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Kinetics of Disulfide Bond Reduction in α-Lactalbumin by Dithiothreitol and Molecular Basis of Superreactivity of the Cys6-Cys120 Disulfide Bond†

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ABSTRACT: Kinetics of disulfide reduction in α-lactalbumin by dithiothreitol are investigated by measuring time-dependent changes in absorption at 310 nm and in CD ellipticity at 270 nm (pH 8.5 or 7.0, and 25 °C). When the disulfide-intact protein is folded, the kinetics are biphasic. The disulfide bond between the half-cystines-6 and -120 is reduced in the fast phase, and the other three disulfide bonds are reduced in the slow phase. The apparent rate constants of the two phases are both proportional to the concentration of dithiothreitol, indicating that both phases are expressed by bimolecular reactions. However, detailed molecular mechanisms that determine the reaction rates are markedly different between the two phases. The slow phase shows a sigmoidal increase in the reaction rate with increasing concentration of a denaturant, urea, and is also accelerated by destabilization of the native state on removal of the bound Ca^{2+} ion in the protein. The disulfide bonds are apparently protected against the reducing agent in the native structure. The fast phase reaction rate is, however, decreased with an increase in the concentration of urea, and the disulfide bond shows extraordinary superreactivity in native conditions. It is 140 times more reactive than normal disulfides in the fully accessible state, and three-disulfide α-lactalbumin produced by the fast phase assumes nativelike structure under a strongly native condition. As ionic strength does not affect the superreactivity of this disulfide bond, electrostatic contributions to the reactivity must be negligible. Inspection of the disulfide bond geometry based on the refined X-ray coordinates of baboon α-lactalbumin [Acharya et al. (1989) *J. Mol. Biol. 208*, 99-127] and comparison of the geometry with those in five other proteins clearly demonstrate that the superreactivity arises from the geometric strain imposed on this disulfide bond by the native structure folding. Relationships of the disulfide strain energy to the protein stability and the disulfide reactivity are discussed.

The disulfide bond is an important factor to stabilize native structures of globular proteins. Experimentally, partial disruption of natural disulfides in a protein often does not radically alter the protein structure, while complete disruption of all the disulfides leads to global unfolding of the protein molecule. The disulfides located on the surface of a protein molecule are often selectively reduced by 2-mercaptoethanol, dithiothreitol (DTT^{SH}_{SH}),[1] or other reducing agents without significant loss of the native structure (DiBella & Liener, 1969; Segawa et al., 1981; Shapira & Arnon, 1969; Sperling et al., 1969; Tamburro et al., 1970; Sondack & Light, 1971; Vincent et al., 1971; Schwarz et al., 1987; Bewley, 1977; Kelley et al., 1987; Pace et al., 1988), but the stability of the folded conformation of the partially disrupted species against thermal or denaturant-induced unfolding is remarkably reduced compared with the disulfide-intact protein. The cleavage of a disulfide cross-link increases the chain entropy of the molecule in the unfolded state, and thereby the stability of the folded state relative to the unfolded one is decreased to such an extent as to be brought about by the entropy increase in the unfolded state (Schellman, 1955; Flory, 1956; Poland & Scheraga, 1965). This aspect of natural disulfides in globular proteins has stimulated recent attempts in protein engineering to enhance protein stability by introducing a new intramolecular disulfide bond (Wells & Powers, 1986; Pantoliano et al., 1987; Villafranca et al., 1987; Matsumura et al., 1989). However, the stability of a protein with a new disulfide bond was not always so enhanced as expected, and, clearly, more studies are required for elucidating the relationship between the disulfide cross-link and the protein stability.

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[1] Abbreviations: 3SS-LA, three-disulfide α-lactalbumin with free cysteinyl residues; CD, circular dichroism; CM-3SS-LA, carboxymethylated three-disulfide α-lactalbumin; DTT^{S}_{S}, oxidized dithiothreitol; DTT^{SH}_{SH}, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; *I*, ionic strength; MOPS, 3-(*N*-morpholino)propanesulfonic acid; TPCK, L-1-(tosylamino)-2-phenylethyl chloromethyl ketone; UV, ultraviolet.

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α-Lactalbumin has four disulfide cross-links, Cys6–Cys120, Cys28–Cys111, Cys61–Cys77, and Cys73–Cys91 (Hill & Brew, 1975). Our previous study (Segawa et al., 1981) and the study by Shechter et al. (1973) have shown that there are fast-reducing and slowly reducing disulfide bonds in α-lactalbumin and that the partially reduced intermediate with one open disulfide bond kinetically accumulates during reduction by dithioerythritol. The fast-reducing bond has been identified as the Cys6–Cys120 disulfide bond although structural factors that lead to the preferential disruption of this bond remain obscured. As the disulfide intermediate can easily be trapped kinetically under mild conditions (see Results), this protein will be a good model for studying the relationship between the disulfide cross-link and the protein stability.

α-Lactalbumin and chicken-type lysozyme are homologous to each other and have the same disulfide bond arrangement (Hill & Brew, 1975). The close similarity in their stereoregular structures has been supported by many physicochemical studies of the two proteins [see Hill and Brew (1975), Kuwajima et al. (1976, 1985), Kuwajima (1989), and Kronman (1989) and other references cited therein]. Their structural similarity previously inferred from model-building (Browne et al., 1969) and energy-refinement studies (Warme et al., 1974) has been confirmed recently by the X-ray crystallographic analysis of baboon α-lactalbumin (Acharya et al., 1989). Nevertheless, the two proteins have been reported to be markedly different in the reactivity of disulfide bonds (Atassi et al., 1970; Iyer & Klee, 1973; Shechter et al., 1973; Segawa et al., 1981). The reduction of disulfides in lysozyme is known to be extremely sluggish in native conditions, while all four disulfide bonds in α-lactalbumin are readily reduced under similar conditions. From comparison of our previous results (Segawa et al., 1981) with the results in Iyer and Klee (1973), the reduction of the fast-reducing disulfide bond in α-lactalbumin in the absence of urea must be more than 200 times faster than the reduction of the corresponding disulfide in lysozyme in 8 M urea. Probably, one interpretation placed to the difference in their reactivities may be given by the difference in their Ca^{2+} binding capacities. α-Lactalbumin is known to bind tightly one Ca^{2+} per molecule with an apparent binding constant of 10^6–10^9 M^{-1} (Hiraoka et al., 1980; Segawa & Sugai, 1983; Mitani et al., 1986; Hamano et al., 1986; Permyakov et al., 1987), while most well-characterized lysozymes do not have such affinity for Ca^{2+}, except recently found cases in equine and pigeon lysozymes (Nitta et al., 1988). As the reactivity of the disulfide bond with a reducing agent has often been studied in the presence of EDTA, the high reactivity of the disulfide in α-lactalbumin might be interpreted in terms of removal of the bound Ca^{2+} by the chelating agent and the resultant destabilization of the protein structure. However, at present, the validity of this interpretation remains to be explored.

In this study, the kinetics of the disulfide reduction in α-lactalbumin by DTT_{SH}^{SH} are investigated under a variety of conditions, i.e., at varying concentrations of DTT_{SH}^{SH}, in the presence and absence of EDTA, at various concentrations of urea, and at various ionic strengths (pH 8.5 or 7.0, and 25 °C). The structures of the three-disulfide species trapped kinetically and its carboxymethylated derivative are investigated by CD measurements. The results show that the Cys6–Cys120 disulfide bond is not essential for maintenance of the folded structure and that the three-disulfide species assumes nativelike structure when the bound Ca^{2+} is present. The difference in reactivity of the disulfide bonds previously observed between α-lactalbumin and lysozyme is only partly interpreted by destabilization of the former by EDTA but shown to be primarily due to an anomalous environment of the Cys6–Cys120 disulfide bond in native α-lactalbumin. This bond is 140 times more reactive than the normal disulfides in the fully unfolded molecule. It is demonstrated that the superreactivity of the Cys6–Cys120 disulfide bond arises from geometric strain imposed on this bond in the native structure. Relationships of the strain energy of the disulfide bond to the protein stability and the disulfide reactivity will be discussed.

