformation in α-lactalbumin have been studied directly by trapping the various species that differ in the number and identities of their disulfide bonds. Intermediates were trapped either by an acid quench, and the trapped intermediates separated by reversed-phase HPLC at pH 2.0, or by covalent modification of all free thiol groups. The former method has the advantage of being rapid and reversible, whereas the latter is irreversible and necessary for structural analysis of the intermediates under conditions similar to those used for the kinetic analysis (Ewbank & Creighton, 1993). Separation of α-lactalbumin by reversed-phase HPLC is complex and not directly related to its conformation or number of disulfides bonds (Katzenstein et al., 1986), so it is most useful when there is a small number of species. In contrast, nondenaturing polyacrylamide gel electrophoresis separates intermediates on a simple basis of net charge and hydrodynamic volume that is especially useful with complex mixtures of species (Goldenberg & Creighton, 1984). Iodoacetamide is a neutral blocking reagent, so these trapped intermediates are separated primarily on the basis of their conformations. Intermediates trapped with iodoacetic acid, on the other hand, have an additional negative charge for each thiol group modified and so are also separated on the basis of the number of disulfide bonds they contain. Use of both blocking groups ensures that all intermediates with different hydrodynamic volumes or numbers of disulfides can be distinguished.

Reduction of αLA by DTT^{SH}_{SH} at High Ca^{2+} Concentrations. The reduction pathway of αLA by DTT^{SH}_{SH} was relatively simple in the presence of 10 mM Ca^{2+}. Very similar results were obtained using acid trapping and separation by HPLC or using covalent modification and then electrophoresis (Figure 2). Electrophoresis was routinely performed in the presence of EDTA, as only under these conditions were the various species resolved (Ewbank & Creighton, 1993).

The process of reduction could be split to a first approximation into two distinct kinetic phases, as observed previously by Kuwajima et al. (1990). In the first, rapid phase, αLA was reduced to give a species with a somewhat increased hydrodynamic volume when trapped with neutral iodoacetamide and an apparent net charge increase when trapped with acidic iodoacetate (Figure 2a). This has been shown to be a specific three-disulfide form, designated 3SS, with the disulfide bond between Cys6 and Cys120 broken (Ewbank & Creighton, 1993), identical to the only reduction intermediate observed previously (Shechter et al., 1973; Kuwajima et al., 1990).

In the second, slower kinetic phase, 3SS was further reduced to give a previously unobserved kinetic intermediate with a somewhat greater hydrodynamic volume. It was identified as having both the Cys6–120 and Cys28–111 disulfides cleaved (Ewbank & Creighton, 1993) and was designated 2SS. Both trapped species $3SS_{cam}$ and $2SS_{cam}$ bound Ca^{2+}, and their electrophoretic mobilities were then indistinguishable from that of αLA.

Further reduction of 2SS produced only fully reduced protein, and no one-disulfide intermediates accumulated to significant levels; reduction of a third disulfide must therefore have been followed by much more rapid reduction of the final disulfide bond. The fully reduced protein does not bind Ca^{2+} under these conditions (Ewbank & Creighton, 1993), and it is likely that Ca^{2+} dissociated from the protein after reduction of Ca^{2+}-bound 2SS (see Discussion).

The rate of each step observed was proportional to the concentration of DTT^{SH}_{SH}, and the pathway of reduction could

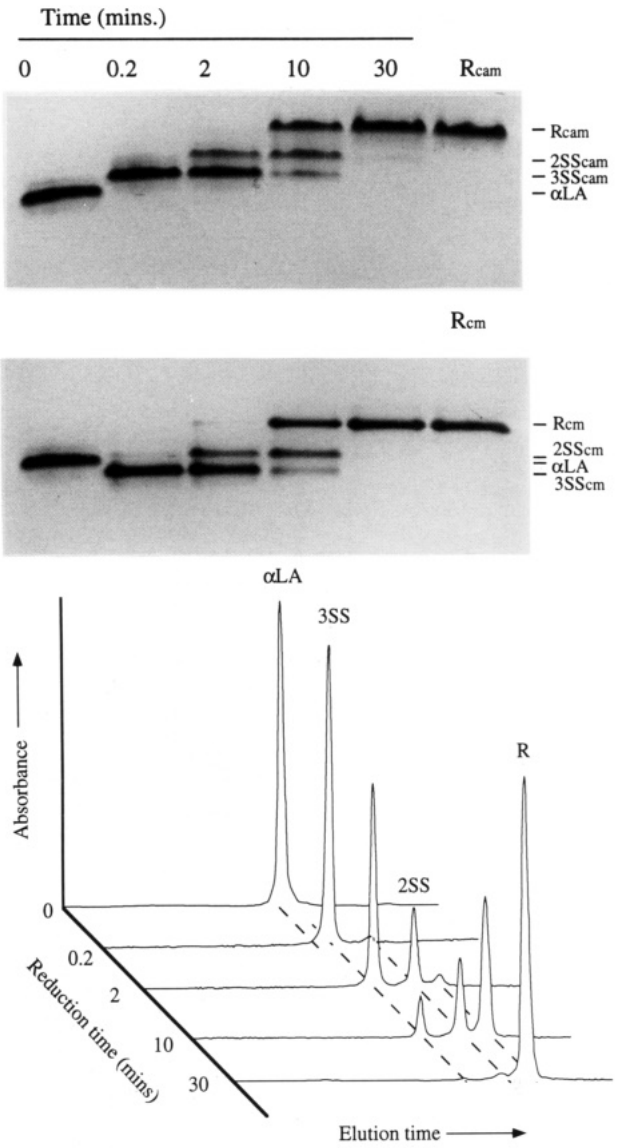


FIGURE 2: Reduction of 20 μM bovine αLA in the presence of 10 mM $CaCl_2$ by 5 mM DTT_{SH}^{SH}. (a, top two panels) Covalent blocking with either iodoacetamide (upper panel) or iodoacetate (lower panel) and separation by nondenaturing gel electrophoresis in the presence of EDTA. (b, bottom) Acid trapping and separation by reversed-phase HPLC.

be described quantitatively by[2]

$$\alpha LA{\cdot}Ca^{2+} \underset{k^4_{f,D}}{\overset{k^4_{r,D}}{\rightleftarrows}} 3SS{\cdot}Ca^{2+} \underset{k^3_{f,D}}{\overset{k^3_{r,D}}{\rightleftarrows}} 2SS{\cdot}Ca^{2+} \underset{k^2_{f,D}}{\overset{k^2_{r,D}}{\rightleftarrows}} \{1SS\} \underset{k^{[1]}_{f,D}}{\overset{k^{[1]}_{r,D}}{\rightleftarrows}} R \quad (6)$$

where $k^n_{r,D}$ and $k^n_{f,D}$ represent the bimolecular rate constants for disulfide bond reduction and formation, respectively, for each of the steps (Table I). Putative intermediate {1SS} was not detected and could not be characterized, so it represents an ensemble of some or all of the 28 possible one-disulfide isomers.

Added DTT_S^S substantially reversed the reduction of the first disulfide bond, giving a value for the reverse rate constant $k^4_{f,D}$. This was the only reverse step that was measurably affected by the addition of DTT_S^S, indicating that the other reverse rates were much lower and providing only a maximum estimate of their values.

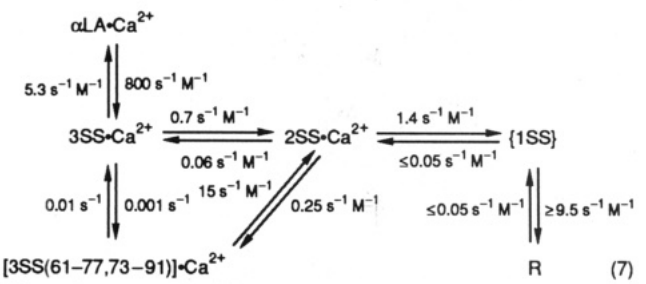
[2] The disulfide and thiol reagents and the free Ca^{2+} (where appropriate) are omitted in these schemes for the sake of clarity.

The isolated Ca^{2+}-bound trapped species $3SS_{cam}$ and $2SS_{cam}$ were reduced with kinetics very similar to those expected from the overall process of equation 6 (Table I).

The scheme of eq 6 accounted for the total flux of protein along the reduction pathway at high DTT_{SH}^{SH} concentrations (Figure 3a,b). At low DTT_{SH}^{SH} concentrations, however, the kinetics indicated that intramolecular disulfide rearrangements in 3SS were significant. The nature of this phenomenon was investigated further by studying the reverse process, disulfide bond re-formation in Ca^{2+}-bond 2SS, using the isolated intermediate. Several three-disulfide species, in addition to 3SS and αLA, were generated in the presence of DTT_S^S, and their appearance preceded that of αLA. The electrophoretic mobilities of these trapped intermediates were altered in the presence of Ca^{2+} and were then indistinguishable from that of αLA (Ewbank, 1992). That all these trapped species bound Ca^{2+} and then had native-like hydrodynamic volumes strongly suggests that the two native disulfide bonds of 2SS (Cys61–77 and Cys73–91) were intact. These species were reduced by DTT_{SH}^{SH} at a rate (15 s^{-1} M^{-1}) more than 20-fold higher than that of reducing Ca^{2+}-bound 3SS (Table I). Hence these three-disulfide species must each have had a third disulfide bond, other than Cys28–111, between different pairs of Cys6, Cys28, Cys111, and Cys120. Only five such disulfide isomers are possible, but no individual species were resolved by HPLC or by electrophoresis (Ewbank, 1992). One of these third disulfides might have been the native pairing Cys6–120, but the majority must have been nonnative third disulfides. Therefore, these intermediates are referred to collectively as [3SS(61–77,73–91)], indicating that they retained the Cys61–77 and Cys73–91 disulfide bonds; they were treated as a single kinetic species for the purposes of kinetic analysis.

The rate of re-formation of the Cys28–111 disulfide in 2SS was measured directly using $2SS^{6/120cam}$ (in which the only thiol groups are those of Cys28 and Cys111) in the presence of DTT_S^S and 10 mM Ca^{2+} (Figure 3f). Species $3SS_{cam}$ was regenerated at a rate (0.06 s^{-1} M^{-1}) that was $^1/_5$ the observed rate of total disulfide formation in 2SS (0.31 s^{-1} M^{-1}). This is consistent with the above conclusion that 2SS forms predominantly disulfides other than Cys28–111, to generate [3SS(61–77,73–91)].

