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Reduction and Renaturation of Hen Egg Lysozyme Containing Carboxymethylcysteine-6 and -127†

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ABSTRACT: The thermodynamic stability of an enzymatically active derivative (sample LH_1) of hen egg lysozyme containing three, presumably, native disulfide bonds and carboxymethylated cysteines at positions 6 and 127 and exhibiting characteristics of the intact enzyme has been studied with respect to the formation of disulfide bonds. The procedure is to measure the equilibrium between the native and nonnative forms, after reequilibrating the disulfide bonds in the presence of β-mercaptoethanol, by gel filtration on Bio-Gel P-30. The native form has a lower hydrodynamic volume than the nonnative forms. To test whether the equilibrium has been reached, three different disulfide bonded forms of sample LH_1 (native, reduced, and "scrambled") are exposed to the same concentration of β-mercaptoethanol (1.5 mM) at pH 8.0 and 37 °C, which permits the disulfide interchange. All three samples show similar ratios (3:2) of native to nonnative forms after 16 h. This ratio is reached within 5 h in the case of native sample LH_1. Consequently the apparent equilibrium appears to be reached. In these same conditions intact lysozyme shows only the native form. Thus, sample LH_1 is less stable than the intact protein with respect to disulfide bond formation, but more stable than the nonnative isomers of sample LH_1 obtained by reshuffling the disulfide bonds. This latter point has been tested by measuring the proportions of all the partially reduced forms of sample LH_1 by trapping the free sulfhydryl groups present in the equilibrium mixture with iodoacetic acid.

Pancreatic ribonuclease A (RNase A) renatures after reduction and reoxidation, indicating that the free energy associated with a native protein containing a set of disulfide bonds is distinctly lower than that associated with its isomers containing nonnative sets of disulfide bonds (Epstein et al., 1963; Anfinsen, 1967). On the other hand, if the rearrangement of disulfide bonds by sulfhydryl-disulfide interchange is not permitted to occur in the reoxidation system of reduced RNase A, only nonnative species are formed (Venetianer & Straub,

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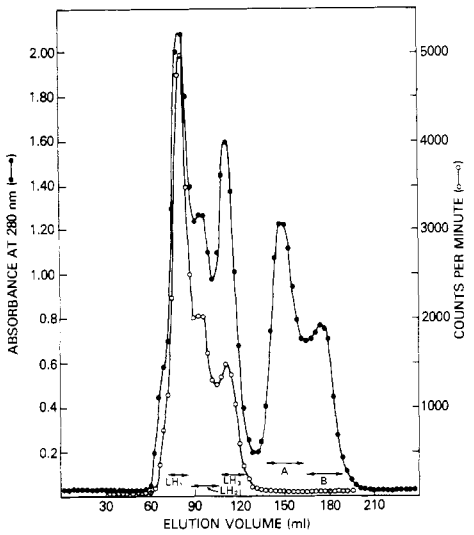

FIGURE 1: Preparative ion-exchange chromatography of the lower hydrodynamic volume form species generated during air-oxidation of reduced lysozyme for 10 min trapped by carboxymethylation with [1-^{14}C]iodoacetic acid. Approximately 50 mg of the lower hydrodynamic volume forms was taken in approximately 2 mL of 0.1% ammonium acetate/0.01 M acetic acid (pH 4.8) and applied to a Bio-Rex 70 column (2.2 × 40 cm) equilibrated and eluted with 0.2 M phosphate buffer, pH 7.15 at 23 °C. The elution of the protein samples was monitored by measuring the absorbance at 280 nm (●—●) and that of the carboxymethylated species by determining the radioactivity of 0.2-mL aliquots (○—○).

1964; Givol et al., 1964; Andria & Taniuchi, 1978). Since in such a system a pair of cysteinyl residues must be in proximity in order to form a disulfide bond, it would follow that the frequency of the native-like fold of reduced protein, permitting the formation of the native set of disulfide bonds (without the following rearrangement of the bonds), is not greater than that of the other folds of the polypeptide chain resulting in the formation of nonnative sets of disulfide bonds. Then it would further follow that, only after the formation of one or more disulfide bonds, the free energy associated with the native or native-like fold of the polypeptide chain (containing native disulfide bonds) is distinctly lower than that associated with the nonnative fold, i.e., the isomers containing nonnative disulfide bonds.

