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Contribution of the 6–120 Disulfide Bond of α-Lactalbumin to the Stabilities of Its Native and Molten Globule States†

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ABSTRACT: The unfolding and refolding of a derivative of α-lactalbumin, in which the disulfide bond between Cys6 and Cys120 is selectively reduced and S-carboxymethylated, are investigated by equilibrium and kinetic circular dichroism measurements. The native conformation of this derivative is known to be essentially identical to that of intact α-lactalbumin. The equilibrium unfolding of the derivative involves a stable intermediate, which is also similar to the molten globule state of the disulfide intact protein. The results of stopped-flow circular dichroism experiments show that the same intermediate is formed rapidly as a transient intermediate in kinetic refolding. The conformational stabilities for the native and intermediate states have been estimated and compared with the stabilities for the corresponding states of intact α-lactalbumin. The stabilization of the native state by the disulfide has been interpreted in terms of a decrease in chain entropy in the unfolded state and elimination of the strain imposed on the disulfide bond in the native state. The molten globule state is also stabilized by the disulfide bond, although the degree of stabilization of the molten globule state is smaller than of the native state. The results suggest that, in the molten globule state, some ordered structures are present within the loop moiety formed by the 6–120 disulfide.

In order to elucidate the relationship between a protein sequence and the three-dimensional structure, it is important to evaluate the contribution of an individual interaction to the conformational stability of the protein. In the present study, we have studied the contribution of the 6–120 disulfide bond to the conformational stability in the native (N)[1] state of bovine α-lactalbumin (BLA). BLA has four disulfide bonds, Cys6–Cys120, Cys28–Cys111, Cys61–Cys77, and Cys73–Cys91. One of them (Cys6–Cys120) has unusually high reactivity to thiol reagents, and hence it can be selectively reduced and S-carboxymethylated (Shechter et al., 1973; Kuwajima et al., 1990). The resultant derivative (2CM-3SS-BLA) is known to assume a native-like conformation as evidenced by antibody binding (Shechter et al., 1973), CD (Kuwajima et al., 1990), and NMR spectra (Ikeguchi et al., unpublished results). Therefore, the contribution of the 6–120 disulfide bond to the stability of the native structure of BLA can be evaluated by comparing the stability between intact BLA and 2CM-3SS-BLA.

The contribution of the 6–120 disulfide bond to the stability of the secondary structure in the molten globule (A) state, which is known to be identical with an early intermediate in kinetic refolding of disulfide intact BLA (Ikeguchi et al., 1986a; Kuwajima, 1989), has also been investigated in this study. As proteins fold to their specific native conformations through a series of identifiable intermediate stages (Kuwajima, 1989; Kim & Baldwin, 1990), it is important to know whether or not the interaction that stabilizes the ultimate native structure is present in the folding intermediate. Although the disulfide bond itself is present throughout the refolding reaction of a disulfide intact protein, its contribution to the stability is expected to change depending on the conformational state of the protein. The previous study on a derivative of BLA in which all of the four disulfide bonds are reduced and S-carboxymethylated (8CM-BLA) has shown that, under an appropriate condition, it can assume secondary structure similar to that found in the molten globule state of the disulfide intact protein, although it can not acquire the native conformation in any conditions (Ikeguchi & Sugai, 1989). The secondary structure in 8CM-BLA is less stable than that in the molten globule state of intact BLA, indicating that at least a part of the four disulfide bonds have a significant contribution to the stability of the molten globule conformation. It remains to be seen, however, to what extent each disulfide bond contributes to the stability of the secondary structure formed in the molten globule state.

MATERIALS AND METHODS

Materials. Holo-2CM-3SS-BLA was prepared from purified holo-BLA by the method previously described (Kuwajima et al., 1990) with the following modifications. 2CM-3SS-BLA was separated from reagents, used for reduction and S-carboxymethylation, by passing through a Sephadex G-25 column equilibrated and eluted with 5 mM NH_4HCO_3

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[1] Abbreviations: BLA, bovine α-lactalbumin; 2CM-3SS-BLA and 2CAM-3SS-BLA, derivatives of bovine α-lactalbumin in which disulfide bond between cysteine 6 and cysteine 120 is selectively reduced and then S-carboxymethylated and S-carboxyamidomethylated, respectively; 8CM-BLA, a derivative of bovine α-lactalbumin in which all of the four disulfide bonds are reduced and S-carboxymethylated; CD, circular dichroism; Cys, cysteine; EDTA, ethylenediaminetetraacetic acid; GdnHCl, guanidine hydrochloride; NMR, nuclear magnetic resonance. The N, A, and U states denote the native, intermediate, and fully unfolded states, respectively. Although we had used D as the abbreviation to mean the fully unfolded state previously, we use U to indicate the same state in this paper.