Given the rate constants for direct interconversion of 3SS and 2SS, the rates of rearrangement of [3SS(61–77,73–91)] to and from 3SS could be estimated by simulating the kinetics of appearance of the latter and of the level of accumulation of [3SS(61–77,73–91)] during disulfide formation in 2SS. Thus, disulfide bond reduction and formation with dithiothreitol at the two- and three-disulfide stages could be more fully described, with the appropriate rate constants, as[2]



(7)

The values measured directly in this way are consistent within a factor of 2 of the thermodynamic requirement that there be no change in free energy around the circular part of eq 7, even though this requirement was not imposed during the kinetic fitting. These results explain the kinetics of reduction; at high concentrations of DTT_{SH}^{SH}, the vast majority of 3SS was

Table I: Kinetic Parameters of Disulfide Reduction in αLA (pH 8.7, 0.1 M Tris, 0.2 M KCl, 25 °C)

	10 mM Ca^{2+}					
step	$k_{r,D}$ (s^{-1} M^{-1})	$k_{f,D}$ (s^{-1} M^{-1})	k_{intra}[a] (s^{-1})	$k_{r,G}$ (s^{-1} M^{-2})	$k_{f,G}$ (s^{-1} M^{-1})	k_{intra}[b] (s^{-1})
αLA ↔ 3SS	800	5.3	265	5	6.7	400
3SS ↔ 2SS	0.7, 0.55[c]	≤0.05, 0.06[d]	≤2.5, 3[d]	0.18	≤1	9.7
2SS ↔ {1SS}	1.4, 0.5,[c] 0.2[e]	≤0.05[c,e,f]	≤2.5[c,e,f]	0.5		7.0
{1SS} ↔ R	≥9.5	≤0.05[c,e,f]	≤2.5[c,e,f]	≥5		

	1 mM EDTA			8 M urea	
step	$k_{r,D}$ (s^{-1} M^{-1})	$k_{f,D}$ (s^{-1} M^{-1})	step	$k_{r,D}$ (s^{-1} M^{-1})	$k_{f,D}$ (s^{-1} M^{-1})
αLA ↔ 3SS	650	0.03[g]	αLA ↔ [3SS]^u	42	≤0.025
3SS ↔ [2SS]	18, 15[c]	≤0.05[c]	[3SS]^u ↔ [2SS]^u	32	≤0.025
[2SS] ↔ [1SS]	22	≤0.05[c,e]	[2SS]^u ↔ [1SS]^u	24	≤0.025
[1SS] ↔ R	10	≤0.05[c,e]	[1SS]^u ↔ R	11	≤0.025
[3SS] ↔ [2SS]	30	≤0.05			

[a] $k_{intra} = k_{f,D}/2K_D^{MD}$; K_D^{MD} = (100 M)$^{-1}$. [b] $k_{intra} = k_{ex}k_{r,D}/4k_{r,G}$; k_{ex} = 10 s^{-1} M^{-1} (Creighton & Goldenberg, 1984; Darby & Creighton, 1993); uncorrected for the number of participating thiol groups. [c] Value measured using isolated $3SS_{cam}$. [d] Value measured using $2SS^{6/120cam}$. [e] Value measured using $2SS_{cam}$. [f] Assuming minimum value for $k_{r,D}$. [g] Determined indirectly from the effect of disulfide bond reduction on Ca^{2+} affinity; see text for details.

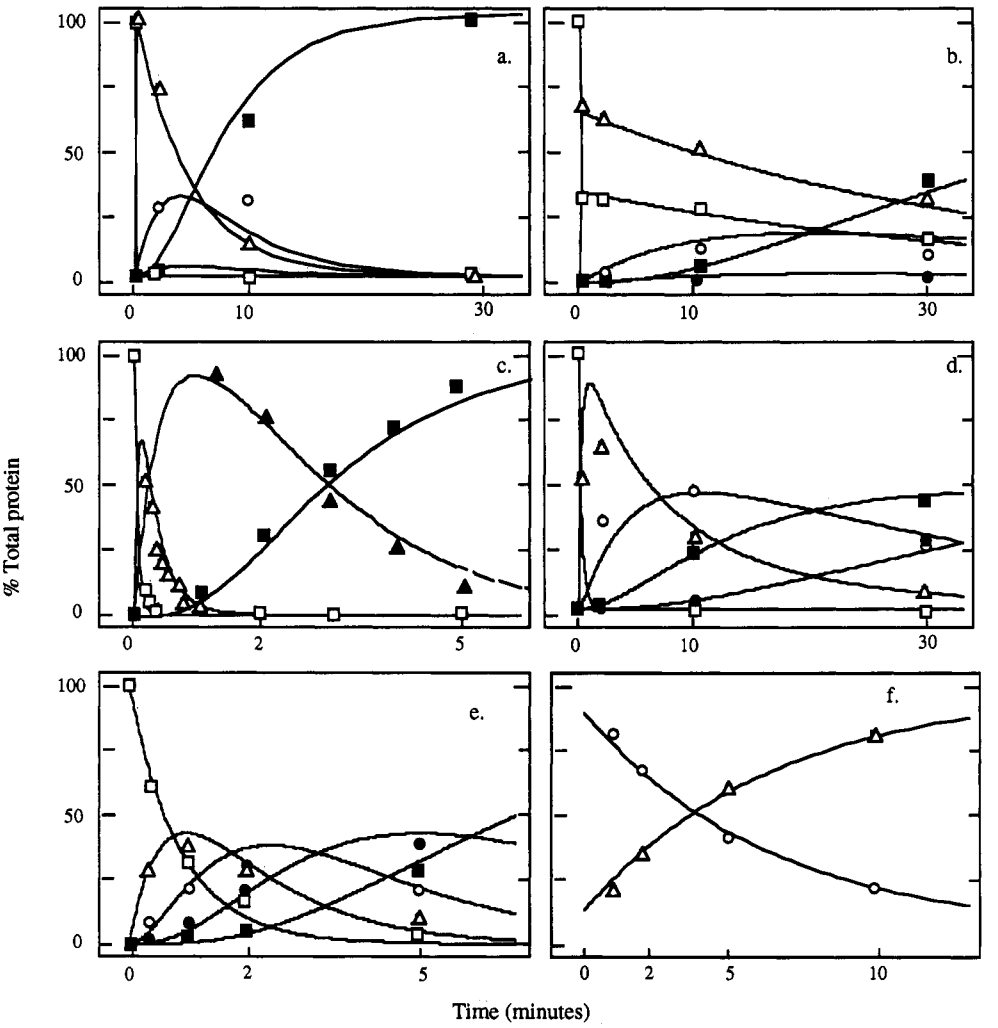


FIGURE 3: Kinetic simulations of disulfide bond reduction and formation with dithiothreitol in bovine α-lactalbumin. Reduction of αLA in the presence of 10 mM $CaCl_2$: (a) 5 mM DTT_{SH}^{SH}; (b) 1 mM DTT_{SH}^{SH}, 80 mM DTT_S^S. Reduction of αLA in the presence of 1 mM EDTA: (c) 1 mM DTT_{SH}^{SH}; (d) 100 μM DTT_{SH}^{SH}. Reduction of αLA in the presence of 8 M urea: (e) 500 μM DTT_{SH}^{SH}. Formation of Cys28–111 in $2SS^{6/120cam}$ in the presence of 10 mM $CaCl_2$: (f) 100 μM DTT_{SH}^{SH}, 40 mM DTT_S^S. The data give the proportions of each species present at the time of trapping. Those in (d) and (e) are from disulfide-counting gels and in the others are from HPLC analyses of acid-trapped species. The curves were generated using the rate constants given in Table I. The symbols used are (a–e) □, αLA; ■, R; (a and b) △, 3SS; ○, 2SS; ●, {1SS}; (c) △, 3SS; ▲, [3SS] + [2SS] + [1SS]; (d) △, 3SS + [3SS]; ○, [2SS]; ●, [1SS]; (e) △, [3SS]^u; ○, [2SS]^u; ●, [1SS]^u; (f) △, $3SS_{cam}$; ○, $2SS^{6/120cam}$.

reduced directly. With slower rates of reduction, however, [3SS(61–77,73–91)] become populated to a small extent, and the alternative pathway became significant.

These data also make it possible to estimate the rate at which the Cys28–111 disulfide might be formed last to regenerate αLA, from the hypothetical species with the three

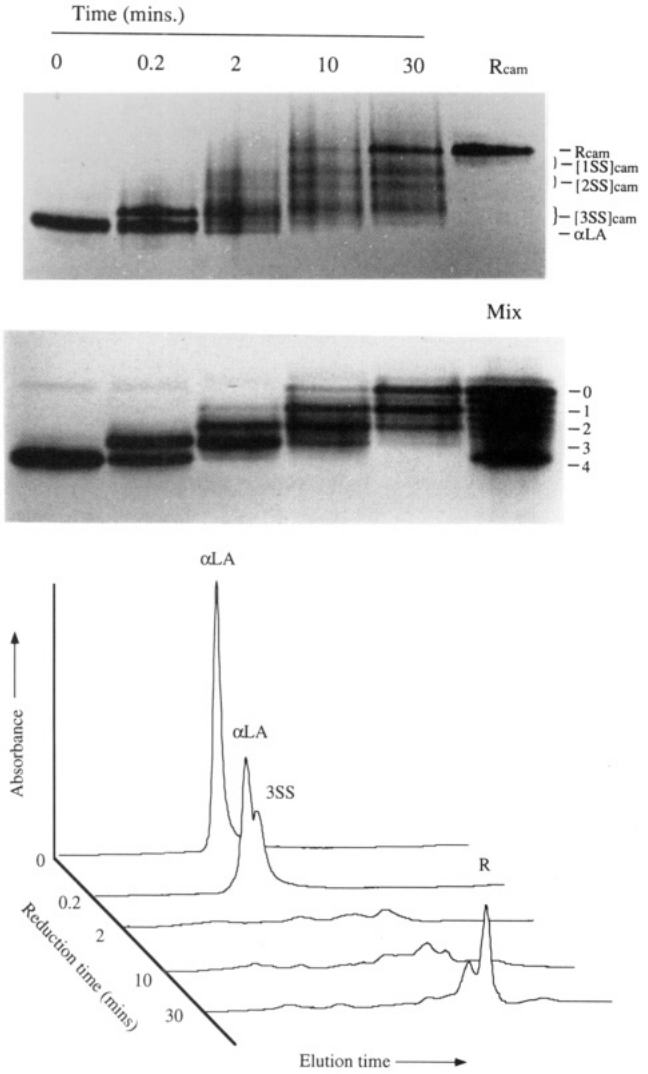


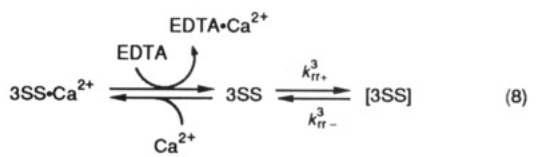
FIGURE 4: Reduction of 10 μM αLA in the presence of 1 mM EDTA by 100 μM DTT_{SH}^{SH}. (a, top two panels) Covalent trapping by reaction with iodoacetamide. Upper panel: direct analysis of trapped intermediates using nondenaturing gel electrophoresis in the presence of EDTA. Lower panel: disulfide-counting; the same samples blocked with iodoacetamide were fully reduced and then trapped with iodoacetate. Electrophoresis was in the presence of 8 M urea. The right-hand lane is a mixture of fully reduced αLA blocked with different ratios of iodoacetamide and iodoacetic acid to generate molecules containing between zero and eight acidic cm groups. (b, bottom) Acid trapping and separation by reversed-phase HPLC.

native disulfides Cys6–120, Cys61–77, and Cys73–91 in [3SS-(61–77,73–91)]. If the Cys28–111 disulfide bond would be reduced in αLA at the same rate as it is in 3SS, 0.7 s^{-1} M^{-1}, the rate of its re-formation can be calculated from the thermodynamic cycle linking αLA, 3SS, and [3SS(61–77,73–91)] in eq 7 to be 0.05 s^{-1} M^{-1}. This rate is only 0.01 of that for forming the Cys6–120 disulfide in the more stable 3SS; consequently, forming the Cys28–111 disulfide last to regenerate αLA would not be kinetically significant.