Though in the case of RNase A (Anfinsen, 1967) or hen egg lysozyme (Isemura et al., 1961; White, 1962; Epstein & Goldberger, 1963; Saxena & Wetlaufer, 1970), the species containing four native disulfide bonds is of lowest free energy, it is not clear at the moment whether the species containing less than four native disulfide bonds also represent species of lowest free energy as compared with the corresponding nonnative isomers. Thus, it will be of interest to know the differences in the free energy between the intermediate species in the renaturation of reduced RNase A or lysozyme (namely, those containing one or two or three native disulfide bonds and six, four, and two sulfhydryl groups, respectively) and their isomers (containing nonnative sets of the same number of disulfide bonds and sulfhydryl groups). This difference in free energy should determine the extent or probability of the renaturation (mediated by sulfhydryl–disulfide interchange) at given oxidation–reduction levels of sulfhydryl group–disulfide bond of the protein (in a hypothetical situation in which no "flow" of the species from and to the different oxidation–reduction levels occurs).

Creighton (1974, 1975, 1977b) has reported that a species of pancreatic trypsin inhibitor containing one particular native disulfide bond and four sulfhydryl groups and another containing two particular native disulfide bonds and two sulfhydryl groups are thermodynamically favorable over their isomers. Light & Sinha (1976) have shown that a trypsinogen derivative containing carboxymethylcysteine-179 and -203 renatures in the yield of 50% after reduction and oxidation. We have observed (Acharya & Taniuchi, 1977) that a native-like lower hydrodynamic volume structure is formed from the derivatives of hen egg lysozyme in which the formation of one of the four native disulfide bonds (one at a time) is blocked. These studies have given an insight to the thermodynamic stability of the intermediate species as compared with their isomers at the same oxidation–reduction level. However, since these experiments are designed primarily for the investigation of the renaturation process, in these experimental systems the equilibrium between the species at the same oxidation–reduction level could be perturbed by the flow of the species from or to the different oxidation–reduction levels or the equilibrium of the system is reached often from only one starting state.

In this communication, we have investigated the thiol-catalyzed equilibration between a derivative of hen egg lysozyme containing, presumably, three native disulfide bonds and carboxymethylcysteine-6 and -127 and its isomers containing nonnative sets of three disulfide bonds, using three different disulfide bonded forms exposed to the same final conditions permitting the disulfide interchange. The combined results of the present studies and those reported previously (Acharya & Taniuchi, 1977) are interpreted as implying the thermodynamic stability of native lysozyme containing one open disulfide bond over its individual isomers containing nonnative sets of three disulfide bonds and one open disulfide bond.

Materials and Methods

The procedures for the preparation of reduced hen egg lysozyme, gel filtration of the reoxidation products on Bio-Gel P-30 columns, and ion-exchange chromatography of lysozyme and its derivatives on Bio-Rex 70 columns have been described previously (Acharya & Taniuchi, 1976). The analytical methods employed have also been described previously (Acharya & Taniuchi, 1976) unless specified otherwise.

Denatured lysozyme containing presumably nonnative disulfide bonds and showing a higher hydrodynamic volume than native lysozyme was prepared by reoxidation of reduced lysozyme at pH 8.0, 37 °C, in the presence of 4×10^{-5} M Cu^{2+}. The reoxidized material was fractionated on columns of Bio-Gel P-30 to isolate the higher hydrodynamic volume form (Acharya & Taniuchi, 1976). Ultracentrifugal analysis at 20 °C by the high-speed sedimentation equilibrium method of Yphantis (1964) using a Beckman-Spinco Model E analytical centrifuge of these higher hydrodynamic volume forms (concentration 0.1 mg/mL in 0.01 M acetic acid/1% ammonium acetate, pH 4.8; Acharya & Taniuchi, 1976) showed that these materials are a monomeric form.