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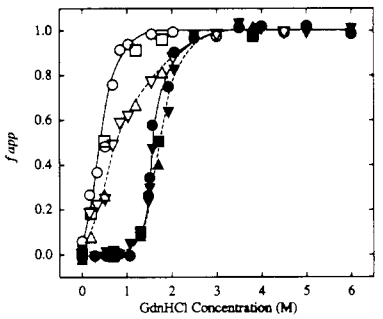

FIGURE 1: Normalized unfolding-transition curves of 2CM-3SS-BLA and 2CAM-3SS-BLA induced by GdnHCl (50 mM cacodylate buffer (pH 7.0), $[Na^+]$ = 0.1 M, 4.5 °C). The apparent fractional extent of unfolding, f_{app}, was calculated from $[\theta]_{270}$ (○, ●), $[\theta]_{222}$ (▽, ▼) of 2CM-3SS-BLA and $[\theta]_{270}$ (□, ■), $[\theta]_{222}$ (△, ▲) of 2CAM-3SS-BLA as previously described (Ikeguchi et al., 1986b). Open symbols and closed symbols refer to the values obtained in the presence of 1 mM EDTA and 1 mM $CaCl_2$, respectively. The protein concentration was 28–33 μM.

at 4 °C, and then lyophilized. This was found to be useful to avoid precipitation of a part of the protein, which occurred in the previous method (Kuwajima et al., 1990). A derivative of bovine α-lactalbumin in which the disulfide bond between cysteine 6 and cysteine 120 is selectively reduced and S-carboxyamidomethylated (2CAM-3SS-BLA) was prepared by the same method except for the use of monoiodoacetamide instead of monoiodoacetic acid. Apoproteins were prepared by gel filtration of the holoprotein dissolved in metal-free 10 mM HCl on a column of Sephadex G-25 equilibrated and eluted with the same solvent at 4 °C. Reagents used were the same as reported previously (Kuwajima et al., 1985, 1990).

Methods. The concentrations of 2CM-3SS-BLA and 2CAM-3SS-BLA were determined spectrophotometrically with the same molar extinction coefficient at 280 nm as that of intact BLA (Kuwajima et al., 1985). Equilibrium and kinetic CD measurements were done as described previously (Ikeguchi et al., 1986b; Kuwajima et al., 1987; Sugawara et al., 1991).

RESULTS

Equilibrium Unfolding of 2CM-3SS-BLA. In order to estimate the stability of both the N and A states of 2CM-3SS-BLA, the equilibrium unfolding transition induced by GdnHCl was investigated by CD spectra. Since the stability of 2CM-3SS-BLA is expected to depend on Ca^{2+} concentration as observed for intact BLA (Ikeguchi et al., 1986b), experiments were done both in the absence and in the presence of Ca^{2+}. The results are shown in Figure 1. As observed previously for intact BLA (Kuwajima et al., 1976; Ikeguchi et al., 1986b), the unfolding transition curves obtained from the ellipticity changes at 270 nm and at 222 nm do not coincide with each other, demonstrating that there is at least one stable intermediate in the equilibrium unfolding of 2CM-3SS-BLA. According to the previous results of intact BLA, we employ a three-state model with the A state as the intermediate for analysis of the unfolding equilibria of 2CM-3SS-BLA. It is necessary, however, for such an analysis to know the ellipticities at 270 and 222 nm ($[\theta]_{270}$ and $[\theta]_{222}$) for the pure A state. For intact BLA, the pure A state is observed at acid pH at equilibrium, and the same state is observed also transiently in kinetic refolding. It has been known for the pure A state of intact BLA that the $[\theta]_{270}$ is approximately identical to that in the fully unfolded (U) state, and the $[\theta]_{222}$ is approximately identical to that in the N state (Kuwajima et al., 1976, 1985; see also Figure 3). It is not yet known, however, whether or not these are valid for 2CM-3SS-BLA as well. To investigate this, the intermediate of 2CM-3SS-BLA has been further studied by kinetic refolding and acidification experiments.