Materials and Methods

Materials. Bovine α-lactalbumin was prepared from fresh milk as described previously and contained one bound Ca^{2+} per molecule (Kuwajima et al., 1976; Hiraoka et al., 1980). Its concentration was determined spectrophotometrically using a molar extinction coefficient at 280 nm of 28 500 M^{-1} cm^{-1} (Kuwajima et al., 1985). TPCK-treated trypsin was obtained from Sigma Chemical Co. DTT_{SH}^{SH}, 5,5′-dithiobis(2-nitrobenzoic acid), sodium iodoacetate, and urea were specially prepared reagent grade from Nakarai Chemical Ltd., Kyoto. The stock solution of urea was deionized on a mixed-bed column of ion-exchange resin and used within a day. The concentration of DTT_{SH}^{SH} was determined by Ellman's method using a molar extinction coefficient for 2-nitro-5-thiobenzoate of 1.415×10^4 M^{-1} cm^{-1} at 412 nm (Riddles et al., 1979).

CD Measurements. CD spectra of the protein and its derivatives, partially reduced three-disulfide α-lactalbumin (3SS-LA) and its carboxymethylated form (CM-3SS-LA), were measured in Jasco J20 and J500A spectropolarimeters (Kuwajima et al., 1985).

Kinetic Measurements. The disulfide reduction in α-lactalbumin was initiated by rapid mixing of the two solutions of the protein and DTT_{SH}^{SH}, and the absorption at 310 nm was monitored (Iyer & Klee, 1973; Creighton, 1975; Segawa et al., 1981). Unless otherwise specified, the final solutions contained 10^{-5}–10^{-4} M protein, 0.18 M KCl, and an appropriate amount of buffer salt, MOPS or Tris. DTT_{SH}^{SH} added was always in large excess and at least 10 times as large in concentration as the protein disulfide. The time-dependent absorption changes were measured in a stopped-flow spectrophotometer (a Union Giken Model RA-1100 or a specially constructed apparatus by Unisoku, Inc., Osaka) or in a Union Giken SM-401 spectrophotometer that was equipped with a mixing device of a magnetic stirring mixer, depending on the time interval when the reaction occurred (Segawa et al., 1981; Kuwajima et al., 1985; Harushima & Sugai, 1989). The absorption change due to autoxidation of DTT_{SH}^{SH} was corrected by using the same DTT_{SH}^{SH} solution without the protein as a reference solution. The conformational unfolding of the protein associated with the disulfide reduction was followed by the ellipticity at 270 nm in a Jasco J500A spectropolarimeter with the mixing device mentioned above. Only the reactions that occurred in a time longer than 10 s were investigated by the CD measurement. The reduction of the L-cystine disulfide was initiated by rapid mixing of the L-cystine (0.4 mM) and DTT_{SH}^{SH} (20 mM) solutions with a volume ratio of 1:1 in the stopped-flow apparatus, and the reaction was followed by monitoring the absorption change at 283 nm.

As the disulfide reduction occurred in large excess of DTT_{SH}^{SH}, all the reaction curves in this study were expressed as a sum of pseudo-first-order reactions as

$$y(t) = y(0) + \sum_i \Delta y_i (1 - e^{-k_{app,i}t}) \quad (1)$$

where $y(t)$ represents the absorption or the ellipticity at time t and Δy_i and $k_{app,i}$ are the amplitude and the apparent

first-order rate constant, respectively, of the ith phase. The values of Δy_i and $k_{app,i}$ were obtained by the nonlinear least-squares analysis of the observed reaction curve.

Preparation of CM-3SS-LA. CM-3SS-LA, in which the Cys6–Cys120 disulfide bond was selectively reduced and carboxymethylated, was prepared as follows: 2.5 mL of 2% α-lactalbumin in a buffer solution of 0.1 M Tris-HCl plus 1 mM $CaCl_2$ at pH 7.0 was mixed with the same volume of 6 mM DTT_{SH}^{SH} in the same buffer, and the disulfide reduction was allowed to proceed for 2 min at 25 °C; only the Cys6–Cys120 disulfide bond is cleaved under these conditions (see Results). The free thiol groups in 3SS-LA were carboxymethylated by adding 5 mL of 10 mM sodium iodoacetate in 0.9 M Tris-HCl plus 1 mM $CaCl_2$ (pH 8.0) and by allowing the reaction to proceed for 30 min under dark at 25 °C. Two hundred microliters of acetic acid was added, and the reaction mixture was dialyzed against 0.1 M ammonium acetate (pH 5.0) and then dialyzed against distilled water. A slight amount of precipitate occasionally formed was discarded by centrifugation, and the supernatant was lyophilized. The sample prepared by the procedure gave a single band in polyacrylamide gel electrophoresis and had a mobility larger than intact α-lactalbumin (pH 7.5). The selective reduction and carboxymethylation of the sample obtained were confirmed by amino acid analysis. The intact protein and CM-3SS-LA (1%) were treated with TPCK-trypsin (0.025%) for more than 24 h at pH 7.5 (50 mM Tris-HCl) and 25 °C in the presence of 2 mM EDTA and 2 M urea. The peptide fragments in the digests were separated by reversed-phase HPLC on a Finepak SIL C_{18}-S column (Jasco TRIROTAR VI HPLC system) with a linear gradient from 0.1% trifluoroacetic acid/water to 0.07% trifluoroacetic acid/60% acetonitrile. The amino acid analysis was made by the method of postcolumn derivatization with *o*-phthalaldehyde/NaClO in a Jasco OPA amino acid analyzer system. Two peptide fragments (Cys6–Arg10, Leu115–Leu123) linked by the disulfide Cys6–Cys120 in the intact protein were found to be carboxymethylated in the digest of CM-3SS-LA, and no other (carboxymethyl)cysteines were found.

Calculations of Disulfide Bond Geometry and Torsion Energy. The atomic coordinates of baboon α-lactalbumin and 5 other high-resolution proteins providing the coordinates for 23 intramolecular disulfide bonds (Table III) were obtained from the Brookhaven Protein Data Bank at Osaka University (Bernstein et al., 1977). The bond length of each disulfide and the torsion angles for the five successive covalent bonds of the cystine side chain were calculated, and the torsion energy of the disulfide bond was estimated (see Discussion). The X-ray data stored in a magnetic tape were transformed into the data compatible with MS-DOS, at Hokkaido University Computing Center, and the calculations were made in an NEC 9801 microcomputer (Microsoft FORTRAN77).

Results

Disulfide Reduction in α-Lactalbumin. Figure 1a shows typical reaction curves of the disulfide bond reduction in the Ca^{2+}-bound (holo) form of α-lactalbumin measured by UV absorption at 310 nm (pH 8.5, 10 mM DTT_{SH}^{SH}, and 25 °C). The absorption increase at this wavelength has been used as a measure to follow the kinetics of disulfide reduction in proteins by DTT_{SH}^{SH} and reflects mainly the increase in concentration of DTT_S^S that is generated by the disulfide reduction (Iyer & Klee, 1973; Creighton, 1975), but as shown later, the absorption change due to protein unfolding may not be negligible at 310 nm when the protein unfolds during the reduction. The reaction kinetics are biphasic with two pseudo-first-order reactions. The fast phase of the biphasic kinetics has an apparent rate constant ($k_{app,F}$) of 8 s^{-1} and was observed by the stopped-flow measurement. The slow phase has a rate constant ($k_{app,S}$) of 0.007 s^{-1} and could be measured by manual mixing with a magnetic stirring mixer.