Preparation of Sample LH_1. The lysozyme derivative, sample LH_1, containing three, presumably, native disulfide bonds and [1-^{14}C]carboxymethylcysteine-6 and -127 was prepared from reduced lysozyme incubated for 10 min for air-oxidation (no thiol groups added) and carboxymethylated using an 80-fold molar excess [1-^{14}C]iodoacetic acid at pH 8.0 (no EDTA added) by the methods described previously (Acharya & Taniuchi, 1976). Figure 1 presents the pattern of the preparative ion-exchange chromatography of the lower (native) hydrodynamic volume form species. The material contains three radioactive species, samples LH_1, LH_2, and LH_3 (Acharya & Taniuchi, 1976) and a major and a minor nonradioactive species. The major nonradioactive species eluted

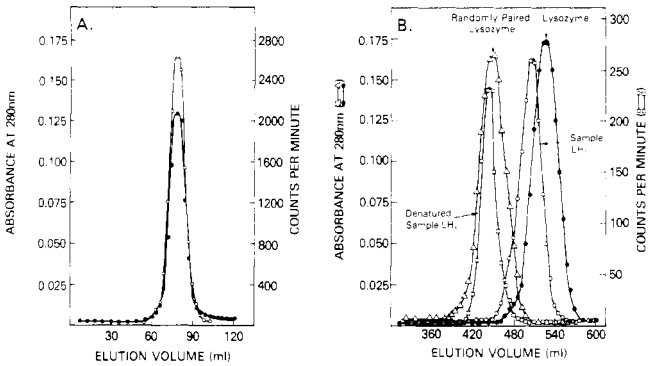

FIGURE 2: (A) Rechromatography of sample LH_1 obtained in the experiment described in Figure 1. The methods of ion-exchange chromatography are the same as those described in Figure 1. Absorbance at 280 nm (●—●); radioactivity (○—○). (B) Gel filtration of sample LH_1 and denatured sample LH_1. A Bio-Gel P-30 column (2 × 200 cm) was equilibrated and eluted with 0.1% ammonium acetate/0.01 M acetic acid, pH 4.8. Approximately 0.5 mg of sample LH_1 (○—○) mixed with 5 mg of intact lysozyme was applied to the Bio-Gel column. Similarly, denatured sample LH_1 (0.4 mg) (□—□) was also mixed with monomeric, denatured lysozyme. Sample LH_1 was detected by measuring the radioactivity (2-mL aliquots in A and 1-mL aliquots in B) and lysozyme by measuring the absorbance at 280 nm.

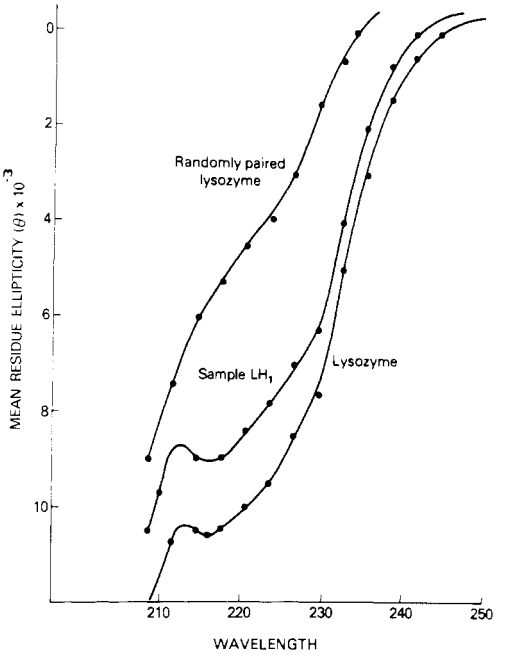

FIGURE 3: Circular dichroism spectra of sample LH_1, native and denatured lysozyme. All spectra were taken at a protein concentration of 0.05 mg/mL in 0.1% ammonium acetate/0.01 M acetic acid at pH 4.8 at 25 °C. Molecular ellipticities [θ] are calculated on the basis of average residue weights.

at the position corresponding to native lysozyme and the minor species eluted slightly after the major species. Both the major and the minor species exhibited the enzymic activity similar to that of native lysozyme. The amino acid composition of this minor species was the same as that of native lysozyme. On rechromatography, most of the minor species eluted at the position of native lysozyme with only a small amount of the material eluting at the original position as a hump of the main peak. Thus, it appears that the major nonradioactive species is renatured metastable form and can be converted to the native form.

The radioactive sample LH_1 (Figure 1) was desalted by gel filtration on a Sephadex G-25 column, lyophilized, rechromatographed on the same Bio-Rex 70 column (Figure 2A), again desalted and lyophilized. This rechromatographed radioactive sample is referred to as sample LH_1 below. Sample LH_1 was homogeneous by disc electrophoresis in the presence of 8 M urea (Acharya & Moore, 1973).