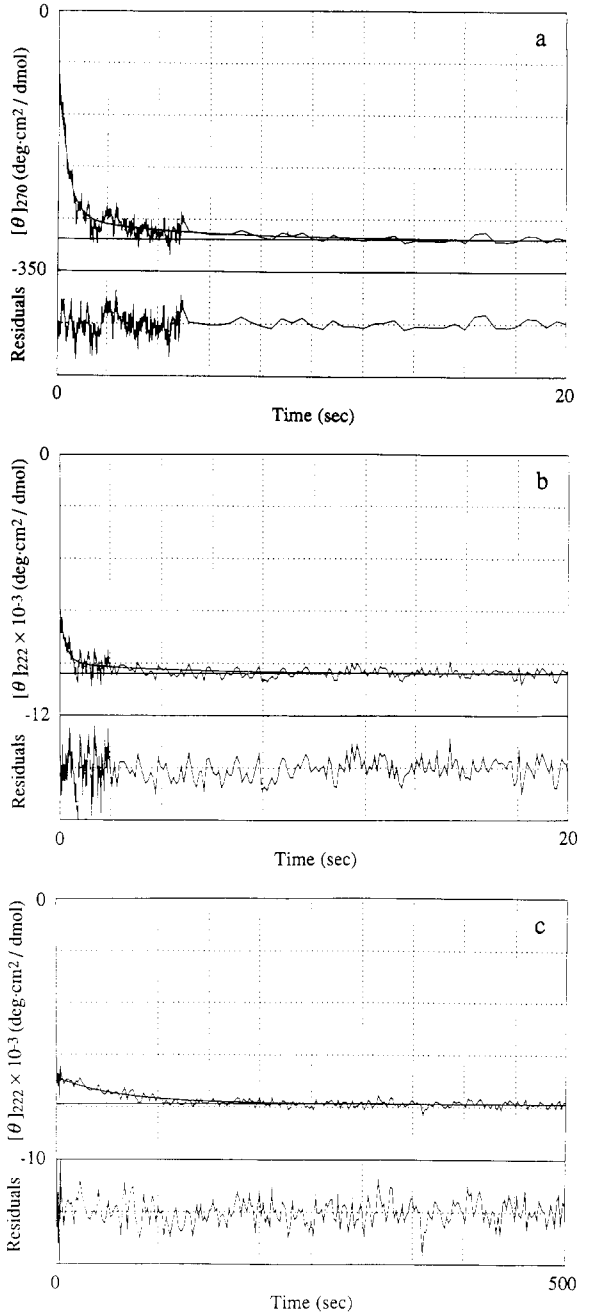

FIGURE 2: Kinetic progress curves of the refolding of 2CM-3SS-BLA at 0.35 M GdnHCl observed by CD at 270 nm in the presence of 1 mM Ca^{2+} (a, top), at 222 nm in the presence of 1 mM Ca^{2+} (b, middle), and at 222 nm in the presence of 1 mM EDTA (c, bottom). Other conditions were the same as in Figure 1. The protein concentrations were 50.7 μM (a), 31.2 μM (b), and 24.5 μM (c). Full scales of residuals are 50 deg·cm²/dmol (a), 1000 deg·cm²/dmol (b), and 500 deg·cm²/dmol (c).