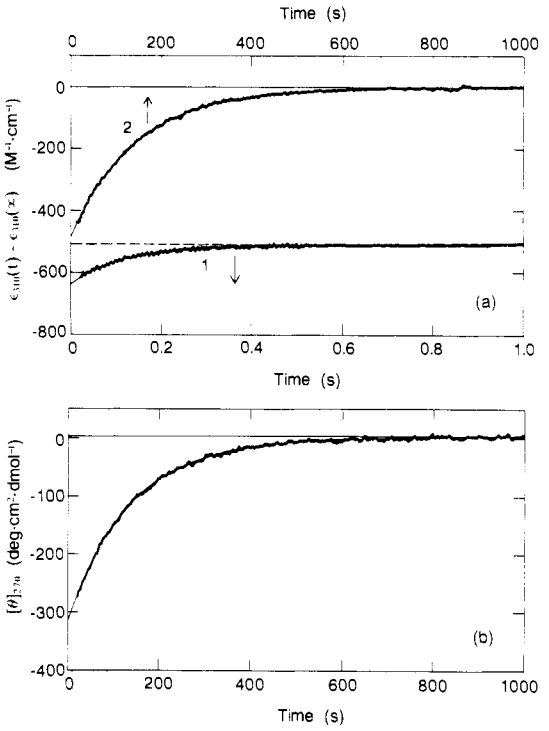


FIGURE 1: Kinetic progress curves of disulfide bond reduction in holo-α-lactalbumin by DTT_{SH}^{SH} at pH 8.5 and 25 °C (0.2 M Tris-HCl plus 0.18 M KCl, I = 0.25 M). (a) Measured by UV absorption at 310 nm; curve 1 represents the fast phase (10.7 mM DTT_{SH}^{SH} and 35 μM protein) and curve 2 the slow phase (9.7 mM DTT_{SH}^{SH} and 37 μM protein). (b) Measured by CD ellipticity at 270 nm; only the slow phase reaction curve is shown (9.8 mM DTT_{SH}^{SH} and 35 μM protein).

The disulfide reduction of the protein was followed by the CD spectra at 270 nm under the same condition as shown above. The time-dependent change in the mean residue ellipticity, $[\theta]_{270}$, reflects the conformational unfolding of the protein during the disulfide reduction. Most of the change in $[\theta]_{270}$ occurs in the slow phase, and the CD kinetics measured by manual mixing are shown in Figure 1b. The apparent rate constant of the kinetics is identical with the $k_{app,S}$ measured by UV absorption. Extrapolation of the curve of Figure 1b to zero time gives a value for $[\theta]_{270}$, −310 deg·cm²/dmol, which is only slightly larger than the value known for native α-lactalbumin (−340 deg·cm²/dmol), suggesting that 3SS-LA has a nativelike folded conformation. The $[\theta]_{270}$ value extrapolated to infinite time is essentially zero, indicating that the protein is unfolded after full reduction of the disulfide bonds.

Kinetic Parameters of Disulfide Reduction. The rate-limiting step of the reaction is essential for elucidating the reaction mechanism. When disulfide interchange between the protein and DTT_{SH}^{SH} is rate-limiting, the observed reaction is a bimolecular process. When the conformational unfolding that allows the protein disulfides to be accessible to the reducing agent is rate-limiting, the observed reaction is a unimolecular process and does not depend on the concentration of DTT_{SH}^{SH}. The apparent rate constants, $k_{app,F}$ and $k_{app,S}$, of the two phases were thus measured at various concentrations of DTT_{SH}^{SH}. The results are shown in Figure 2a. Both apparent rate constants are proportional to DTT_{SH}^{SH} concentration, demonstrating that the two phases observed are bimolecular processes. Because

Table I: Kinetic Parameters of Disulfide Bond Reduction in Holo-α-lactalbumin by DTT^{SH}_{SH} Measured by Absorption at 310 nm and CD Ellipticity at 270 nm (pH 8.5, 25 °C, and I = 0.25 M)

	k_F (s^{-1} M^{-1})	k_S (s^{-1} M^{-1})	$\Delta\epsilon_F$ (M^{-1} cm^{-1})	$\Delta\epsilon_S$ (M^{-1} cm^{-1})	$\Delta[\theta]_S$ (deg·cm² $dmol^{-1}$)
native	760 ± 8	0.62 ± 0.01	121 ± 5	528 ± 22	315 ± 9
unfolded in 7.2 M urea		5.5 ± 0.7		480 ± 36	

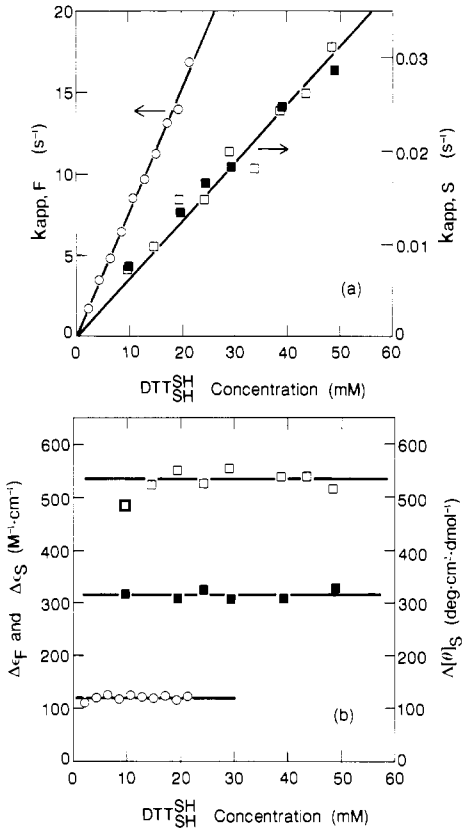


FIGURE 2: Apparent rate constants ($k_{app,F}$ and $k_{app,S}$) (a) and amplitudes ($\Delta\epsilon_F$, $\Delta\epsilon_S$, and $\Delta[\theta]_S$) (b) of the kinetics of disulfide bond reduction in holo-α-lactalbumin as a function of DTT^{SH}_{SH} concentration at pH 8.5 and 25 °C (0.2 M Tris-HCl plus 0.18 M KCl). (○) Fast phase measured by UV absorption at 310 nm; (□) slow phase measured by UV absorption at 310 nm; (■) slow phase measured by CD ellipticity at 270 nm.

the molar concentrations of DTT^{SH}_{SH} employed are more than 10 times the concentration of the protein disulfides, the bimolecular rate constants, k_F and k_S, for the fast and slow phases, respectively, are given by the slopes of the respective linear plots in Figure 2a. The amplitudes represented by the changes in the molar extinction coefficient with respect to the molar concentration of the protein at 310 nm, $\Delta\epsilon_F$ and $\Delta\epsilon_S$, for the fast and slow phases, respectively, and the amplitude represented by the change in $[\theta]_{270}$ for the slow phase, $\Delta[\theta]_S$, are shown as a function of DTT^{SH}_{SH} concentration in Figure 2b. All these amplitudes are independent of DTT^{SH}_{SH} concentration.