Reduction and Reoxidation of Sample LH_1. Sample LH_1 was reduced in the presence of 8.0 M urea by incubation with 0.3 M β-mercaptoethanol at 23 °C for 16 h. The reduction mixture was acidified to pH 4.0 with acetic acid and desalted by gel filtration using 0.1 M acetic acid. This desalted reduced sample LH_1 (0.025 mg/mL) was air-oxidized in 0.1 M Tris/acetic acid, pH 8.0, containing 1.5 mM β-mercaptoethanol at 37 °C for 24 h. Then, the reoxidation mixture was adjusted to pH 5.0, dialyzed against 0.1 M acetic acid at 23 °C, lyophilized, desalted, and relyophilized. This radioactive material was referred to as reduced–reoxidized sample LH_1.

Circular Dichroism Measurements. The CD spectra of solutions of sample LH_1 and the lysozyme species (0.05 mg/mL) were measured with a Cary Model 6001 recording spectropolarimeter equipped with a CD attachment using a 3-mL quartz cuvette of 1-cm light path.

Results

Properties of Sample LH_1. Sample LH_1 elutes in the elution position of the lower (native) hydrodynamic volume form by gel filtration and exhibits approximately 35% of enzymic activity of lysozyme (Acharya & Taniuchi, 1976). However, it is to be noted that the elution position of sample LH_1 is slightly earlier than that of lysozyme (Figure 2B).

The ultraviolet absorption spectrum from 230 to 300 nm of sample LH_1 in 0.2 M phosphate buffer (pH 7.0) is similar to that of native protein except for a slightly higher absorbance of sample LH_1 in the region of 250 nm. The uncorrected fluorescence emission spectrum of sample LH_1 exhibits the maximum around 338 nm, at pH 7.0 (see Figure 4, the pattern of fluorescence spectrum at zero time) and is similar to that of intact lysozyme (Steiner, 1964; Lehrer & Fasman, 1967). Addition of 6 M guanidine hydrochloride to sample LH_1 increased the λ_{max} to 350 nm with a concomitant increase in the fluorescence intensity (approximately 35%) as is observed with intact lysozyme treated in the same way.

The CD spectra of sample LH_1, and denatured lysozyme (see Materials and Methods) from 210 nm to 240 nm are compared with that of native lysozyme in Figure 3. While denatured lysozyme shows a featureless spectrum, sample LH_1 exhibited a negative ellipticity in the region of 220 nm characteristic of the helical structure (Greenfield & Fasman, 1969). The magnitude of this ellipticity is close to that of native lysozyme. These physicochemical and enzymic characteristics of sample LH_1 indicate that the folding of the polypeptide chain of sample LH_1 is similar to that of native lysozyme. On this basis, the disulfide bonds of sample LH_1 are assumed to be of native nature.

Proteolysis of Sample LH_1 with Trypsin. Native lysozyme is resistant to proteolysis (Imoto et al., 1972; Atassi et al., 1973). No change in the tryptophan emission spectrum of lysozyme was observed even after 5 h incubation with trypsin (an enzyme to substrate ratio, 1:60) at pH 7.0, 23 °C. On the contrary, when sample LH_1 was incubated with trypsin under similar conditions, the emission maximum of the fluorescence shifted from 338 nm to a higher wavelength with a concomitant decrease in fluorescence intensity as a function of time (Figure 4) (after 3 h incubation a maximum around 350 nm). If the extent of this decrease in the fluorescence intensity at 330 nm (at which the maximum difference fluorescence intensity between the original sample and the 3 h digest is observed) is

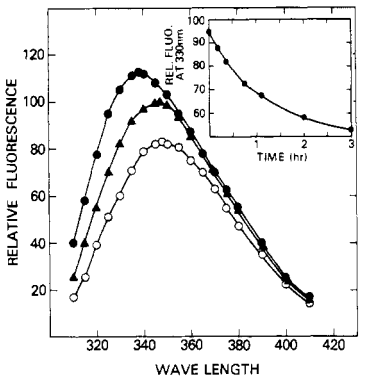

FIGURE 4: Changes of tryptophan fluorescence of sample LH_1 incubated with trypsin as a function of time. Sample LH_1 was incubated (at a concentration of 0.2 mg/mL) at 23 °C with trypsin in 0.2 M phosphate buffer, pH 7.0 at an enzyme to substrate ratio by weight of 1:60. Zero time (●—●); 40 min (▲—▲); and 3 h (○—○). The inset shows the kinetics of the fluorescence changes at 330 nm (see text) on incubation with trypsin. The excitation was carried out at 290 nm.