The A State Observed in Kinetic Refolding and Acidification Experiments. Refolding of 2CM-3SS-BLA initiated by a GdnHCl concentration jump from 4 M to 0.35 M was monitored by CD at various wavelengths. Typical kinetic refolding curves of 2CM-3SS-BLA are shown in Figure 2. As previously observed for intact BLA (Kuwajima et al., 1985), refolding kinetics of 2CM-3SS-BLA at 0.35 M GdnHCl are monophasic in the absence of Ca^{2+} and are biphasic in the presence of 1 mM Ca^{2+}. The rate constants of refolding

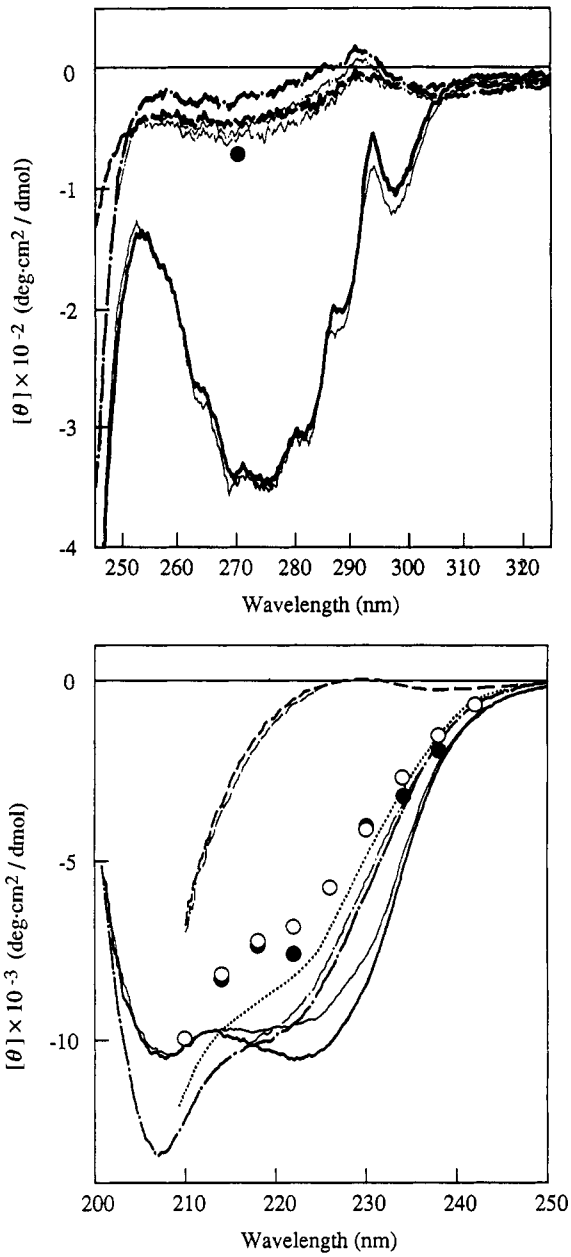

FIGURE 3: CD spectra of BLA (thin lines) and 2CM-3SS-BLA (thick lines) at 4.5 °C. Solid lines, dot-dash lines, and broken lines indicate the spectra of the N state (pH 7.0), the A state (0.1 M KCl, pH 2.0), and the U state (6 M GdnHCl, pH 7.0), respectively. Open and closed circles denote the spectra of the transient folding intermediate of 2CM-3SS-BLA in the presence of 1 mM EDTA and 1 mM $CaCl_2$, respectively. They are the zero-time ellipticities obtained from kinetic refolding curves as shown in Figure 2 (see text). Protein concentrations were 20–50 μM. The dotted line shows the spectrum of the transient refolding intermediate of intact BLA (Kuwajima et al., 1985).

reactions are independent of the wavelength measured. At 222 nm, the ellipticity obtained by extrapolation of the kinetic curve to zero time ("zero-time ellipticity") does not coincide with the ellipticity of the U state expected at 0.35 M GdnHCl, which has been obtained by linear extrapolation of the dependence of the ellipticity on the GdnHCl concentration observed in a region after the unfolding transition. Thus, the zero-time ellipticity in kinetic refolding may reflect formation of a transient intermediate occurring within the dead time of a stopped-flow measurement. The zero-time ellipticities measured at various wavelengths give a CD spectrum of the transient intermediate. In Figure 3, the spectrum of the transient intermediate of 2CM-3SS-BLA is compared with