Reduction of αLA by DTT_{SH}^{SH} in the Presence of EDTA. The anomalously high rate of reduction of the Cys6–120 disulfide bond in αLA was nearly as great in the presence of EDTA. The resulting intermediate 3SS rapidly disappeared, however, and a great number of poorly resolved intermediates were observed by both electrophoresis and reversed-phase HPLC (Figure 4). This contrasts with the stability of 3SS and the very small number of intermediates present during reduction in the presence of Ca^{2+} (Figure 2).

To facilitate interpretation of these complex patterns, a modification of the straightforward trapping procedure was used, a disulfide-counting method, that quantified intermediates simply on the number of disulfide bonds they contained, not their identities (Hirose et al., 1988). The protein trapped with iodoacetamide was separated from excess iodoacetamide and then fully reduced. All free thiol groups thus generated were blocked with iodoacetate. This introduced two negatively charged cm groups for every disulfide bond that had been present in the intermediates before the full reduction step. The intermediates then had no disulfide bonds and differed primarily in the number of neutral and acidic blocking groups they contained. They could be readily resolved and quantified by electrophoresis in 8 M urea, where the mobility depended solely on charge. This procedure greatly simplified the interpretation of the reduction time course, and the overall rates of reduction of the collections of three-, two-, and one-disulfide intermediates could be measured. The rate constants measured in this way (Table I) are complex totals of all the microscopic steps occurring, averaged over all the species present.

The rapid disappearance of 3SS in the absence of Ca^{2+} could not be attributed to reduction of its disulfide bonds, for it occurred at a rate that was largely independent of the DTT_{SH}^{SH} concentration and was not accompanied by the appearance of two-disulfide species (Figure 4). Instead it was likely to be due to intramolecular thiol–disulfide interchange, which was confirmed using isolated 3SS. Ca^{2+}-bound 3SS was relatively stable to reduction and thiol-disulfide interchange (Figure 2, eq 7). When the liganded Ca^{2+} was removed from 3SS by the addition of EDTA, however, the single 3SS species rapidly disappeared and a large number of other three-disulfide species were generated [Figure 4 in Ewbank and Creighton (1993)] and designated collectively as [3SS]. The subsequent addition of excess Ca^{2+} resulted in the slow reappearance of 3SS. This was interpreted in terms of the scheme



(8)

where [3SS] represents the mixture of rearranged species containing three disulfides, other than the specific species 3SS, and k_{rr+}^{3} and k_{rr-}^{3} are the apparent rate constants for rearrangement in the indicated directions. The equilibrium position for the reaction in the presence of excess EDTA lies well to the right, with no detectable 3SS remaining at equilibrium, indicating that the value of k_{rr-}^{3} was at least 25 times less than that of k_{rr+}^{3}. Binding of Ca^{2+} by EDTA is much more rapid than the disulfide rearrangements observed (Ewbank & Creighton, 1991). As expected, the kinetics of the initial disappearance of 3SS by rearrangement were well fitted by a first-order reaction (Figure 5). The kinetics of the reappearance of 3SS from [3SS] upon addition of excess Ca^{2+} were also well fitted by a first-order reaction (Figure 5). As only a few species in [3SS] are likely to be immediate precursors of 3SS, this suggests that most of the [3SS] species were in rapid equilibrium. The rate of disulfide bond rearrangement of 3SS was virtually the same when it was fully unfolded in 8 M urea (Table II).

Isolated intermediates 2SS and $2SS^{6/120cam}$ were also observed to undergo reversible intramolecular disulfide re-

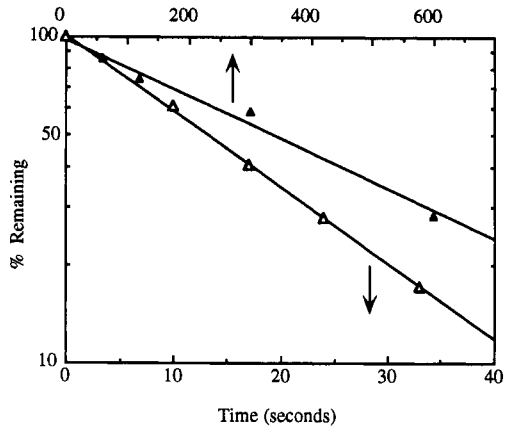


FIGURE 5: Rate of rearrangement of three-disulfide bovine α-lactalbumin in 0.1 M Tris (pH 8.7), 0.2 M KCl, 25 °C. The partially reduced species 3SS was generated by reduction of Ca^{2+}-bound αLA and diluted into buffer containing an excess of EDTA. Aliquots were removed at the indicated times, trapped with acid, and the proportion of 3SS (△) remaining was measured by HPLC analysis. After 2 min, an excess of Ca^{2+} was added and the amount of [3SS] (▲) remaining as a function of time was determined in a similar manner.

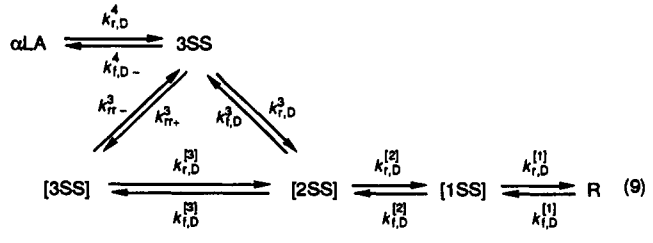
Table II: Rates of Rearrangement of Partially Reduced Forms of αLA in the Presence and Absence of 8 M Urea[a]

species	no urea k_{rr+} (s^{-1})	no urea k_{rr-}[b] (s^{-1})	8 M urea k_{rr+} (s^{-1})
3SS	0.053	1.9×10^{-3}	0.060
2SS	0.045	8.0×10^{-4}	0.031
$2SS^{6/120cam}$	0.022	1.0×10^{-3}	0.020

[a] 0.1 M Tris (pH 8.7), 0.2 M KCl, 1 mM EDTA, 25 °C. [b] In the presence of 10 mM $CaCl_2$.

arrangements upon removal of Ca^{2+}, with rates similar to that of 3SS (Table II).

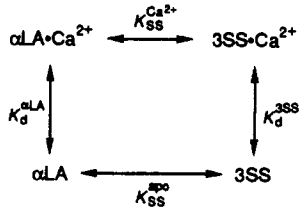
During reduction of apo-αLA, many two- and one-disulfide intermediates were observed, in addition to the multiple three-disulfide forms, and no specific intermediates were resolved. Reduction of the rearranged three-disulfide species apparently gave sequentially mixtures of two- and one-disulfide intermediates that could also undergo disulfide bond rearrangements and were designated [2SS] and [1SS], respectively. The kinetics of reduction of apo αLA were consistent with the following pathway: the Cys6–120 disulfide bond was specifically broken to yield 3SS, which could either be reduced directly by DTT_{SH}^{SH} or undergo intramolecular thiol–disulfide interchange to give intermediates containing three disulfide bonds, which were then further reduced[2] as shown below.



where [*n*SS] represents the mixture of isomers containing *n* disulfide bonds obtained both directly by reduction of the previous intermediate and by intramolecular rearrangement of disulfide bonds, and $k_{r,D}^{[n]}$ and $k_{f,D}^{[n]}$ are the rate constants for reduction of, and oxidation to, [*n*SS] involving DTT_{SH}^{SH} and DTT_S^S, respectively. All [*n*SS] intermediates with the same number of disulfide bonds appeared to be in rapid equilibrium, and no single intermediate predominated in any case. If the equilibrium is not extremely rapid, there would be kinetic competition between disulfide bond reduction and intramolecular thiol–disulfide interchange, so the precise nature of [*n*SS] would depend on the concentration of DTT_{SH}^{SH}. At very high concentrations of DTT_{SH}^{SH}, reduction would tend to be through intermediates with native disulfides, while at low DTT_{SH}^{SH} concentrations intermediates with nonnative disulfides would predominate. Such potential complexities were not apparent here, however, and [3SS], [2SS], and [1SS] were each treated as single populations for the purposes of kinetic simulations.

The rate of disulfide bond cleavage in apo-$3SS_{cam}$ could be measured accurately, as it was unable to undergo thiol–disulfide interchange due to the thiol groups of Cys6 and Cys120 being blocked. The rate constant determined was very similar to that estimated for direct reduction of apo-3SS from the kinetic simulations (Table I), although the latter value was not considered very accurate. Given rates for direct reduction of apo-3SS and for its rearrangement, the other rate constants of eq 9 could be estimated by kinetic simulations of the process of reduction by DTT_{SH}^{SH} in the presence and absence of added DTT_S^S (Table I; Figure 3c,d).

The value of $k_{f,D}^4$, the rate constant for re-formation of the Cys6–120 disulfide in apo-3SS, could not be measured, due to the high rate of rearrangement of apo-3SS. Even high concentrations of DTT_S^S (up to 40 mM) caused no detectable effect on the rate of the initial reduction of αLA, although it did promote intermolecular disulfide bond formation, as described further below. An indirect estimate could be made for $k_{f,D}^4$, however, since there should be linkage between the equilibrium constants for disulfide bond breakage (horizontal arrows) and Ca^{2+} binding (vertical arrows):[2]



where K_d^{3SS} and $K_d^{\alpha LA}$ are the apparent dissociation constants for Ca^{2+} of 3SS and αLA, respectively, and $K_{SS}^{Ca^{2+}}$ and K_{SS}^{apo} are the equilibrium constants for reducing and re-forming the Cys6–120 disulfide bond, given by $k_{r,D}^4/k_{f,D}^4$, in Ca^{2+}-bound and apo-αLA, respectively. It is a thermodynamic necessity that

$$\frac{K_{SS}^{Ca^{2+}}}{K_{SS}^{apo}} = \frac{K_d^{\alpha LA}}{K_d^{3SS}} \qquad (11)$$

The apparent Ca^{2+} affinities of αLA and $3SS_{cam}$ were known most accurately (Ewbank & Creighton, 1993; see below) and with the measured value of $k_{r,D}^4$ could be used to give a value for $k_{f,D}^4$ in apo-3SS of 0.03 s^{-1} M^{-1}. This value is 2 orders of magnitude lower than when Ca^{2+} was bound.

A comparison of the kinetics of appearance and disappearance of the different species observed by the disulfide-counting technique and by the standard trapping procedure allowed tentative assignment of the number of disulfide bonds present in the numerous trapped species separated by electrophoresis. In general, disulfide bond reduction was ac-

companied by the expected decrease in electrophoretic mobility, corresponding to an increase in hydrodynamic volume, although there were exceptions (Ewbank & Creighton, 1993).

Using covalent trapping and gel electrophoresis, it was readily apparent when intermolecular disulfide bonds, reflecting aggregation processes, were present in the reduction intermediates. Such oligomers typically gave numerous bands, in a ladder-like manner with decreasing electrophoretic mobilities, and nonintegral numbers of disulfide bonds when counted (Ewbank, 1992). Intermolecular disulfides were observed when apo-αLA, even at low protein concentrations of 10–25 μM, was incubated with very low concentrations of DTT_{SH}^{SH}, such that reduction stopped at the three-disulfide form, or when very high concentrations of DTT_S^S were also present. They probably resulted from the time-dependent aggregation of partially reduced intermediates and disulfide rearrangement between them. Such aggregation did not occur in the kinetic experiments analyzed here.