The values of the kinetic parameters obtained above are summarized in Table I. If there is no absorption change other than the absorption change due to the production of DTT^{S}_{S}, the observed change in the extinction coefficient must be proportional to the number of disulfide bonds cleaved in the protein molecule. The molar extinction coefficient of DTT^{S}_{S} has been reported to be 110 M^{-1} cm^{-1} (Iyer & Klee, 1973). The value of $\Delta\epsilon_F$ (120 M^{-1} cm^{-1}) is, thus, consistent with the fact that only one disulfide bond (Cys6–Cys120) is cleaved in the fast phase. The value of $\Delta\epsilon_S$ (530 M^{-1} cm^{-1}), however, exceeds significantly the value that would be expected from reduction of the remaining three disulfide bonds. The increase in the extinction coefficient at 310 nm due to the unfolding of α-lactalbumin has been estimated to be ~130 M^{-1} cm^{-1} (Segawa et al., 1981). Evidently, this increase in the extinction coefficient is involved in $\Delta\epsilon_S$ under the present condition.

The bimolecular rate constant of reduction of fully unfolded α-lactalbumin in 7.2 M urea was examined by absorption measurements (Table I). In the fully unfolded state, the kinetics were found to be monophasic, indicating that the four disulfide bonds are equally reactive to DTT^{SH}_{SH} (data not shown). The rate constant of the urea-unfolded protein is approximately 10 times larger than the k_S for native α-lactalbumin. Therefore, the three disulfide bonds reduced in the slow phase in the absence of urea are at least partly protected against the reducing agent. However, the k_F for the native protein is more than 100 times larger than the rate constant of the urea-unfolded protein. Apparently, the Cys6–Cys120 disulfide bond reduced in the fast phase must be extraordinary superreactive to DTT^{SH}_{SH}. As all four disulfide bonds are equally reactive in the urea-unfolded state, such superreactivity of the disulfide bond may not arise from the primary sequence of α-lactalbumin.

Nativelike Structure of 3SS-LA. The results above have suggested that 3SS-LA has a nativelike folded conformation. Because of the known pH dependence of the rate of disulfide interchange (see eq 2), 3SS-LA must be kinetically more stable at a lower pH. The reduction was thus performed at 1 mM DTT^{SH}_{SH} at pH 7.0 and 25 °C in the presence of 1 mM $CaCl_2$. The fast phase was complete in 200 s ($k_{app,F}$ = 0.02 s^{-1}), while the slow phase was too slow to measure at 1 mM DTT^{SH}_{SH}; $k_{app,S}$ is expected to be on the order of 10^{-5} s^{-1} (see Table II). 3SS-LA thus kinetically trapped was subjected to CD measurements. The CD measurements were started at 4 min after mixing of the protein with DTT^{SH}_{SH} and finished within 30 min, so that more than 97% of the protein is in the form of 3SS-LA. The CD spectra of 3SS-LA are very similar to the spectra of the intact native species (Figure 3). The fine structures in the near-UV spectra of the intact species are all preserved in the spectra of 3SS-LA, indicating that the aromatic residues are in similar environments in the two species. The difference CD spectra between the native intact protein and the nativelike three-disulfide species show broad negative bands between 250 and 310 nm and a positive peak at 227 nm (Figure 3c and 3d). The shape and intensities of the difference CD bands are consistent with those observed experimentally and also predicted theoretically as the inherent CD spectra of protein disulfides [see Menendez-Botet and Breslow (1975), Bewley (1977), Kahn (1979), Rauk (1984), Kishore et al. (1988), and Hider et al. (1988) and other references cited therein]. The difference CD spectra may thus arise from the disulfide bond cleaved in the fast phase and may not be associated with a conformational change.

The two thiol groups in 3SS-LA were carboxymethylated by iodoacetic acid (see Materials and Methods), and the CD spectra of CM-3SS-LA in the Ca^{2+}-bound form were measured at pH 7.0 and 25 °C in the presence and absence of excess Ca^{2+} (Figure 3). The spectra of CM-3SS-LA are essentially identical with the spectra of 3SS-LA described above. Thus, the nativelike structure of the three-disulfide species is not disrupted by carboxymethylation under the conditions.

Table II: Kinetic Parameters of Disulfide Bond Reduction in α-Lactalbumin by DTT^{SH}_{SH} in the Presence and in the Absence of EDTA (pH 7.0, 25 °C, and I = 0.25 M)

	k_F (s^{-1} M^{-1})	k_S (s^{-1} M^{-1})	$\Delta\epsilon_F$ (M^{-1} cm^{-1})	$\Delta\epsilon_S$ (M^{-1} cm^{-1})	$\Delta[\theta]_F$ (deg·cm² dmol⁻¹)	$\Delta[\theta]_S$ (deg·cm² dmol⁻¹)
1.0 mM EDTA	22 ± 1	0.2–0.7	239 ± 5	250–350	248 ± 4	83–112
0 mM EDTA	22 ± 3	0.006–0.02	115 ± 24	490–740	27 ± 4	312–492

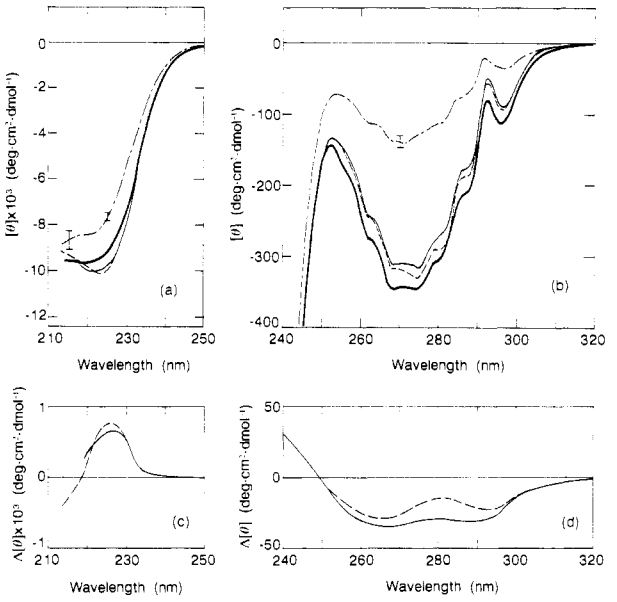


FIGURE 3: CD spectra of α-lactalbumin and its three-disulfide derivatives at pH 7.0 and 25 °C (50 mM MOPS plus 0.2 M KCl). (a and b) Peptide and aromatic CD spectra for the disulfide-intact protein at 1 mM $CaCl_2$ (thick line), 3SS-LA at 1 mM $CaCl_2$ (—), CM-3SS-LA at 1 mM $CaCl_2$ (– –), and CM-3SS-LA at 1 mM EDTA without excess Ca^{2+} (–·–). 3SS-LA was kinetically trapped by partial reduction of the protein at 1 mM DTT^{SH}_{SH} (see text). The spectra of the intact protein and CM-3SS-LA, both in the holo form, were also measured in the absence of excess Ca^{2+}, and the same spectra as at 1 mM $CaCl_2$ were observed. (c and d) Difference CD spectra at 1 mM $CaCl_2$: the intact protein – 3SS-LA (—); the intact protein – CM-3SS-LA (– –). The difference spectra were made by graphic subtraction of the original spectra.