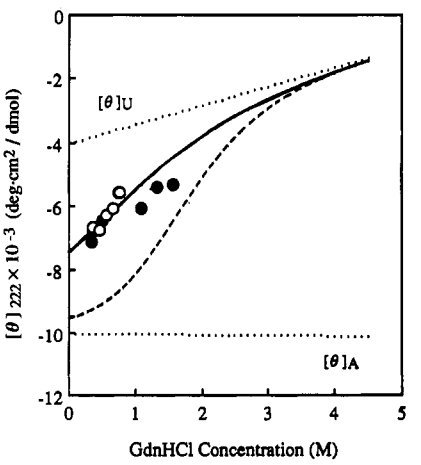

FIGURE 4: GdnHCl-induced unfolding curves of the A state of 2CM-3SS-BLA and intact BLA (pH 7.0, 4.5 °C). The solid and broken lines indicate the A ⇌ U transition curves of 2CM-3SS-BLA and intact BLA, respectively, which were calculated from $\Delta G^{H_2O}_{AU}$ and m_{AU} listed in Table I. Open and closed circles indicate the zero-time ellipticities obtained from kinetic refolding experiments for 2CM-3SS-BLA in the presence of 1 mM EDTA and 1 mM $CaCl_2$, respectively.

that of intact BLA previously obtained (Kuwajima et al., 1985). The spectrum of 2CM-3SS-BLA is similar in shape to but smaller in intensity than that of intact BLA. There are two interpretations for this result. One is that the structure of the intermediate of 2CM-3SS-BLA differs from that of intact BLA. The other is that the intermediate of 2CM-3SS-BLA has the same structure as the A state of intact BLA has but that it is less stable than the A state of intact BLA. The unfolding curve of the transient intermediate and the CD spectrum of 2CM-3SS-BLA in the acid state may indicate the validity of the latter interpretation. Figure 4 shows the unfolding transition of the transient refolding intermediate of 2CM-3SS-BLA which has been obtained from refolding experiments at various GdnHCl concentrations in a manner as described previously (Ikeguchi et al., 1986a). For comparison, the corresponding unfolding curve for intact BLA is also shown in Figure 4. The unfolding curves in Figure 4 indicate that the intermediate of 2CM-3SS-BLA is less stable than that of intact BLA and is partly unfolded even at 0.35 M GdnHCl (pH 7). As shown in Figure 3, in the acid state, the CD spectrum of 2CM-3SS-BLA is essentially identical to that of intact BLA. The acid state of intact BLA has been known to be the pure A state, and the A state is known to be more stable at acid pH than at neutral pH (Kuwajima et al., 1976). Therefore, the identity of the CD spectra of the two proteins at pH 2 suggests that the acid state of 2CM-3SS-BLA is also the pure A state. In the following, the equilibrium unfolding curve of 2CM-3SS-BLA will, thus, be analyzed on the basis of the three-state model as used previously for intact BLA.

Comparison of the Stability of the N and A States between 2CM-3SS-BLA and BLA. The free energy changes of the N ⇌ U and A ⇌ U transitions (ΔG_{NU} and ΔG_{AU}, respectively) calculated in the transition zone linearly depend on GdnHCl concentration and are fitted to the following equation:

$$\Delta G_{ij} = \Delta G^{H_2O}_{ij} - m_{ij}[\text{GdnHCl}] \quad (1)$$

where i and j denote a conformational state, i.e., N, A, or U, and $\Delta G^{H_2O}_{ij}$ means the free energy change of the $i \rightleftharpoons j$ transition in water. The parameter m_{ij} is related to the difference in the protein–solvent interaction between the i and j states and is approximately proportional to the difference

Table I: Comparison of the Conformational Free Energy and the Solvation Parameter of the N and A States between BLA and 2CM-3SS-BLA (pH 7.0, [Na^+] = 0.1 M, 4.5 °C)

	[Ca^{2+}] (mM)	BLA[a]	2CM-3SS-BLA
$\Delta G_{NU}^{H_2O}$ (kcal/mol)	0	3.66 ± 0.25	1.26 ± 0.29
	1	6.98 ± 0.39	3.86 ± 1.20
m_{NU} (kcal/(mol·M))	0	2.27 ± 0.16	2.11 ± 0.62
	1	2.56 ± 0.14	2.30 ± 0.74
$\Delta G_{AU}^{H_2O}$ (kcal/mol)		1.33 ± 0.20	0.15 ± 0.07
		(0.87 ± 0.29)[b]	
m_{AU} (kcal/(mol·M))		0.91 ± 0.11	0.58 ± 0.06
		(0.62 ± 0.17)[b]	