Reduction by DTT_{SH}^{SH} of αLA Fully Unfolded in 8 M Urea. In the presence of 8 M urea, CD spectra indicated that αLA was fully unfolded, as were the trapped intermediates on the reduction pathway (Ewbank & Creighton, 1993). Under these denaturing conditions, the reduction process was complex: the Cys6–120 disulfide bond was not reduced at a rate any greater than that of the other disulfides, as was also observed by Kuwajima et al. (1990); no single three-disulfide species was generated; many three-, two-, and one-disulfide intermediates were observed; and disulfide bond rearrangements occurred at each stage (Figure 6). The simple trapping procedures allowed the rates of disappearance of the disulfide intact protein and of appearance of the fully reduced protein to be determined but were supplemented by the disulfide-counting technique to provide rates for the overall reduction of the ensembles of three-, two-, and one-disulfide isomers (Figure 6a). The addition of DTT_S^S up to 40 mM did not measurably affect the kinetics of reduction, so the reverse rate constants were not significant for this analysis, and only maximum values could be determined. The experimental results fitted the kinetic scheme[2]

$$\alpha\text{LA} \underset{k_{f,D}^{4}}{\overset{k_{r,D}^{4}}{\rightleftarrows}} [3\text{SS}]^{u} \underset{k_{f,D}^{[3]u}}{\overset{k_{r,D}^{[3]u}}{\rightleftarrows}} [2\text{SS}]^{u} \underset{k_{f,D}^{[2]u}}{\overset{k_{r,D}^{[2]u}}{\rightleftarrows}} [1\text{SS}]^{u} \underset{k_{f,D}^{[1]u}}{\overset{k_{r,D}^{[1]u}}{\rightleftarrows}} \text{R} \qquad (12)$$

where $[n\text{SS}]^{u}$ represents the mixture of isomers containing n disulfide bonds generated in 8 M urea, analogous to the situation with apo-αLA (eq 9). The average rate constants were determined as above (Table I and Figure 3e). The addition of Ca^{2+} to 10 mM did not alter the kinetics of reduction (results not shown), consistent with none of the species binding Ca^{2+} in 8 M urea.

The three- and two-disulfide intermediates trapped by iodoacetamide (identified by the kinetics of their appearance) were less compact overall, as measured by their electrophoretic mobilities, than the equivalent intermediates during reduction in the absence of urea (compare Figures 4a and 6a).

Reduction of αLA by DTT_{SH}^{SH} at Intermediate Ca^{2+} Concentrations. Study of the effect of Ca^{2+} concentration on the reduction kinetics has the potential to provide information on the Ca^{2+} affinities of the various species. It was assumed that all Ca^{2+} not bound by the protein was free in solution; there is the potential for Ca^{2+} to interact with the buffer or the thiol reagent, but the magnitudes of such interactions are not known.

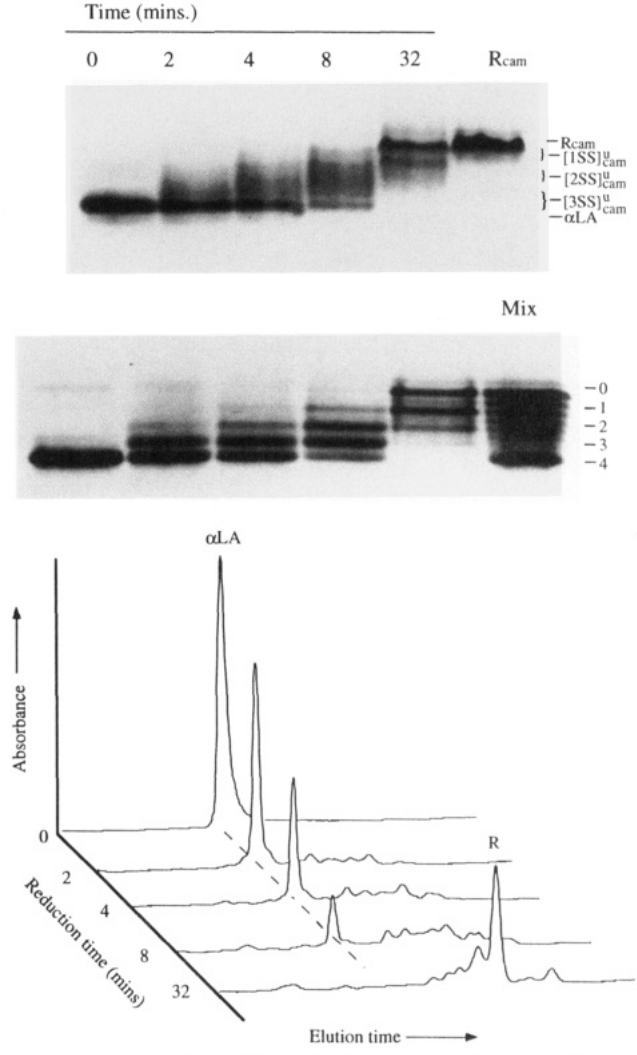


FIGURE 6: Reduction of 15 μM bovine αLA in 8 M urea by 100 μM DTT_{SH}^{SH}. (a, top two panels) Covalent blocking with iodoacetamide. Upper panel: direct analysis of trapped intermediates and separation by nondenaturing gel electrophoresis in the presence of EDTA. Lower panel: disulfide counting after reduction of all protein disulfides and reaction with iodoacetate; electrophoresis was in the presence of 8 M urea. (b, bottom) Acid trapping and separation by reversed-phase HPLC.

The rate of cleavage of the Cys6–120 disulfide bond of αLA was so rapid that it could be measured at pH 8.7 by manual-mixing techniques only at very low concentrations of DTT_{SH}^{SH}. When the pH was lowered to 7.0, however, the rate was sufficiently reduced that the effect of Ca^{2+} could be measured accurately over a range of DTT_{SH}^{SH} concentrations. The rate constant measured at pH 7, 25 s^{-1} M^{-1}, was in very good agreement with the value of 22 s^{-1} M^{-1} obtained by Kuwajima et al. (1990). It was found to be independent of added Ca^{2+} from a stoichiometric concentration (20 μM) up to 10 mM (Ewbank, 1992), consistent with the high affinity of αLA for Ca^{2+} under these conditions, with an estimated value of $K_d^{\alpha LA}$ of 2×10^{-8} M (Hiraoka & Sugai, 1985; Ewbank, 1992).

The overall rates of reduction of 3SS and 2SS were found to depend upon the concentration of added Ca^{2+}; the rate of disappearance of 3SS was increased at very low Ca^{2+} concentrations, but the accumulation of the intermediate 2SS became insignificant, and a number of unresolved species accumulated to very low levels. These results could be explained by a rapid equilibrium between Ca^{2+}-bound and apo forms of the two reduction intermediates, with the latter

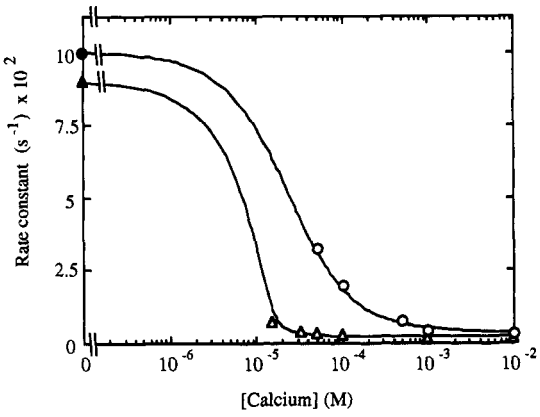
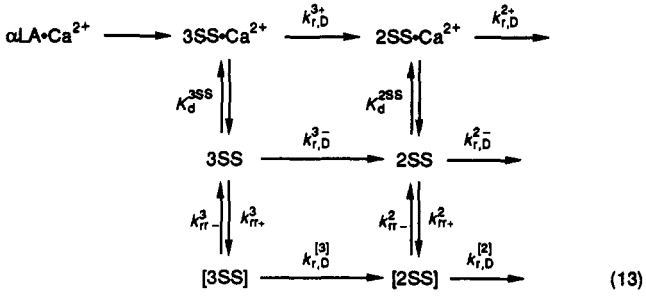


FIGURE 7: Dependence of the observed pseudo-first-order rates of disappearance of 3SS (△) and 2SS (○) on Ca^{2+} concentration at 2.5 mM DTT_{SH}^{SH}. The lines are theoretical curves generated using eq 14, with values of 1.6×10^{-5} M and 2.5×10^{-7} M for K_d^{2SS} and K_d^{3SS} respectively, and the values of second-order rate constants from Table I, taking the value of $k_{r,D}^{[2]}$ as $k_{r,D}^{2-}$, to give the rates of disappearance of 3SS (▲) and 2SS (●) in the absence of Ca^{2+}.

being much more susceptible to disulfide bond reduction and rearrangements. Under these circumstances, the kinetics of reduction will be complex. For example, the observed rate constant for reduction $k_{r,D}^3$ will consist of three components: $k_{r,D}^{3+}$ and $k_{r,D}^{3-}$, corresponding to the rates of reduction of Ca^{2+}-bound and apo-3SS, respectively, plus k_{rr+}^3, corresponding to the rearrangement of apo 3SS; $k_{r,D}^2$ could be similarly split:



(13)

where K_d^{3SS} and K_d^{2SS} are the apparent dissociation constants of Ca^{2+} from 3SS and 2SS, respectively.[2] With excess DTT_{SH}^{SH}, the observed rate of reduction of a disulfide bond of the apo and Ca^{2+}-bound species will be given by the pseudo-first-order rate constants $k_{r,p}^{3-}$ and $k_{r,p}^{3+}$, respectively, each the product of the initial DTT_{SH}^{SH} concentration and the appropriate second-order rate constant. The reverse rearrangement is negligible, so the observed pseudo-first-order rate of reduction of 3SS will depend upon the proportion of molecules with and without Ca^{2+} bound and will be given by

$$k_{obs} = f_{Ca}\, k_{r,p}^{3+} + (1 - f_{Ca})(k_{rr+}^3 + k_{r,p}^{3-}) \qquad (14)$$

where f_{Ca} is the fraction of 3SS in the Ca^{2+}-bound form. The value of f_{Ca} is initially related to the total Ca^{2+} and protein concentrations ([Ca] and [P], respectively) by

$$K_d^{3SS} = ([P] - [P]f_{Ca})\,([Ca] - [P]f_{Ca})([P]f_{Ca})^{-1} \qquad (15)$$

Given values for $k_{r,D}^{3-}$, $k_{r,D}^{3+}$, and k_{rr+}^3 (Tables I and II) and [P], a value for K_d^{3SS} could be derived from the observed dependence of the rate of disappearance of 3SS on the Ca^{2+} concentration at a given excess concentration of DTT_{SH}^{SH}. There was only a small variation in the observed rate of disappearance of 3SS over the experimentally accessible range of Ca^{2+} concentrations, indicating a high affinity of 3SS for Ca^{2+} (Figure 7). No attempt was made to measure the rate at substoichiometric concentrations of Ca^{2+}. Analysis of the kinetics was already complicated by changes in the concentration of free Ca^{2+} due to ligand dissociation from the protein as a result of its reduction. This factor was not included in the simulations but will only have become significant when the total Ca^{2+} concentration was low and may have resulted in an underestimation of K_d^{3SS}. The value of K_d^{3SS} was consequently not well-defined, but the experimental data gave a value of $\approx 2.5 \times 10^{-7}$ M. A 10-fold greater value was measured more directly and substantially more accurately with $3SS_{cam}$ (Ewbank & Creighton, 1993); this value was therefore used for the solution of eq 11.