Effect of EDTA on Disulfide Reduction. The effect of EDTA on the disulfide reduction kinetics of α-lactalbumin was investigated at pH 7.0 and 25 °C. EDTA destabilizes the protein through removal of the bound Ca^{2+}. In the presence of a sufficient amount of potassium or sodium ions (>0.1 M), however, the protein retains the native structure even in the presence of EDTA, at pH 7.0 and 25 °C (Hiraoka et al., 1980; Hiraoka & Sugai, 1985). The kinetic parameters of the disulfide reduction in the presence and absence of EDTA are summarized in Table II. Because the rate constants of the reduction are slower than those at pH 8.5 (eq 2), both the fast and slow phases could be measured by manual mixing.

The pH dependence of the observed bimolecular rate constant, k_{obs}, for the disulfide interchange reaction is known to be expressed by

$$k_{obs} = k/(1 + 10^{pK_a - pH}) \quad (2)$$

where k is the pH-independent part of the rate constant and pK_a refers to DTT^{SH}_{SH} (pK_a = 9.2) (Szajewski & Whitesides, 1980; Fukada & Takahashi, 1980). The rate constant values at the two different pHs, 8.5 and 7.0, in the absence of EDTA satisfy the above relationship (Tables I and II), indicating that the mechanisms of the disulfide reduction in the protein are essentially the same in this pH range. However, the slow phase was too sluggish to measure the whole kinetics at pH 7.0, and the appearance of turbidity often interfered with accurate evaluation of the rate constant. The parameter values for the slow phase in Table II are only approximate estimates and shown as ranges of the values obtained in different experiments.

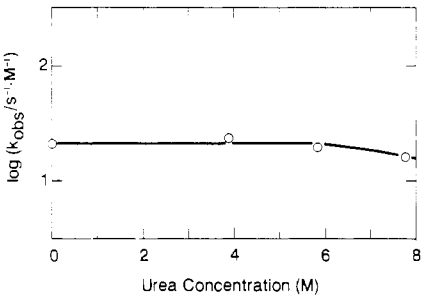


FIGURE 4: Bimolecular rate constant of reduction of the L-cystine disulfide by DTT^{SH}_{SH} as a function of urea concentration at pH 8.5 and 25 °C (0.2 M Tris-HCl plus 0.18 M KCl and 1 mM EDTA).

From Table II, k_F does not depend on EDTA, while k_S is increased at least 10-fold at 1 mM EDTA. This increase in k_S obviously arises from unfolding of 3SS-LA as indicated by a large increase in the amplitude of the fast phase, $\Delta[\theta]_F$, represented by $[\theta]_{270}$, at 1 mM EDTA. The CD spectra of CM-3SS-LA measured at pH 7.0 and 1 mM EDTA show considerable reduction in the intensity in the near-UV region (Figure 3), being consistent with the above results of the disulfide kinetics.

Effect of Urea on Disulfide Reduction. Urea is a popular protein denaturant and will be useful for further assessment of the relationship between protein stability and disulfide reduction kinetics. Urea is superior to guanidine hydrochloride because it has little effect on the chemical interchange step of disulfide reduction. Figure 4 shows the bimolecular rate constant of reduction of the L-cystine disulfide by DTT^{SH}_{SH} as a function of urea concentration. The rate constant is independent of urea concentration up to 6 M. The disulfide reduction kinetics of α-lactalbumin were investigated at various concentrations of urea at pH 8.5 in the absence of EDTA.

Urea-induced equilibrium unfolding of intact α-lactalbumin was first investigated by CD measurements at 270 and 222 nm to correlate the disulfide reduction kinetics with the protein stability. The transition curves expressed by the apparent fractional extent of unfolding as a function of urea concentration are shown in Figure 5. As shown in the previous studies of guanidine hydrochloride induced unfolding of this protein (Kuwajima et al., 1976), the transition curves measured at the two wavelengths do not coincide with each other, demonstrating that an intermediate state that has nativelike secondary structure but has unfolded tertiary structure is populated in the equilibrium unfolding. The midpoint of the transition is located at 4.7 M urea when the unfolding is measured by $[\theta]_{270}$ and at 5.1 M urea when measured by $[\theta]_{222}$.

The values of k_F and k_S of the disulfide reduction of α-lactalbumin are shown as a function of urea concentration in Figure 6. The value of k_F decreases with increasing urea concentration, and the fast phase disappears when the protein is sufficiently unfolded in concentrated urea. On the other hand, k_S increases with increasing urea concentration between

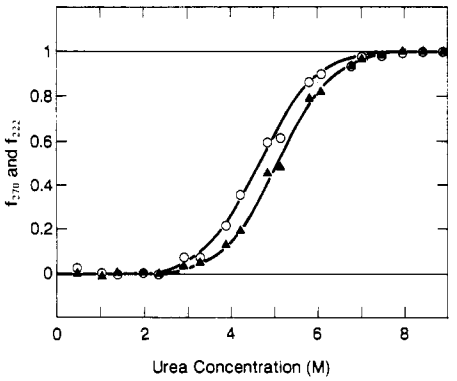
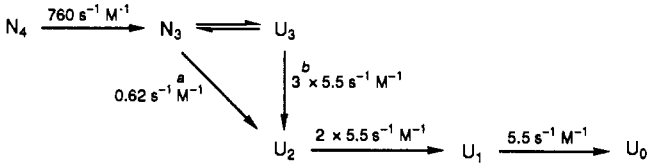


FIGURE 5: Urea-induced transition curves of α-lactalbumin at pH 8.5 and 25 °C (0.2 M Tris-HCl plus 0.18 M KCl). The apparent fractional extents of unfolding (f_{270} and f_{222}) were calculated from the ellipticity changes at 270 nm (○) and at 222 nm (▲).

Scheme I



1 and 3 M, and saturates above 3 M urea. The saturation of k_S suggests that 3SS-LA may be unfolded above 3 M urea, so that the stability of 3SS-LA must be lower than the stability of the disulfide intact protein (see Figure 5). Figure 6 also shows that k_S is independent of urea concentration below 1 M. This indicates that under strongly native conditions, the slow phase kinetics are independent of the protein stability. This can be interpreted in terms of the presence of a direct reduction pathway to cleave the second accessible disulfide bond in nativelike folded 3SS-LA.

Reaction Scheme. The simplest reaction scheme that accounts for all of the results above for the disulfide reduction of α-lactalbumin by DTT^{SH}_{SH} is shown in Scheme I. The subscripts in the scheme indicate the number of disulfide bonds, so that N_4 and N_3 refer to the native intact and the nativelike 3SS-LA species, respectively. U_3, U_2, U_1, and U_0 are the unfolded species. The numerical value on each reaction step indicates the bimolecular rate constant of reduction at pH 8.5 and 25 °C (Table I). The rate constants of the steps from the U states are assumed to be equivalent to the rate constant for the fully unfolded protein in concentrated urea. However, the U states may not necessarily be fully unfolded, but such a partially unfolded state as the intermediate observed in the urea-induced or guanidine hydrochloride induced unfolding might be sufficient to make the disulfides accessible to the reducing agent. The factors 3 and 2 multiplied by the rate constant (5.5 s^{-1} M^{-1}) for the steps from U_3 and U_2 are the statistical factors considering the number of the available disulfide bonds.