[a] Ikeguchi et al. (1986a). [b] The values were recalculated using only the data below 2 M GdnHCl (see text).

in solvent-accessible surface area between the two states (Schellman, 1978, 1987; Alonso & Dill, 1991). The fitted parameters are summarized and compared with the corresponding parameters for intact BLA in Table I. The increments in $\Delta G_{NU}^{H_2O}$ caused by addition of 1 mM Ca^{2+} are 2.6 (±1.2) kcal/mol for 2CM-3SS-BLA and 3.3 (±0.5) kcal/mol for intact BLA and reflect the stabilization of the N state by Ca^{2+}. From these values, the Ca^{2+} binding constants of the proteins in the N state are estimated to be 1.1×10^5 M^{-1} and 4.1×10^5 M^{-1} for 2CM-3SS-BLA and intact BLA, respectively [see Ikeguchi et al. (1986b) for derivation of Ca^{2+} binding constant]. The modification of the 6–120 disulfide does, thus, not affect remarkably the Ca^{2+} binding ability of BLA. The result is consistent with the known structural similarity of 2CM-3SS-BLA and intact BLA. The difference in $\Delta G_{NU}^{H_2O}$ between 2CM-3SS-BLA and intact BLA is 2.4 (±0.4) kcal/mol in the absence of Ca^{2+} and 3.1 (±1.3) kcal/mol in the presence of 1 mM Ca^{2+}. Although these values are smaller than the value expected from an increase in chain entropy brought about by cleavage of the disulfide bond in the U state [4.8 kcal/mol at 4.5 °C (Poland & Scheraga, 1965)], the discrepancy has been interpreted in terms of geometric strain imposed on the 6–120 disulfide bond (Kuwajima et al., 1990).

In Figure 4, the theoretical unfolding curve of the A state of 2CM-3SS-BLA, which has been calculated from $\Delta G_{AU}^{H_2O}$ and m_{AU} listed in Table I, is shown, and it is well coincident with the zero-time ellipticity in kinetic refolding. This coincidence demonstrates the identity between the A state observed in equilibrium unfolding and the transient intermediate trapped in kinetic refolding as previously shown for intact BLA (Ikeguchi et al., 1986a). It is clear from Figure 4 that the A state of 2CM-3SS-BLA is less stable than that of intact BLA. Thus, the 6–120 disulfide bond has a stabilization effect on the secondary structure in the A state. The mechanism by which the 6–120 disulfide bond stabilizes the A state is discussed below.

Influence of Negative Charges of S-Carboxymethyl Groups. In order to ask whether or not the negative charges of *S*-carboxymethyl groups introduced into 2CM-3SS-BLA affect the structure and the stability of this protein, the equilibrium unfolding of 2CAM-3SS-BLA was also investigated by CD spectra. The CD spectrum of 2CAM-3SS-BLA is identical with that of 2CM-3SS-BLA (not shown), indicating close structural similarity between the two proteins. The results of the GdnHCl-induced unfolding experiments for 2CAM-3SS-BLA are included in Figure 1 and compared with those for 2CM-3SS-BLA. The unfolding transition curves of 2CAM-3SS-BLA agree well with those of 2CM-3SS-BLA. Therefore, the effect of the negative charges of *S*-carboxymethyl groups in 2CM-3SS-BLA on the stabilities of both the N and A states is negligible.

DISCUSSION

Contribution of the 6–120 Disulfide to the Stability of the Native State. The contribution of an individual interaction to the conformational stability of a protein can be estimated from the difference in net stability brought about by disruption of that interaction. This is true, however, only when the modifications introduced into the protein molecule for abolishing the interaction do not perturb any other interaction and when the introduced groups do not produce any extraneous interaction. As the stability measured is a relative one against the U state, the interactions present in the U state must also be taken into account. In the following discussion, we distinguish between the intramolecular interaction within the protein molecule and the intermolecular interaction of the protein molecule with the solvent.