For 2SS, the variation in the rate of reduction as a function of Ca^{2+} was more marked and extended over a greater range of Ca^{2+} concentrations (Figure 7), so the value of K_d^{2SS} was better defined. By taking the measured rate constant for the reduction of [2SS] as $k_{r,D}^{2-}$, a best fit of the experimental data was obtained with a value for K_d^{2SS} of 1.6×10^{-5} M. This value is considerably greater than that for 3SS and explains why the accumulation of 2SS during reduction became negligible at low concentrations of Ca^{2+}; 2SS no longer bound Ca^{2+} and was reduced much more rapidly than was 3SS, which retained the Ca^{2+}.

Reduction of αLA by GSH. The kinetics of reduction of protein disulfides by monothiols like GSH give complementary information to that obtained with an intramolecular reagent such as DTT_{SH}^{SH} (see Materials and Methods). With high concentrations of GSH in the presence of 10 mM $CaCl_2$, the same intermediates accumulated as with high concentrations of DTT_{SH}^{SH}. The rates of each step were proportional to the square of the GSH concentration. No mixed-disulfide intermediates, which would have had an increased electrophoretic mobility due to glutathione's negative charge, accumulated to detectable extents. The results were described by the same pathway as with DTT_{SH}^{SH}, but with different rate constants:[2]

$$\alpha\text{LA}\cdot Ca^{2+} \underset{k_{f,G}^4}{\overset{k_{r,G}^4}{\rightleftarrows}} 3\text{SS}\cdot Ca^{2+} \underset{k_{f,G}^3}{\overset{k_{r,G}^3}{\rightleftarrows}} 2\text{SS}\cdot Ca^{2+} \underset{k_{f,G}^2}{\overset{k_{r,G}^2}{\rightleftarrows}} \{1\text{SS}\} \underset{k_{f,G}^{[1]}}{\overset{k_{r,G}^{[1]}}{\rightleftarrows}} \text{R} \qquad (16)$$

The results provided third-order rate constants $k_{r,G}^n$ for reduction of the first, second, and third disulfide bonds (Table I). Added GSSG substantially reversed the reduction of the first disulfide bond, giving a value for the second-order rate constant $k_{f,G}^4$ corresponding to the re-formation of the Cys6–120 disulfide bond. This was the only reverse step that was measurably affected by the addition of low concentrations of GSSG, indicating that the other reverse rates were low (Table I). Similar kinetics of reduction were observed with mercaptoethanol as thiol reagent (Ewbank, 1992).

The ratio of the equilibrium constants for the formation and breakage of a given disulfide bond with glutathione and dithiothreitol $(k_{f,G}/k_{r,G})/(k_{f,D}/k_{r,D})$ gives an indirect measure of the equilibrium constant for the reaction between GSSG and DTT_{SH}^{SH} (eq 5). The value of 202 M measured here using the reversible breakage of the Cys6–120 disulfide is only slightly lower than that determined directly at the same pH, 380 M (Chau & Nelson, 1991; Rothwarf & Scheraga, 1992).

The kinetics of reduction by GSH of apo-αLA and of unfolded αLA in 8 M urea were more complex. During reduction of apo-αLA, there was substantial intermolecular disulfide bond formation, reflecting the aggregation of the

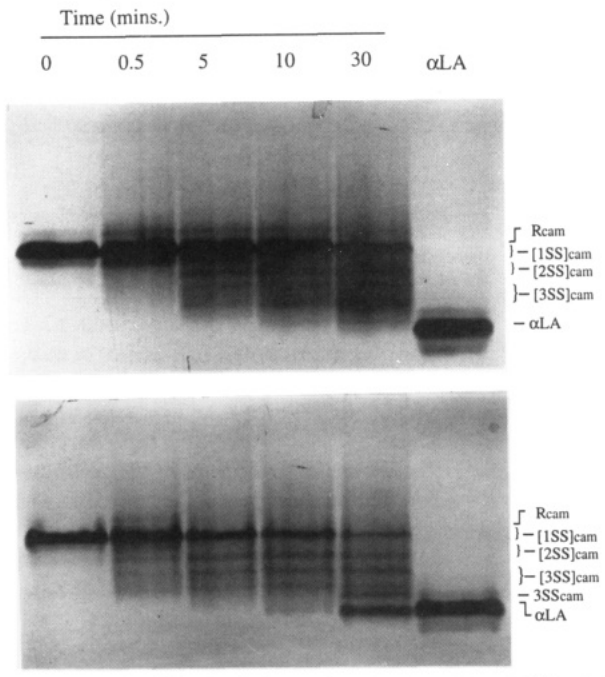


FIGURE 8: Disulfide bond formation by 40 mM DTT_S^S in fully reduced bovine α-lactalbumin, in the presence of 20 μM DTT_{SH}^{SH}, for the indicated times. Disulfide bond formation took place in the presence of 1 mM EDTA (upper panel) or of 10 mM $CaCl_2$ (lower panel). It was followed by covalent blocking with iodoacetamide and nondenaturing gel electrophoresis in the presence of EDTA.

partially reduced protein. In the presence of 8 M urea, many different mixed-disulfide species accumulated, and simulations were not attempted.

Disulfide Bond Formation in Reduced α-Lactalbumin. The kinetics of disulfide bond formation with DTT_S^S starting from fully reduced α-lactalbumin were followed by the same methods (Figure 8). The most striking result was that in the presence of Ca^{2+} very different intermediates accumulated during disulfide bond formation than during disulfide bond reduction (compare Figures 2a and 8). Very many poorly resolved intermediates, similar to those observed in the reduction of apo-αLA, were populated during disulfide formation, both in the presence and in the absence of Ca^{2+}. To make kinetic analysis possible, the disulfide-counting method was used. This demonstrated that there were multiple one-, two-, and three-disulfide intermediates both in the presence and in the absence of Ca^{2+} and allowed a tentative assignment of the number of disulfide bonds present in the trapped intermediates (Figure 8). As expected, disulfide bond formation was accompanied by an increase in electrophoretic mobility of the iodoacetamide-trapped intermediates, reflecting a decreased hydrodynamic volume. The electrophoretic patterns of the trapped intermediates generated during the early stages of disulfide bond formation in the presence and absence of Ca^{2+} were similar (Figure 8). As expected, these intermediates did not bind Ca^{2+} even with low affinity, for their electrophoretic mobilities were unchanged during electrophoresis in the presence of 5 mM Ca^{2+} (Ewbank, 1992).

In the absence of Ca^{2+}, disulfide bond formation did not progress beyond the three-disulfide stage. αLA was regenerated from R only in the presence of Ca^{2+}, confirming the observations of Rao and Brew (1989). The only other prominent species observed during re-formation of the disulfide bonds in the presence of Ca^{2+} accumulated at late stages and appeared from the electrophoretic mobility of its trapped form in the presence and absence of Ca^{2+} to be 3SS.

The kinetics of disulfide bond formation with DTT_S^S in $R^{6/120cam}$ and $R_{28/111cam}^{6/120cam}$ were also measured, to determine whether the thiol groups that were blocked in these species played a crucial role during the re-formation of the native disulfides. During disulfide bond formation in $R^{6/120cam}$ and $R_{28/111cam}^{6/120cam}$, no unique intermediates were observed either in the presence or in the absence of Ca^{2+}, and the small differences in the respective rates are thought to arise primarily from residual low levels of oxidation caused by Ca^{2+}, which could not be accurately quantified. For $R^{6/120cam}$, both in the presence and in the absence of Ca^{2+}, disulfide formation progressed through mixtures of one- and two-disulfide intermediates that were similar, as judged by their electrophoretic patterns, to those obtained when starting from R. Species containing three disulfides accumulated, both in the presence and in the absence of Ca^{2+}, but in neither case was $3SS_{cam}$ generated to a detectable level. The three-disulfide species formed lacked free thiol groups and were therefore unable to undergo disulfide bond rearrangements to $3SS_{cam}$. Analogously, although two-disulfide species were obtained upon disulfide bond reformation starting from $R_{28/111cam}^{6/120cam}$, both in the presence and in the absence of Ca^{2+}, in neither case was $2SS_{cam}$ regenerated (Ewbank, 1992). These results indicate that normally 3SS and 2SS are not generated during refolding by forming directly the native disulfide bonds but arise primarily by intramolecular disulfide rearrangements.

The kinetics of disulfide bond formation in R in the presence of Ca^{2+} were consistent with the following simplest possible scheme, with Ca^{2+} binding to 3SS being fast (Hiraoka & Sugai, 1985):[2]

$$\mathrm{R} \underset{k_{r,D}^{[1]}}{\overset{k_{f,D}^{[1]}}{\rightleftharpoons}} [1SS] \underset{k_{r,D}^{[2]}}{\overset{k_{f,D}^{[2]}}{\rightleftharpoons}} [2SS] \underset{k_{r,D}^{[3]}}{\overset{k_{f,D}^{[3]}}{\rightleftharpoons}} [3SS] \underset{k_{rr+}^{3}}{\overset{k_{rr-}^{3}}{\rightleftharpoons}}$$

$$3SS \underset{}{\overset{K_d^{3SS}}{\rightleftharpoons}} 3SS{\cdot}Ca^{2+} \underset{k_{r,D}^{4}}{\overset{k_{f,D}^{4}}{\rightleftharpoons}} \alpha LA{\cdot}Ca^{2+} \qquad (17)$$

The rate constants for formation of the first, second, and third disulfide bonds were well-defined by the data (Table III), while the values of $k_{r,D}^4$, $k_{f,D}^4$, $k_{r,D}^{[3]}$, $k_{r,D}^{[2]}$, and $k_{r,D}^{[1]}$ were those given by the kinetics of disulfide bond reduction of Ca^{2+}-bound and apo-αLA (Table I) and those for k_{rr+}^3 and k_{rr-}^3 were determined directly (Figure 5, Table II). This pathway is essentially the reverse of that for disulfide reduction of Ca^{2+}-bound αLA (eqs 6 and 13), except for the omission of intermediate 2SS. It could be included in this scheme, being formed by rearrangement from [2SS], but this made little difference to the simulations, for 2SS would not be predicted to accumulate to substantial levels and it does not form disulfides rapidly. The crucial factor for regenerating αLA was the appearance of 3SS by disulfide rearrangements, its tight binding of Ca^{2+} and consequent stabilization, and its rapid formation of the Cys6–120 disulfide bond.

The rates of disulfide bond formation were also determined for R, $R^{6/120cam}$, and $R_{28/111cam}^{6/120cam}$ fully unfolded in 8 M urea (Table III). The observed rates were unaffected by the addition of Ca^{2+} to 10 mM (Ewbank, 1992), indicating that no substantial fraction of the species generated bound Ca^{2+}. The spectrum of intermediates generated in the presence of 8 M urea for each species was distinct from that obtained in the absence of urea. In general, the iodoacetamide-trapped intermediates generated in urea had greater hydrodynamic volumes, as judged by their lower electrophoretic mobilities.