In the native four-disulfide species (N_4), the Cys6–Cys120 disulfide bond is in an anomalous environment and has exceptionally high reactivity toward DTT^{SH}_{SH}. The reduction of N_4 thus rapidly produces N_3 (the fast phase). Under a strongly native condition, e.g., at a urea concentration below 1 M in the absence of EDTA, N_3 is sufficiently stable that the second accessible disulfide in N_3 is reduced in the slow phase, and once the second disulfide is cleaved, the protein rapidly unfolds to form U_2. Step *a* in Scheme I is thus rate-limiting for reduction in the slow phase. Under a marginally native condition, e.g., at 3 M urea (pH 8.5) or at 1 mM EDTA in the absence of urea (pH 7.0), once the first disulfide is cleaved,

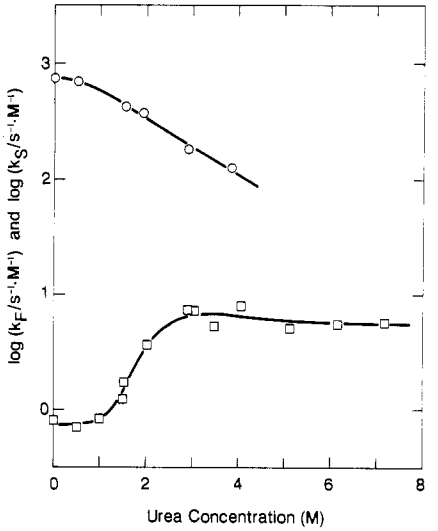


FIGURE 6: Bimolecular rate constants for the fast and slow phases (k_F and k_S) of disulfide bond reduction in holo-α-lactalbumin by DTT^{SH}_{SH} as a function of urea concentration at pH 8.5 and 25 °C (0.2 M Tris-HCl plus 0.18 M KCl): (○) k_F; (□) k_S. The reactions were carried out at ~10 mM DTT^{SH}_{SH} and were followed by UV absorption at 310 nm. The $k_{app,F}/[DTT^{SH}_{SH}]$ (or $k_{app,S}/[DTT^{SH}_{SH}]$) was taken as k_F (or k_S), where $[DTT^{SH}_{SH}]$ is the molar concentration of DTT^{SH}_{SH}.

N_3 rapidly unfolds, so that the reduction in the slow phase occurs through step *b*. Under an intermediate condition, where N_3 and U_3 are both significantly populated, the stability of N_3 determines how much population of 3SS-LA is reduced through step *b*, so that the rate constant of the slow phase depends on urea concentration (Figure 6). However, in this case, the intramolecular disulfide interchange within U_3 may further complicate the slow phase kinetics. At least three kinetic processes, the bimolecular disulfide reduction from U_3, the intramolecular disulfide interchange, and the conformational transition between N_3 and U_3, are expected to be associated with the slow phase.

Superreactivity of the Cys6–Cys120 Disulfide Bond. There are at least two alternative sources that bring about the superreactivity of the disulfide bond. (i) The electrostatic effect exerted by positively charged groups located close in space to the disulfide increases the effective concentration of reactive thiolate anion and enhances the apparent reactivity of the disulfide bond. The reactivity of the disulfide in thioredoxin from *Escherichia coli* is known to be 10^2–10^3 times larger than the reactivity of normal disulfides, presumably owing to the presence of a positively charged group of a nearby lysyl residue (Holmgren, 1985). The electrostatic environments have been shown to provide a means of achieving a 10^6-fold range in the rate constants of the disulfide interchange reactions in a number of small peptide fragments (Snyder et al., 1981). (ii) The strain of the disulfide bond geometry imposed by structural folding around the disulfide bond in the native state will increase its rate of reduction. Because rotation around a disulfide bond is largely hindered with the preferred dihedral angle of the CS–SC linkage, the disulfide bond is destabilized when constrained to form unfavorable geometry (see Discussion). The enhanced reactivity of the strained disulfide bond has been demonstrated in the five-membered ring of lipoic acid (Creighton, 1975), in neurophysin (Menendez-Botet & Breslow, 1975), and in short model peptides that contain small disulfide loops (Zhang & Snyder, 1989).

In order to evaluate the electrostatic contribution to the reactivity of the Cys6–Cys120 disulfide bond in native α-lactalbumin, the fast phase reaction rate of the disulfide reduction was measured at various ionic strengths while adding

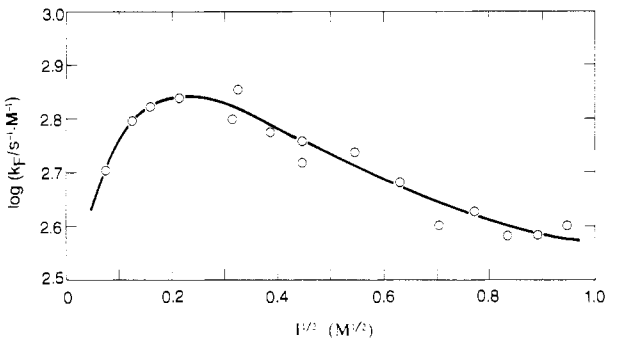


FIGURE 7: Dependence of the k_F of holo-α-lactalbumin on the square root of ionic strength ($I^{1/2}$) at pH 8.5 and 25 °C. The solutions contained at least 20 mM Tris-HCl and varying amounts of KCl.

varying amounts of KCl, and the results are shown in Figure 7. The rate constant k_F first increases and then gradually decreases with increasing ionic strength. The total net charge of the protein under the condition is known to be −7 to −6 (Robbins et al., 1967; Kuwajima et al., 1975), so that the first increase in k_F may be due to electrostatic screening of the long-range repulsion between $DTT^{S^-}_{SH}$ and the negatively charged protein molecule. The secondary salt effect due to the ionic strength dependence of the pK_a of DTT^{SH}_{SH} may also contribute to the increase in k_F. The decrease in k_F at a higher ionic strength might thus be ascribable to the local electrostatic effect exerted by a positively charged group close to the disulfide bond. However, at a sufficiently high ionic strength (>0.5 M), where the electrostatic contributions must be negligible, the observed k_F is still 100 times as large as the rate of the normalized disulfide in concentrated urea. Obviously, the superreactivity of the Cys6–Cys120 disulfide bond in native α-lactalbumin is not due to the electrostatic effect.

Discussion

The present results are summarized as follows. (i) The kinetics of disulfide bond reduction of α-lactalbumin by DTT^{SH}_{SH} are biphasic, and both phases are rate-limited by the intermolecular disulfide interchange. The biphasic character of the reduction is due to the superreactivity of the Cys6–Cys120 disulfide bond and the relative inaccessibility of the other three disulfide bonds to the reducing agent in the native state. (ii) The Cys6–Cys120 disulfide bond, 140 times more reactive than the normal disulfide, must be in an anomalous environment in the native protein structure. The local electrostatic effect is not the primary source of the superreactivity. (iii) Fully reduced α-lactalbumin is unfolded, and whether the global structural unfolding is associated with the fast phase or the slow phase depends on the stability of 3SS-LA. Under a strongly native condition, 3SS-LA assumes the nativelike structure, and the slow phase is rate-limited by reduction of the second accessible disulfide bond. Under a marginally native condition, e.g., at 3 M urea or in the presence of EDTA, the unfolding occurs during the fast phase, and 3SS-LA is already unfolded.

α-Lactalbumin and lysozyme have been reported to be markedly different in the reactivity of the disulfide bonds. The difference was thought to not be easily compatible with the postulated similarity of the two proteins and interpreted in terms of either (i) the structure of the α-lactalbumin molecule, in the vicinity of the disulfide bonds, being appreciably more expanded than that of lysozyme or (ii) the degree of flexibility of the two proteins being markedly different (Atassi et al., 1970; Iyer & Klee, 1973; Shechter et al., 1973). All these previous studies of the disulfide reduction of α-lactalbumin were, however, made before the Ca^{2+} binding character of this protein was established, and the disulfide reactivities of the two proteins were compared in the presence of a millimolar concentration of EDTA. Therefore, the first suggestion was that α-lactalbumin was selectively destabilized by removal of the bound Ca^{2+} by EDTA, which made the disulfide bonds much more reducible than those in lysozyme. The present results show that the high reducibility of α-lactalbumin reported previously is, in fact, partly due to the destabilization of the protein by EDTA. However, the Cys6–Cys120 disulfide bond reduced in the fast phase is extraordinarily superreactive, and the reduction of this disulfide bond does not depend on the presence of absence of EDTA. Because the local electrostatic effect is not the primary source of the superreactivity, a geometric strain imposed on this disulfide bond in native α-lactalbumin may be responsible for the superreactivity.