First, we consider the influence of the disulfide reduction and S-carboxymethylation on the intramolecular interactions in the N and U states. In the N state, the intramolecular interaction directly correlates with the coordinates of each atoms that compose the protein molecule. Although the three-dimensional structure of 2CM-3SS-BLA has not yet obtained, spectroscopic evidences indicate that the native conformation of 2CM-3SS-BLA is essentially identical with that of intact BLA. We assume, therefore, that the influence of the modification on the intramolecular interactions other than the 6–120 disulfide bond, if any, is negligible in the N state. Besides the influence of the modification on the interactions originally present in intact BLA, the possibility that the introduced groups produce some extraneous interactions without any large conformational changes must be taken into account. That is, the negative charges of *S*-carboxymethyl groups attached to Cys6 and Cys120 are expected to be in close proximity in the N state of 2CM-3SS-BLA, and then they may repel each other and lead to destabilization of the native conformation. However, such an effect is also negligible, because the equilibrium unfolding transition of 2CM-3SS-BLA coincides with that of 2CAM-3SS-BLA. It has been known, on the other hand, that the U state of intact BLA is regarded as a randomly coiled conformational state (Fish et al., 1970; Izumi et al., 1983; Dolgikh et al., 1985). As the CD spectrum of the U state of 2CM-3SS-BLA is identical with that of intact BLA (Figure 3), the U state of 2CM-3SS-BLA may also be a randomly coiled state. In the U state, therefore, there is no specific intramolecular interaction for both intact BLA and 2CM-3SS-BLA.

Although the intermolecular interaction between the protein and solvent molecules in the N or U state is not measured directly in the present study, the difference in the interaction between the N and U states can be estimated from the m_{NU} value (Schellman, 1978, 1987; Alonso & Dill, 1991). The m_{NU} value of 2CM-3SS-BLA is close to that of intact BLA both in the absence and in the presence of Ca^{2+} (Table I). Although the m_{NU} value of 2CM-3SS-BLA is slightly smaller than that of intact BLA both in the absence and in the presence of Ca^{2+}, this small difference is due to the difference in GdnHCl concentration at which the m_{NU} value has been determined. For both intact BLA and 2CM-3SS-BLA, the m_{NU} value tends to increase slightly with GdnHCl concentration [compare the m_{NU} values in the absence and presence of Ca^{2+}, and see also Ikeguchi et al., (1986b)]. Therefore, the m_{NU} values of intact BLA and 2CM-3SS-BLA should be compared at the same GdnHCl concentration. The m_{NU} of 2CM-3SS-BLA

in the presence of 1 mM Ca^{2+}, which has been determined at 1.5–1.9 M GdnHCl, agrees well with the m_{NU} of intact BLA in the absence of Ca^{2+}, which has been determined at 1.1–2 M GdnHCl. The difference in protein–solvent interactions between the N and U states is, therefore, similar for both proteins. This is consistent with the fact that the side chains of Cys6 and Cys120 of α-lactalbumin are exposed to solvent in the native conformation (Acharya et al., 1989, 1990). Exposure of these side chains suggest that the carboxymethyl groups introduced in 2CM-3SS-BLA are exposed in both the N and U states and exert no influence upon the difference in the protein–solvent interaction between the N and U states. Recently, Doig and Williams (1991) have proposed the effect of the disulfide bond to stabilize the native state by decreasing the solvent-accessible surface area in the unfolded state. As intact BLA and 2CM-3SS-BLA assume essentially the same conformation in the N state, the solvent accessible area in the N state is essentially identical for both proteins. If the 6-120 disulfide bond of intact BLA reduces the solvent-accessible surface area in the U state, the m_{NU} value for intact BLA should be smaller than that of 2CM-3SS-BLA. The present results are inconsistent with this expectation, so that the solvent-accessible surface area may be identical for both intact BLA and 2CM-3SS-BLA. In conclusion, the difference in $\Delta G_{NU}^{H_2O}$ between intact BLA and 2CM-3SS-BLA is attributed to a decrease in chain entropy in the U state and elimination of the strain imposed on the 6–120 disulfide in the N state of BLA (Kuwajima et al., 1990).