Table III: Rate Constants for Disulfide Bond Formation in Reduced Forms of Bovine α-Lactalbumin Using DTT_S^S [a]

	R			$R^{6/120cam}$			$R_{28/111cam}^{6/120cam}$		
disulfide formed	10 mM Ca^{2+}	1 mM EDTA	8 M urea	10 mM Ca^{2+}	1 mM EDTA	8 M urea	10 mM Ca^{2+}	1 mM EDTA	8 M urea
first	0.05	0.05	0.01	0.03	0.02	0.005	0.02	0.018	0.005
second	0.03	0.03	0.01	0.02	0.008	≤0.002	0.01	0.006	<0.002
third	0.02	0.02	<0.005	<0.005	<0.001				

[a] 0.1 M Tris (pH 8.7), 0.2 M KCl, 25 °C. All rate constants are second order (s^{-1} M^{-1}) and corrected where possible for residual nonspecific oxidation. The values were obtained by simulating the experimental data, using the appropriate rate constants for disulfide bond reduction and for forming αLA from 3SS (Table I) and the rates of rearrangement to 3SS (Table II).

The extent to which disulfide bond formation progressed toward completion was changed by urea. No three-disulfide intermediates were observed in 8 M urea starting from R, while $R^{6/120cam}$ and $R_{28/111cam}^{6/120cam}$ formed second disulfides only slowly.

DISCUSSION

Pathways of Disulfide Bond Reduction and Formation. The pathway of disulfide bond reduction and re-formation for α-lactalbumin has been examined directly, using methods to trap and identify the kinetic intermediates. The results obtained are wholly consistent with those obtained previously using indirect methods (Iyer & Klee, 1973; Segawa et al., 1981; Rao & Brew, 1989; Kuwajima et al., 1990) and considerably extend those studies, since the intermediates were detected directly and the rates of rearrangement and of reduction and re-formation of individual disulfide bonds were measured in specific intermediates. As a result, a variety of intermediates, including a newly-identified specific two-disulfide intermediate, 2SS, have been detected and characterized. As in any complex kinetic situation, only the most prominent steps and most stable intermediates can be observed directly. Unstable intermediates can be vital for a particular step, but not accumulate to detectable levels.

Kinetic studies of disulfide bond reduction and re-formation were carried out under standard conditions of pH, ionic strength, and temperature used with the other proteins studied in this way: BPTI (Creighton & Goldenberg, 1984; Darby & Creighton, 1993), RNase A (Creighton, 1977b; Wearne & Creighton, 1988), and RNase T_1 (Pace & Creighton, 1986), although there were slight differences in concentrations of salts and buffers in some instances. Consequently, the rates of disulfide bond formation, breakage, and rearrangement in the various proteins can be compared quantitatively.

α-Lactalbumin Unfolded in 8 M Urea. αLA appears to be fully unfolded in 8 M urea and did not bind Ca^{2+} detectably, even with 10 mM Ca^{2+}. Its disulfides would be expected to be reduced randomly under these conditions, and the ratios of the observed average rate constants $k_{r,D}^4:k_{r,D}^{[3]u}:k_{r,D}^{[2]u}:k_{r,D}^{[1]u}$ were found to be very close to the expected values of 4:3:2:1, reflecting the number of disulfide bonds (Table I). Thus, the rate of reduction by DTT_{SH}^{SH} of the average disulfide bond in αLA unfolded in 8 M urea at pH 8.7 was 11 s^{-1} M^{-1}. This value is in good agreement with the value of 5.5 s^{-1} M^{-1} obtained by Kuwajima et al. (1990) at the slightly lower pH of 8.5, where thiol reactivity would be expected to be approximately 1.6-fold lower, and with the value of k_{ex} in the absence of urea. Urea affects the rate of thiol–disulfide interchange with different model compounds to varying small degrees (Creighton, 1977c; Kuwajima et al., 1990). It will be assumed for the following discussion that 8 M urea altered only the conformation of the protein.

The first disulfide bonds were formed in fully reduced α-lactalbumin in 8 M urea with intramolecular rates (Table III) and stabilities similar to those observed in other comparable reduced proteins (Creighton, 1988) and about a factor of 5 slower than in the absence of urea. If R in 8 M urea were a totally random polypeptide chain, the rates of disulfide bond formation between cysteine residues would be expected to be approximately proportional to $n^{-3/2}$, where n is the number of residues between the two cysteine residues in the polypeptide chain. On this basis, and excluding the contribution of the formation of a disulfide between Cys73 and Cys77, which are unfavorably spaced to form a disulfide easily (Zhang & Snyder, 1989), blocking the Cys6 and Cys120 thiols in $R^{6/120cam}$ would be expected to lower the observed rate of disulfide bond formation by a factor of ≈1.6, whereas also blocking Cys28 and Cys111 in $R_{28/111cam}^{6/120cam}$ would be expected to reduce the rate by a factor of ≈2.4; the measured rates were reduced by a factor of ≈2 in both cases (Table III).

The rates of forming further disulfide bonds become progressively sluggish, undoubtedly due to the unfavorable conformational constraint on the fully unfolded state of increasing numbers of intramolecular cross-links. To make three small disulfide loops would require the participation of Cys120, which might explain why no three-disulfide species were obtained from $R^{6/120cam}$. Even with all eight cysteine residues, formation of a third disulfide was very slow. This is analogous to the situation in reduced RNase A under nondenaturing conditions, where disulfide bond formation is initially random but becomes kinetically and energetically unfavorable after the two-disulfide stage (Creighton, 1977b; Wearne & Creighton, 1988).

Disulfide Bond Reduction of Ca^{2+}-Bound α-Lactalbumin. Starting with Ca^{2+}-bound αLA in the native conformation, the first disulfide bond to be cleaved was that between Cys6 and Cys120, which is the most accessible of these disulfides (Figure 1) and also is hyperreactive. Its rate of reduction by DTT_{SH}^{SH} was more than 70-fold greater than for a normal disulfide bond. This was also observed in other studies (Segawa et al., 1981; Kuwajima et al., 1990) and interpreted to be a consequence of strain in the disulfide bond, as had been observed in model disulfide compounds (Creighton, 1975); a 70-fold increase in rate corresponds to a strain energy of 10 kJ/mol at 25 °C.

The intramolecular rate of re-formation of the Cys6–120 disulfide was found here also to be very rapid. The rate constant measured with DTT_S^S, 5.3 s^{-1} M^{-1}, is similar to that for formation of the relatively unstrained Cys14–38 disulfide during the refolding of BPTI, 5.7 s^{-1} M^{-1} (Creighton & Goldenberg, 1984), when the Cys14 and Cys38 thiol groups are held in proximity on the surface of the protein. The kinetics with both DTT_S^S and GSH gave large values of k_{intra} for re-forming the Cys6–120 disulfide bond in 3SS that were in satisfactory agreement (Table I). The kinetic results are therefore consistent with the Cys6 and 120 thiol groups in 3SS being held in proximity by the native conformation of the protein and consequently re-forming the disulfide bond rapidly, in agreement with structural studies (Kuwajima et al., 1990;

Ewbank & Creighton, 1993). Relatively little if any strain in the Cys6–120 disulfide is present in the transition state for making or breaking it (Kuwajima et al., 1990).

The Cys6–120 disulfide is reduced anomalously rapidly in all other α-lactalbumins that have been studied, those from the milk of baboon (Smith et al., 1987), human (Ewbank & Creighton, 1991), and guinea pig (Ewbank, 1992). The corresponding disulfide is reduced most readily in homologous hen lysozyme, to give a corresponding three-disulfide species (Radford et al., 1991), but it does not appear to be hyperreactive.

It was inferred indirectly by Kuwajima et al. (1990) that reduction at high concentrations of DTT_{SH}^{SH} of folded 3SS involved direct cleavage of one of the remaining native disulfide bonds. This inference has been confirmed here: the Cys28–111 disulfide bond was specifically reduced at high concentrations of Ca^{2+} and thiol reagent, to give 2SS. The Cys28–111 disulfide is only very slightly exposed in the native structure (Figure 1), and it was reduced at only 0.06 the rate expected for an exposed disulfide bond. The intermediate 2SS retained α-lactalbumin's Ca^{2+} binding ability, implying that it retained the Cys61–77 and Cys73–91 disulfide bonds. These two remaining disulfides flank the Ca^{2+} binding site in the most rigid part of the αLA molecule (Acharya et al., 1989, 1990, 1991) and are fully buried, which should make them the least susceptible to reduction. They did not appear to be reduced in 3SS at substantial rates, for the kinetics of reduction of αLA at high concentrations of Ca^{2+} were consistent with all the molecules being reduced through 2SS.

Breakage of the Cys28–111 disulfide greatly facilitated cleavage of the remaining disulfide bonds, since the observed rate of reduction of Ca^{2+}-bound 2SS by DTT_{SH}^{SH} or GSH was 2-fold greater than the rate at which it was generated by cleavage of Cys28–111 (Table I). Considering that the other two disulfides were not reduced directly in 3SS at substantial rates, their rate of reduction must have been increased in 2SS by a factor of at least 40-fold. The measured rate was only 0.1 that measured for ordinary accessible disulfide bonds, however, so the two remaining disulfides were probably still relatively inaccessible in 2SS. Reduction of one of the two remaining disulfides caused the final disulfide to be reduced very rapidly, at least as rapidly as when the protein was fully unfolded in 8 M urea. Consequently, it was not possible to determine whether either the Cys73–91 or Cys61–77 disulfide was preferentially reduced in Ca^{2+}-bound 2SS or to detect or characterize the one-disulfide intermediate(s).

Partial Unfolding of the Native Conformation of Ca^{2+}-Bound α-Lactalbumin. Ca^{2+}-bound 3SS maintained the native-like conformation, but it did tend to rearrange the Cys28–111 disulfide bond transiently, with a half-time of about 12 min, to others involving Cys6 and Cys120. This rearrangement was reversed an order of magnitude more rapidly, so the native-like species with the Cys28–111 disulfide bond was at least 6 kJ/mol more stable than any of the other isomers. Analogous disulfide rearrangements in the folded state have not been observed with BPTI, RNase A, RNase T_1, or other proteins. Such a reversible disulfide rearrangement in the folded state indicates that the native conformation in that part of the 3SS molecule containing these four cysteine residues (Figure 1) is not very stable.

This observation is confirmed by the properties of intermediate 2SS. While part of the 2SS molecule appeared to retain its native-like conformation and two disulfides when Ca^{2+} was bound, the remainder of the molecule appeared to be disordered. In particular, 2SS formed a variety of disulfide bonds between Cys6, Cys28, Cys111, and Cys120 at rates and with stabilities like those observed in the fully unfolded protein (eq 7, Table I). No particular disulfide was favored, not even the native Cys6–120 or Cys28–111. The crystal structure of αLA (Figure 1) indicates that formation of a nonnative disulfide between any pair of these four cysteine residues must be accompanied by considerable structural perturbation of the C- and N-terminal regions of the polypeptide chain. Structural characterization of Ca^{2+}-bound $2SS_{cam}$ confirmed that it has only part of the molecule in a native-like conformation and the remainder in a collapsed unstructured conformation (Ewbank & Creighton, 1993).