Disulfide Bond Geometry. The geometries of disulfide bonds in many globular proteins with known X-ray structures have been analyzed by Thornton (1981) and Richardson (1981) and more recently by Katz and Kossiakoff (1986). The distribution analysis of the dihedral angle (χ_3) about the CS–SC bond has indicated that this angle is so restricted that there are only two groups with respect to χ_3, the left-handed disulfides ($\chi_3 \sim -90°$) and the right-handed disulfides ($\chi_3 \sim 90°$), with approximately equal numbers of population for each. However, a disulfide bond and the nearby covalent bonds in a protein often adopt geometries not found in small-molecule studies in order to fit in with the protein structure. An empirical relationship between the dihedral angles and the torsion energy ($E_{torsion}$) for the five successive covalent bonds of a cystine side chain in a protein is known to be given by

$$E_{torsion}\ (\text{kcal/mol}) = 2.0\{(1 + \cos 3\chi_1) + (1 + \cos 3\chi_1')\} + 1.0\{(1 + \cos 3\chi_2) + (1 + \cos 3\chi_2')\} + 0.6(1 + \cos 3\chi_3) + 3.5(1 + \cos 2\chi_3) \quad (3)$$

where χ_1 and χ_1' are the dihedral angles about the C_α–C_β bonds of the two half-cystine residues that form the disulfide bond and χ_2 and χ_2' are those about the C_β–S_γ bonds (Weiner et al., 1984; Katz & Kossiakoff, 1986). The $E_{torsion}$ values and the conformational parameters for the disulfide bonds based on the refined X-ray structure of baboon α-lactalbumin are presented in Table III (Acharya et al., 1989). For comparison, the $E_{torsion}$ and the parameter values for disulfide bonds of five other proteins, for which the disulfide reduction has been well studied, are also included. As the accuracy of the atomic coordinates of a protein is very important for reliability of the $E_{torsion}$ calculated, only the proteins for which the high-resolution X-ray data are available can be used for the calculation. All the X-ray data of the proteins listed have a resolution at least at 1.8 Å with sufficiently small crystallographic residuals (R value) (≤0.2). The $E_{torsion}$ of the Cys6–Cys120 disulfide bond (6.5 kcal/mol) of α-lactalbumin is remarkably higher than those of the other three disulfide bonds ($E_{torsion}$ = 1.0–3.4 kcal/mol) in the protein and also the $E_{torsion}$ of any disulfide bond in human lysozyme ($E_{torsion}$ = 1.0–2.4 kcal/mol). All the disulfide bonds in the other four proteins, except the Cys136–Cys201 of trypsin, have $E_{torsion}$ values less than 5 kcal/mol, indicating that the $E_{torsion}$ of the Csy6–Cys120 bond in α-lactalbumin is extraordinary high. All the disulfide bonds in these proteins are known to be much less reactive than the Cys6–Cys120 bond of α-lactalbumin (Sondack & Light, 1971; Creighton, 1975, 1979; Sperling et al., 1969; Pace & Creighton, 1986). We have also calculated the geometric energies ($E_{geometry}$) of the cystine side chains, which involve the bond stretching and bending energies as well as the torsion energy, using the force field parameters of Weiner et al.

Table III: Geometries and Torsion Energies of Disulfide Bonds in α-Lactalbumin and in Five Other Globular Proteins, Calculated from Their Atomic Coordinates in the X-ray Crystal Structures[a]

disulfide bond	bond length (Å)	dihedral angle (deg)					torsion energy (kcal/mol)
		χ_1	χ_2	χ_3	χ_2'	χ_1'	
baboon α-lactalbumin (1ALC)[b]							
Cys6–Cys120	2.04	−96	−59	102	123	164	6.5
Cys28–Cys111	2.09	171	−82	−74	−86	−38	3.4
Cys61–Cys77	2.06	53	92	97	−65	−56	2.2
Cys73–Cys91	2.02	−64	173	71	64	−179	1.0
human lysozyme (1LZ1)							
Cys6–Cys128	2.07	−69	−52	−65	−44	−60	1.9
Cys30–Cys116	2.06	−175	−96	−96	−72	−58	2.4
Cys65–Cys81	2.08	63	81	95	−58	−71	1.7
Cys77–Cys95	2.04	−71	178	83	48	−176	1.0
bovine trypsin (2PTN)							
Cys22–Cys157[c]	2.04	−61	−72	105	67	−82	2.9
Cys42–Cys58	2.02	−85	−145	−84	−85	−72	4.4
Cys128–Cys232	2.03	−61	−70	96	141	64	2.5
Cys136–Cys201	2.05	−53	−108	98	−91	−40	5.0
Cys168–Cys182	2.04	−168	55	72	74	−64	1.5
Cys191–Cys220	2.05	−154	47	88	−176	−53	2.5
bovine pancreatic trypsin inhibitor (5PTI)							
Cys5–Cys55	2.04	−62	−76	−83	−66	−64	0.9
Cys14–Cys38	2.03	−72	106	95	−114	61	4.9
Cys30–Cys51	2.02	−72	−103	−90	−96	178	3.9
bovine ribonuclease A (7RSA)							
Cys26–Cys84	2.00	−71	−87	−80	−56	−62	1.7
Cys40–Cys95	2.01	−53	−57	−82	−66	−57	0.7
Cys58–Cys110	1.98	−65	−62	−83	−122	−59	2.6
Cys65–Cys72	1.97	−54	−62	109	91	−84	4.4
ribonuclease T_1 from *Aspergillus oryzae* (2RNT)							
Cys2–Cys10	2.01	−50	−71	−116	84	−178	3.7
Cys6–Cys103	2.02	−64	−60	109	−149	−59	2.9

[a] References of the X-ray analysis of the respective proteins are as follows: α-lactalbumin, Acharya et al. (1989); lysozyme, Artymiuk & Blake (1981); trypsin, Walter et al. (1982); trypsin inhibitor, Wlodawer et al. (1984); ribonuclease A, Wlodawer et al. (1988); and ribonuclease T_1, Koepke et al. (1989). [b] The filenames in the Protein Data Bank are given in parentheses. [c] The numbering of amino acid residues in trypsin is based on the residue number of chymotrypsinogen.

(1984). Again, the Cys6–Cys120 of α-lactalbumin was found to be most extraordinary ($E_{geometry}$ = 7.6 kcal/mol) whereas two bonds, Cys28–Cys111 of α-lactalbumin and Cys65–Cys72 of ribonuclease A, also showed a relatively high value (7.1 kcal/mol for both); all other cystine side chains have $E_{geometry}$ between 1.3 and 6.1 kcal/mol (data not shown). The Cys28–Cys111 bond is less accessible to solvent than the Cys6–Cys120 bond in native α-lactalbumin (Acharya et al., 1989). As a result, it is suggested that the geometric strain imposed on the Cys6–Cys120 disulfide bond in native α-lactalbumin may be an important factor that determines the reactivity of this disulfide bond.