Contribution of the 6–120 Disulfide to the Stability of the Molten Globule State. From the similarity of the CD spectra, 2CM-3SS-BLA and intact BLA may have similar backbone structures in the A state. For human α-lactalbumin, the three-disulfide derivative and the disulfide-intact protein are known to have similar compact conformations in the A state (Ewbank & Creighton, 1991). Nevertheless, the m_{AU} of 2CM-3SS-BLA is significantly smaller than that of intact BLA. This apparent discrepancy of the results may also be explained by the deviation from the linear dependence of ΔG_{AU} on GdnHCl concentration, as already seen in the dependence of ΔG_{NU} above. The m_{AU} value as well as the m_{NU} value increases with GdnHCl concentration, as indicated by a slightly concave shape of the plot of $-\Delta G_{AU}$ against GdnHCl concentration [see Figure 4b of Ikeguchi et al. (1986b)]. Therefore, the difference in GdnHCl concentration at which the m_{AU} has been obtained (0.3–2 M for 2CM-3SS-BLA and 1.3–2.5 M for intact BLA) must lead to the difference in m_{AU} between 2CM-3SS-BLA and intact BLA. We have, thus, recalculated the m_{AU} of intact BLA using only the data below 2 M GdnHCl. The recalculated parameters are also included in Table I. The m_{AU} thus obtained is in good agreement with the m_{AU} of 2CM-3SS-BLA, being consistent with the close similarity in the A state conformation between 2CM-3SS-BLA and intact BLA.

From the present study, the 6–120 disulfide has a stabilization effect on the secondary structure in the A state. The stabilization of the A state by the disulfide bond may be due, at least in part, to reduction of the chain entropy of the protein molecule in the U state. Although the polypeptide chain in the A state has a considerably larger degree of conformational freedom than in the N state, the degree of freedom must be restricted when compared with that in the U state, as indicated by disulfide rearrangement studies of human α-lactalbumin (Ewbank & Creighton, 1991). Therefore, the decrease in the chain entropy brought about by the disulfide cross-linking must be smaller in the A state than in the U state. It is unknown whether or not the 6–120 disulfide has only localized effects on the structures formed in the A state. Taking into account the lack of the specific tertiary interactions in the A state, however, it is very likely that the 6–120 disulfide affects only the structure present in the loop formed by the disulfide. In the N state, the A helix (residues 5–11), half of the B helix (23–34), and 3_{10}-helices (12–16, 17–21, and 115–119) are present in the loop formed by the disulfide (Acharya et al., 1989, 1990). If some of them are formed in the A state, their stability may be affected by the disruption of the 6–120 disulfide. Efforts to identify the structural elements affected by the 6–120 disulfide and to exploit the mechanisms of stabilization by the disulfide are in progress.

Comparison with Lysozyme. Recently, two groups have studied the structure and stability of the three-disulfide derivative of hen egg white lysozyme, in which the disulfide bond between Cys6 and Cys127 is reduced and S-carboxymethylated (Denton & Scheraga, 1991; Radford et al., 1991; Cooper et al., 1992). The 6–127 disulfide bond of lysozyme corresponds to the 6–120 disulfide of the homologous protein, α-lactalbumin. This derivative of lysozyme assumes the same native structure as intact lysozyme, and the stability of the native structure of the derivative is significantly reduced. These results are similar to the present results for α-lactalbumin and may be consistent with the structural homology of the two proteins (Acharya et al., 1989, 1990). Owing to the reduced stability of the native structure, the derivative of lysozyme is found to unfold at pH 2 in contrast to intact lysozyme. However, the acid unfolded state does not show the properties that have been seen in CD and NMR spectra of the molten globule state of α-lactalbumin (Radford et al., 1991). On the other hand, our previous results have shown that intact lysozyme can assume the molten globule state that can be identified in kinetic refolding experiments at pH 1.5 (Kuwajima et al., 1985; Ikeguchi et al., 1986a). However, this apparent discrepancy of the results may be explained if the 6–127 disulfide of lysozyme has some stabilization effects on the molten globule state of this protein like the 6–120 disulfide of α-lactalbumin does. As shown in our previous study (Ikeguchi et al., 1986a), the molten globule state of intact lysozyme is less stable than that of α-lactalbumin. Therefore, a small destabilization of the molten globule state by the cleavage of the 6–127 disulfide may be enough so that the molten globule state becomes no longer detectable for the three-disulfide derivative of lysozyme.

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