Such a partially-folded structure may be similar in some respects to that observed in the BPTI intermediate with one disulfide bond linking Cys30 and Cys51 (van Mierlo et al., 1992; Ewbank & Creighton, 1993). In both cases, nonnative disulfides are formed readily between cysteine residues in the unfolded portion of the molecule and subsequently rearrange intramolecularly to a species with the native-like conformation. The BPTI intermediate accumulates to substantial levels only in the process of disulfide formation and refolding, whereas 2SS was apparent only during disulfide reduction of αLA. This difference is due to the difference in energetics of the two processes (see below).

Disulfide Rearrangements and Dissociation of Ca^{2+}. α-Lactalbumin's native conformation made the Cys6–120 disulfide hyperreactive but protected the other native disulfides from further reduction. The latter disulfides were most readily reduced after disulfide bond rearrangements. For example, rearrangement of the Cys28–111 disulfide bond with Cys6 and Cys120 occurred spontaneously, but reversibly, in Ca^{2+}-bound 3SS, when the resulting nonnative disulfides were reduced much more rapidly than was Cys28–111 (eq 7); this became a significant pathway of reduction at low DTT_{SH}^{SH} concentrations.

The disulfides of 3SS and 2SS rearranged and were reduced most rapidly after the bound Ca^{2+} ion dissociated from the protein (Table I). Successive reduction of α-lactalbumin's native disulfide bonds progressively decreases the affinity of the protein for Ca^{2+}, which reflects the decreased stability of the native conformation and its consequent decreased population in the apo form of the protein (Ewbank & Creighton, 1993). Whether during reduction of Ca^{2+}-bound αLA the Ca^{2+} dissociated after cleavage of the first, second, third, or fourth disulfide bond depended upon the free Ca^{2+} concentration. Dissociation of Ca^{2+} was probably why the final disulfide was reduced so rapidly even at high concentrations of Ca^{2+}. The apparent Ca^{2+} affinity was decreased ≈60-fold by the reduction of the Cys28–111 disulfide bond in 3SS to generate 2SS (Figure 7). Reduction of a further disulfide would be expected to lower the Ca^{2+} affinity sufficiently that a significant fraction of molecules with one disulfide bond would not have it bound, even in 10 mM Ca^{2+}. The fully reduced protein R had negligible tendency to adopt the native conformation and did not detectably bind Ca^{2+} even at a free Ca^{2+} concentration of 10 mM.

Disulfide Bond Reduction in Apo-αLA. Apo-αLA tends to adopt the fully native conformation, when the Cys6–120 disulfide is reduced at the same rate as in the Ca^{2+}-bound protein (Kuwajima et al., 1990). Under the conditions used here, only ≈80% of apo-αLA molecule will be in the native conformation (Ewbank & Creighton, 1993), and the value of $k_{r,D}^4$ was decreased accordingly (Table I). Reduction of the hyperreactive Cys6–120 disulfide through the native conformation dominated the kinetics, so it was not possible to measure

the rate of reduction of the disulfides of αLA in the ≈20% of molecules that were in the molten globule conformation.

Apo-3SS is largely in a molten globule conformation (Ewbank & Creighton, 1993), so its observed rates of disulfide bond reduction and formation are of particular interest. The rate constant for the reduction by DTT_{SH}^{SH} of apo-$3SS_{cam}$ (15 s^{-1} M^{-1}), which will be the sum for each of its three disulfide bonds, could be determined accurately and was consistent with the rate constant determined directly for apo-3SS (18 s^{-1} M^{-1}). If all three disulfides were reduced at similar rates, the average rate of reduction would be approximately 5 s^{-1} M^{-1}, consistent with them being about half-exposed. This contrasts with Ca^{2+}-bound 3SS, for which the most exposed disulfide was approximately 10 times more protected.

The value of 0.03 s^{-1} M^{-1} estimated for the rate of re-formation of the Cys6–120 disulfide with DTT_S^S in apo-3SS (eq 11) is almost 200-fold lower than when the protein is in the native conformation with Ca^{2+}-bound (Table I) and is comparable to the rates of forming disulfide bonds in the fully reduced protein (Table III). The molten globule conformation of apo-3SS does not keep Cys6 and Cys120 in proximity, in the way that the native conformation does.

The most remarkable aspect of the molten globule state of 3SS is the rapid rate at which it spontaneously rearranges its three disulfide bonds to a number of alternative pairings (Table II). The rate of rearrangement was very close to that observed for the fully unfolded protein in 8 M urea, as has been demonstrated previously for the human apo-3SS, in which the molten globule conformation is even more predominant (Ewbank & Creighton, 1991). Rearrangement in the absence of urea cannot be attributed solely to rearrangement through the fully unfolded state, with which the molten globule state is in equilibrium, as the observed rate was too rapid. Also, the rearrangement products were distinct in the two cases, and those generated in the absence of denaturant tended to maintain the molten globule conformation much more than those from 8 M urea (Ewbank & Creighton, 1991, 1993). The same phenomenon of rapid thiol–disulfide interchange was observed for apo-2SS, which also exists as an equilibrium mixture of molecules in the molten globule and fully unfolded states (Ewbank & Creighton, 1993). Blocking the Cys6 and Cys120 thiols halved the rate of rearrangement of 2SS (Table II), and a large number of products that did not retain α-lactalbumin's Ca^{2+}-binding site were generated by the rearrangements of 3SS and 2SS (Ewbank & Creighton, 1993). Therefore, the disulfide rearrangements appeared to involve all the cysteine residues.

Pathway of Disulfide Bond Formation and Refolding in Fully Reduced α-Lactalbumin. The fully reduced species R, $R^{6/120cam}$, and $R_{28/111cam}^{6/120cam}$ have similar conformational properties that can be interpreted as an equilibrium between ≈70% of molecules in the unfolded state and ≈30% in the molten globule state (Ewbank & Creighton, 1993). The rate of formation of a first disulfide was increased ≈5-fold in each case relative to that in 8 M urea; assuming this to be due to the 30% molten globule state, the increase would be expected to be about 15-fold with a fully populated molten globule. This increase in rate is probably a consequence of the compact molten globule conformation keeping the cysteine residues in greater proximity than in the fully unfolded state.

Second disulfides were formed at rates slightly lower than the first, consistent with the number of cysteine thiols being diminished and the initial disulfide restricting the conformational flexibility of the protein. While formation of third disulfide bonds was negligible in the presence of 8 M urea, third disulfides were generated from R and $R^{6/120cam}$ at significant rates in its absence, presumably due to the presence of the molten globule conformation. The molten globule conformation appears to keep the cysteine residues in suitable proximity for forming disulfide bonds, even with various combinations of two disulfide bonds already present.

Only at the three-disulfide stage of refolding did Ca^{2+} have a significant effect. None of the initial species generated during disulfide bond formation (R, [1SS], [2SS], [3SS]) appeared to bind Ca^{2+}, and neither their rates of formation nor their equilibrium populations were altered by the presence of even 10 mM free Ca^{2+}. The native-like conformation of the Ca^{2+}-binding portion of the polypeptide chain, which comprises only residues 79–88, must not have been populated to an extent greater than 10^{-6} in these species. The effects of Ca^{2+} on refolding were shown to be due to the presence of significant, but low, quantities of the relatively unstable native-like species 3SS, which binds Ca^{2+} tightly and consequently becomes relatively stable. Furthermore, the native conformation of the Ca^{2+}-bound 3SS causes formation of the final disulfide to be very fast, so refolded αLA was regenerated readily in the presence of Ca^{2+}. Intermediate 2SS also binds Ca^{2+}, but it does not cause subsequent disulfide bond formation to be rapid, and kinetic simulations indicated that its stabilization by Ca^{2+} would not be expected to alter substantially the kinetics of disulfide bond formation. It also was not predicted to accumulate to substantial levels.

These observations explain why Rao and Brew (1989) found addition of Ca^{2+} to be necessary to regenerate native αLA from the reduced protein. Indeed the addition of Ca^{2+} can also produce dissociation of intermolecularly disulfide-bonded oligomers and the recovery of the native protein (Ewbank, 1992). α-Lactalbumin is not unique in its requirement for Ca^{2+} for efficient reformation of its native disulfide bonds; the presence of ligand is also essential for the renaturation of another reduced Ca^{2+}-binding protein, Taka-amylase A (Takagi & Isemura, 1965).

The precise pathway followed during unfolding and refolding of α-lactalbumin depended upon the Ca^{2+} concentration and on the relative rates of disulfide bond formation, breakage, and rearrangement. Disulfide bond formation and breakage are intermolecular processes, and their rates will depend upon the concentrations of disulfide and thiol reagents, while disulfide rearrangements are intramolecular and thus occur at rates independent of the reagents. Detailed dissection of a disulfide folding pathway requires determination of the kinetic effects of removing individual cysteine thiols and of the intramolecular rates constants for all the possible steps. Some such studies were made here by blocking irreversibly the thiols of Cys6, Cys28, Cys111, and Cys120.

The pathways of disulfide bond breakage and re-formation appeared to be the reverse of each other, as would be expected. The rate-limiting intramolecular steps in both directions were the disulfide rearrangements of species with two and three disulfide bonds, between those with the native disulfide bonds and at least part of the native conformation, including a Ca^{2+} binding site, and those with nonnative disulfides and conformations and no Ca^{2+} binding site. This is illustrated in the overall pathway of Figure 9, which compares the free energies of the various species and the transition states of the intramolecular steps interconverting them under one set of conditions. The highest free energy barriers are the steps involving disulfide bond rearrangements. Both paths involving rearrangements to and from 3SS and 2SS have very similar free energies under these particular conditions, so neither path

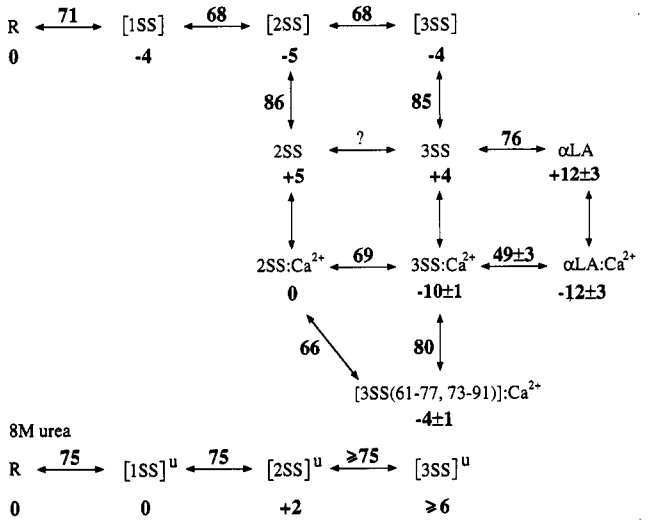


FIGURE 9: Free energy diagram of the intermediates and intramolecular transition states in the pathway of disulfide unfolding and refolding of bovine α-lactalbumin. All free energies are given in kilojoules per mole relative to the fully reduced protein, R. Transition-state free energies are given next to the arrow indicating the transition; those of intermediates are given below their designation. The rate of the intramolecular step in forming a disulfide bond is given by the corresponding value of k_{intra}. The free energy barriers corresponding to these rate constants and to those for intramolecular disulfide rearrangements were calculated using classical transition state theory: $\Delta G^{\ddagger} = -RT \ln (k_{intra}\, h/k_B\, T)$ where R is the gas constant, T is the temperature, h is Plank's constant, and k_B is Boltzmann's constant. The values of k_{intra} used were from Table I for the interconversion of 2SS·Ca^{2+} and 3SS·Ca^{2+}, from Table II for the interconversions of 2SS and 3SS to [2SS] and [3SS], respectively, from Table III for the interconversions of R, [1SS], [2SS], and [3SS] in the absence and presence of urea, and from eq 7 for those involving [3SS(61–77,73–91)]; no value for the interconversion of 2SS and 3SS is known. Many of the observed rate constants are the sum of numerous individual rate constants, and intermediates such as [nSS] are mixtures of many species; in these cases, the free energies of the individual transition states and intermediates will be greater than those indicated here. The stabilities of the disulfide bonds were set by assigning a ratio of DTT_S^S to DTT_{SH}^{SH} of 10^3 to 1; changing the intrinsic disulfide stability uniformly alters the free energy of each species by the same amount per disulfide bond it contains. The free energy of each Ca^{2+}-bound form relative to the apo form is defined by the Ca^{2+} concentration and the affinity of the apo form. The dissociation constants of 2SS, 3SS, and αLA were taken as 1.6×10^{-5} M, 2.5×10^{-7} M, and 2×10^{-8} M, respectively. The free Ca^{2+} concentration was set at 10^{-4} M; changing the Ca^{2+} concentration uniformly alters the free energies of the Ca^{2+}-bound species relative to the apo forms. No free energy barriers to binding Ca^{2+} are given, as this is very fast relative to the other steps. The other conditions were those used in the experiments here: 0.1 M Tris (pH 8.7) and 0.2 M KCl at 25 °C. The bottom scheme relates to disulfide bond formation in the presence of 8 M urea. The indicated uncertainties in some of the values arise because of slight inconsistencies obtained using different transitions.