Strain Energy of the Disulfide Bond Relates to the Conformational Stability and the Disulfide Reactivity. The stabilities of 3SS-LA and CM-3SS-LA are remarkably reduced as indicated by the inability to form the folded structures in the presence of EDTA and by saturation of $k_{app,S}$ above 3 M urea where the disulfide-intact protein is still almost fully folded (Figure 6). The cleavage of a disulfide bond is expected to increase the chain entropy in the unfolded state and thereby to destabilize the native structure relative to the unfolded one. Thus, we first consider if such an entropic effect can account for the observed decrease in stability of three-disulfide α-lactalbumin. The destabilization of 3SS-LA can be analyzed in terms of the following thermodynamic cycle:

$$\begin{array}{ccc} N_4 & \xrightarrow{\Delta G_U(\text{intact})} & U_4 \\ \Big\Vert\!\Big\downarrow \Delta G_R(N) & & \Big\Vert\!\Big\downarrow \Delta G_R(U) \\ N_3 & \xrightarrow{\Delta G_U(\text{3SS-LA})} & U_3 \end{array} \qquad (4)$$

where ΔG_U(intact) and ΔG_U(3SS-LA) are the free energy changes of unfolding of the disulfide-intact (N_4) and the three-disulfide species (N_3), respectively. From eq 4, the difference in the free energy of stabilization, $\Delta\Delta G$, between 3SS-LA and the disulfide-intact protein should be related to the free energy changes of reduction of the Cys6–Cys120 disulfide bond, $\Delta G_R(N)$ and $\Delta G_R(U)$, in the native and the unfolded states, respectively, as

$$\begin{aligned} \Delta\Delta G &= \Delta G_U(\text{3SS-LA}) - \Delta G_U(\text{intact}) \\ &= \Delta G_R(U) - \Delta G_R(N) \end{aligned} \qquad (5)$$

In general, ΔG_R consists of two parts: the intrinsic chemical stability of the disulfide bond, $\Delta G_{R,chem}$, and the conformational part, $\Delta G_{R,conf}$. We are only interested in $\Delta G_{R,conf}$, and the $\Delta G_{R,chem}$ is assumed to be the same for both native and unfolded α-lactalbumin. Then, the major component of $\Delta G_{R,conf}(U)$ is thought to derive from the increase in the chain entropy, ΔS_{chain}, caused by cleavage of the disulfide bond in the unfolded state, and the rearrangement of eq 5 leads to

$$-\Delta G_{R,conf}(N) = \Delta\Delta G + T\Delta S_{chain} \qquad (6)$$

where T denotes the absolute temperature. The $-\Delta G_{R,conf}(N)$ in eq 6 may include all the free energy contributions that stabilize or destabilize the native disulfide bond, e.g., the rigidity of the molecule around the disulfide bond as a stabilizing contribution or the geometric strain imposed on the disulfide bond as a destabilizing contribution [see Creighton (1988)]. When the decrease in stability of 3SS-LA is solely due to the entropic effect, $\Delta G_{R,conf}(N)$ must be zero.

The ΔS_{chain} can be estimated on the basis of the polymer theory by calculating the probability that the ends of a polymer chain will simultaneously occur in the same volume element, v_s (Schellman, 1955; Flory, 1956; Poland & Scheraga, 1965). Different researchers have used different values for this pa-

rameter to calculate the ΔS_{chain}; e.g., v_s = 2.83 Å^3 according to Schellman (1955) while 57.9 Å^3 was employed by Pace et al. (1988); however, the significance of v_s in calculating ΔS_{chain} decreases with an increase in the size of a loop formed by the cross-link. The ΔS_{chain} calculated in this way has been known to well interpret the experimentally observed $\Delta\Delta G$ caused by reduction of a disulfide bond or by introduction of an extrinsic cross-link in a number of globular proteins (Goto & Hamaguchi, 1979; Pace et al., 1988; Johnson et al., 1978; Lin et al., 1984; Ueda et al., 1985). In the present case, the loop formed by the Cys6–Cys120 disulfide bond consists of 33 amino acid residues, and the theoretical ΔS_{chain} is estimated to be 13–18 eu considering the range of the v_s value mentioned above. Thus, $T\Delta S_{chain}$ is estimated to be 3.8–5.5 kcal/mol. On the other hand, the $\Delta\Delta G$ can be estimated experimentally from comparison of the stabilities of intact and three-disulfide α-lactalbumin. Because the unfolding transition of CM-3SS-LA is free from complication of the intramolecular disulfide interchange and known to be reversible, the unfolding equilibrium of CM-3SS-LA has been studied and compared with the known results of the intact protein [Ikeguchi et al., unpublished results; see also Ikeguchi et al. (1986)]. The $\Delta\Delta G$ between CM-3SS-LA and the intact protein has been shown to be −2.4 kcal/mol. The $-\Delta G_{R,conf}(N)$ evaluated from these data is thus. 1.4–3.1 kcal/mol. Because the presence of the carboxymethyl groups is expected to shift the transition to the unfolded side, this value is thought to be a minimal estimate.

Experimentally, the strain energy of a disulfide bond, E_{strain}, may be evaluated from the reduction rate of the disulfide bond. The rotational hindrance around the disulfide bond is known to be primarily due to a strong dependence on the CS–SC dihedral angle of the energy of the repulsive interactions between lone 3p electron pairs on the adjacent sulfur atoms (Bergson, 1958; Boyd, 1972). The rate-limiting step of the reduction of the Cys6–Cys120 disulfide bond in α-lactalbumin by DTT^{SH}_{SH} is the disulfide interchange between the protein disulfide bond and the reducing agent. The thiol–disulfide interchange reaction is known to occur by a simple S_N2 pathway involving no kinetically distinguishable metastable intermediate, and the transition state for the reaction has half-negative charges on the attacking and leaving sulfur atoms with half-bond order for each sulfur to sulfur bond (Wilson et al., 1977; Szajewski & Whitesides, 1980). Therefore, it is a reasonable assumption that the geometric strain of the disulfide bond may be sufficiently relaxed in the transition state. The E_{strain} is thereby simply given by the ratio of the reduction rate, k_{strain}, for the strained disulfide bond and the reduction rate, k_0, of the fully accessible disulfide bond under a strain-free condition as

$$E_{strain} = RT \ln (k_{strain}/\alpha k_0) \quad (7)$$

where R is the gas constant, and α stands for the accessibility of the disulfide bond to the reducing agent and may be close to unity for the Cys6–Cys120 bond that is sufficiently exposed in native α-lactalbumin (Acharya et al., 1989). The acceleration of the reduction rate and the strain energy of the disulfide bond in the five-membered ring of lipoic acid are known to satisfy the above relationship (Creighton, 1975). As it has been shown that the introduction of cross-links into unfolded protein need not introduce strain (Johnson et al., 1978), the reduction rate in the unfolded state in concentrated urea may be taken as k_0 in the present case. The 140-fold higher rate of reduction of the Cys6–Cys120 bond in α-lactalbumin therefore corresponds to an E_{strain} of 2.9 kcal/mol, which is in good agreement with the $-\Delta G_{R,conf}(N)$ estimated above.

Concluding Remarks. Superreactivity of the Cys6–Cys120 disulfide bond arises from the geometric strain imposed on the disulfide bond in native α-lactalbumin. The present results provide an example that the strained disulfide bond is accommodated to the folded protein structure owing to the relative stabilization of the native state by a large entropy loss in the unfolded state.

Acknowledgments

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Registry No. L-Cys, 56-89-3; Ca, 7440-70-2; dithiothreitol, 3483-12-3.

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