is greatly preferred.

The intermediate 3SS appeared to be most important for regaining the four native disulfide bonds of αLA, for the kinetics indicated that virtually all molecules refolded through this intermediate (eq 17). This is undoubtedly because 3SS was the most stable three-disulfide intermediate in the presence of Ca^{2+} and because the Cys6–120 disulfide bond was reformed in it very rapidly.

The disulfide folding pathway of α-lactalbumin resembles those of other proteins, in that the many conformations of the unfolded, reduced protein generate many different disulfide pairings. Nonrandom disulfides predominate during folding only when they stabilize sufficiently a folded conformation that also stabilizes them. The rate-limiting intramolecular steps of disulfide re-formation separate those species with the stable native-like conformation from all other species. The known disulfide-coupled folding pathways differ primarily in the energetics of the various intermediates and transition states. The disulfide folding pathway of α-lactalbumin differs particularly in the rapid rates of disulfide reduction and rearrangement in the folded state, the occurrence of the molten globule conformation, the role of Ca^{2+} binding, and the absence of a clearly-defined rate-limiting step (Figure 9). The last factor is a consequence of the relatively low cooperativity of the folded state of α-lactalbumin.

Disulfide Bonds, Ca^{2+} Binding, and Cooperativity of Folding. There was a marked differences between Ca^{2+}-bound αLA and apo-αLA in the cooperativity of reduction of their disulfides and in the number of isomers that populated the respective pathways.

Reduction of Ca^{2+}-bound αLA involved only a few intermediates and was somewhat cooperative, in that reduction of one disulfide bond increased the rate of reduction of further disulfides. The hyperreactivity of the Cys6–120 disulfide bond made it impossible to determine the effect its reduction had on the rate of reduction of the remaining disulfide bonds, but reduction of Cys28–111 in Ca^{2+}-bound 3SS facilitated the breakage of one or the other of the remaining disulfides by at least 40-fold. And that promoted the final cleavage by a factor of more than 5-fold. So with respect to cleavage of the same disulfide in 3SS, the final reduction step was at least 200-fold enhanced. Some proteins show considerably greater cooperativity during reduction. In RNase A, for example, with four buried disulfide bonds, reduction is an all-or-none process, such that no intermediates accumulate between the native and fully reduced forms (Wearne & Creighton, 1988). In part, this is due to the slowness of the reduction of the first disulfide in RNase A, which occurs at a rate 10^4-fold slower than expected for an accessible disulfide bond.

The reduction of Cys6–120 in αLA has its counterparts in those proteins with exposed disulfides, reduction of which results in little change in the conformation but a decrease in stability [e.g., the two-disulfide form of BPTI with the Cys14–38 disulfide cleaved (Creighton, 1975; Stassinopoulou et al., 1984); for further examples, see the references in Kuwajima et al (1990)]. Whereas such folded species lacking one disulfide bond are usually relatively stable, however, that of α-lactalbumin was stable only in the presence of Ca^{2+}; otherwise, it adopted the molten globule state and rearranged its disulfides, which could then be rapidly reduced. The kinetics of reduction of the subsequent disulfides in Ca^{2+}-bound α-lactalbumin were also intrinsically different from those of the other proteins. Thus, a second, largely buried disulfide bond, Cys28–111, could be selectively reduced to give an intermediate that was relatively resistant to reduction. This occurred only at high Ca^{2+} concentrations, however, and resulted from the bound Ca^{2+} stabilizing the native-like conformation in part of the molecule. The single bound Ca^{2+} ion is very important for the stability of the native conformation of α-lactalbumin, and the unusual partly-folded species 2SS was evident only because the contribution of this interaction could be increased in magnitude by high free Ca^{2+} concentrations.

Reduction of αLA was much less cooperative in the absence of Ca^{2+}, and an enormous number of intermediates were populated, perhaps as many as are seen the presence of 8 M urea when all cooperative interactions are abolished. All the disulfides were reduced at comparable rates, except for the hyperreactive Cys6–120. The enhancement of the final step of reduction was at most 2-fold relative to cleavage of the

same disulfide in apo-3SS, and the overall rate of reduction was close to that of the fully unfolded protein in 8 M urea. This was a consequence of the relatively rapid rate of reduction of the first disulfide and of the native conformation becoming unstable after reduction of just one disulfide bond. Despite the fact that apo-3SS and its rearranged products are compact and contain secondary structure, i.e., tend to adopt the molten globule conformation, they behave with regard to their disulfide chemistry as if they are almost fully unfolded.

Disulfide bond formation in fully reduced α-lactalbumin exhibited very little cooperativity initially, in that formation of one disulfide bond did not stabilize a conformation that brings two further cysteine residues together so that the next disulfide is made more rapidly. This was the case even in the presence of Ca^{2+}, which is a consequence of the nonspecific conformations, the absence of a Ca^{2+} binding site, and the near-equivalent stability of the many intermediates. Only after three native disulfides had been formed, to give 3SS, and it had been stabilized by Ca^{2+} binding, was a further disulfide formed rapidly. Disulfide bond formation is also noncooperative in the early stages of refolding of reduced RNase T_1 (Pace & Creighton, 1986), RNase A (Wearne & Creighton, 1988), α-conotoxin (Zhang & Snyder, 1991), apamin (Chau & Nelson, 1992), and BPTI (Creighton & Goldenberg, 1984). Cooperativity generally occurs only at late stages of disulfide bond formation, when the fully-folded state becomes populated. The partly-folded state of Ca^{2+}-bound 2SS did not favor rapid formation of native disulfide bonds, nor does to a great extent the analogous Cys30–51 one-disulfide intermediate in BPTI refolding (van Mierlo et al., 1992).

Each of the four native disulfide bonds of α-lactalbumin substantially stabilizes its native conformation, although to varying extents throughout the molecule, as indicated by the partly folded state of Ca^{2+}-bound 2SS, and not as much as in other proteins. For example, apo-αLA with four disulfides and the native conformation is 12 kJ/mol *less* stable than R in Figure 9, whereas, with the same intrinsic disulfide stability, the native conformation of BPTI with three disulfide bonds is 22 kJ/mol *more* stable than its reduced state (Creighton & Goldenberg, 1984[3]). Even taking into account the apparent strain of about 10 kJ/mol in the Cys6–120 disulfide bond, the native conformation of αLA is substantially less stable relative to R than is that of BPTI. Thermodynamic linkage implies that the native conformation of α-lactalbumin stabilizes its disulfides correspondingly less than do other proteins, and this was apparent in the relatively rapid rates at which they were reduced and rearranged in α-lactalbumin. The compensating factor in the case of α-lactalbumin is the binding of Ca^{2+}, which further stabilizes the native conformation to an extent determined by the free Ca^{2+} concentration (Figure 9).

Molten Globule State and Intermediates in Protein Folding. The molten globule state substantially increased the initial rate of disulfide bond formation in reduced α-lactalbumin, apparently by making the polypeptide chain compact and bringing cysteine residues distant in the primary structure into closer proximity. It also made the initial intermediates more stable than when the polypeptide chain was unfolded in 8 M urea (Figure 9). But the rates of forming the disulfide bonds were much more comparable to those observed in unfolded polypeptide chains than in folded proteins, indicating that the molten globule does not maintain a fixed conformation with the cysteine residues held in close proximity.

The disulfide bonds preferred by the molten globule state appear to be those that constrain the dimensions of the unfolded polypeptide chain, i.e., are primarily between cysteine residues distant in the primary structure (Ewbank & Creighton, 1991, 1993). The molten globule conformation did not, however, favor the native-like topology or disulfide bonds, nor did it detectably bind Ca^{2+} in any of its forms with or without disulfide bonds.

The native disulfides do not appear to contribute significantly to the stabilization of the molten globule conformation of α-lactalbumin, just as the molten globule conformation does not stabilize the native disulfides to a great extent (Figure 9). Indeed, nonnative disulfides are energetically more favorable in both 2SS and 3SS, but even these do not stabilize the molten globule state to a great extent (Ewbank & Creighton, 1993) and they are reduced at rates like those in the unfolded state. These results further support the picture of α-lactalbumin's molten globule state as a collapsed unfolded form, bereft of cooperative interactions, rather than an expanded form of the folded state. αLA may adopt a relatively specific molten globule conformation when constrained by its four native disulfide bonds (Baum et al., 1989), but in the absence of such restraints, many different partially folded conformations appear to be accessible.

It has been suggested that the equilibrium molten globule state resembles the conformation adopted by a refolding protein (Ikeguchi et al., 1986; Ptitsyn et al., 1990), i.e., the unfolded protein under refolding conditions prior to the rate-limiting step in refolding (Creighton, 1990). The results of detailed structural studies of refolding proteins, for those both with and without disulfide bonds (Serrano et al., 1992; Radford et al., 1992; Jeng et al., 1990; Udgaonkar & Baldwin, 1990), have been interpreted in terms of intermediates possessing relatively specific conformations and a substantial proportion of their native secondary structure, with the remainder of the polypeptide chain disordered. Such a type of conformation was also observed in the partly folded (30–51) intermediate of BPTI (van Mierlo et al., 1992) and is analogous to the structure seen in Ca^{2+}-bound 2SS. In contrast, α-lactalbumin's molten globule appears from the results presented here to be a nonspecific collapsed form of an unfolded protein [see also Ewbank and Creighton (1991, 1993)]. Consequently, α-lactalbumin illustrates both types of conformations (Baldwin, 1991) and may point toward a unified picture of protein folding.

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[3] The ratio of DTT_S^S to DTT_{SH}^{SH} is stated erroneously to be 10^{-3} rather than 10^{+3} in the legend to Figure 7 of Creighton and Goldenberg (1984).

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