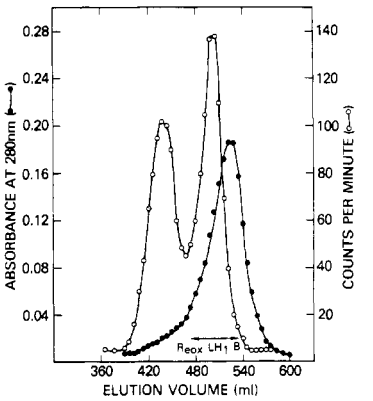

FIGURE 5: Gel filtration of reduced-reoxidized sample LH_1. The reduced-reoxidized sample LH_1 was mixed with carrier intact lysozyme and applied to a Bio-Gel P-30 (2 × 200 cm) column. For other experimental details, see the legend to Figure 2B. Counts per minute, radioactivity of a 0.5-mL aliquot.

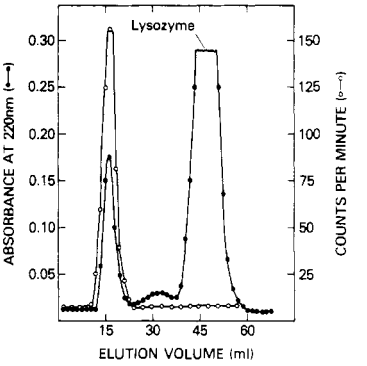

FIGURE 6: Ion-exchange chromatography of the lower hydrodynamic volume form material from reduced-reoxidized sample LH_1 containing carrier intact lysozyme on a Bio-Rex 70 column (0.9 × 35 cm) at pH 7.1. Elution profile of protein material was obtained by measuring the absorbance at 220 nm (●—●) and that of sample LH_1 by the radioactivity (0.5-mL aliquots) (○—○).

assumed to approximate the degree of proteolysis, the digestion of sample LH_1 appears to proceed at a uniform rate from the beginning (Figure 3, inset).

Incubation of sample LH_1 at pH 7 with trypsin (an enzyme to substrate ratio, 1:100) resulted also in progressive loss of the enzymic activity (50% loss in 3 h at 23 °C, or in 45 min at 37 °C). Although the enzymic activity of sample LH_1 incubated with trypsin at 37 °C decreases without a lag phase, in the 23 °C incubation experiment a small lag phase (approximately 30 min) was observed. The reason for the apparent difference in the presence and absence of a lag phase between the fluorescence measurement and the enzymic activity assay of sample LH_1 incubated with trypsin at 23 °C is unknown with the exception that the ratio (by weight) of trypsin to sample LH_1 was greater in the former experiment than in the latter experiment. On the other hand, the enzymatic activity of native lysozyme did not change on incubation with trypsin at 23 °C after 24 h. It is known that trypsin releases only the COOH-terminal leucine of lysozyme (Atassi & Habeeb, 1969) and that removal of the two COOH-terminal residues has no influence on the enzymatic activity and the ability of the protein to refold after reduction and oxidation (Morgan & Riehm, 1968; Johnson et al., 1976).

Characterization of the Renatured Material Formed after Reduction and Reoxidation of Sample LH_1. Reduced-reoxidized (radioactive) sample LH_1 was mixed with native lysozyme. Upon gel filtration of this mixture approximately 60% of the radioactivity eluted at the position of lower (native) hydrodynamic volume form and the rest 40% eluted at a position corresponding to the higher hydrodynamic volume form (Figure 5). When this lower hydrodynamic volume form material was isolated and subjected to ion exchange chromatography (Figure 6), all the radioactivity eluted at the position expected for original sample LH_1. This radioactive material exhibited a tryptophan fluorescence emission maximum around 338 nm (see above) and approximately the same level of enzymatic activity as that of sample LH_1.

Reoxidation (pH 8.0, 37 °C, 16 h) in the presence of β-mercaptoethanol (1.5 mM) of reduced lysozyme formed approximately 95 and 5% of the lower and the higher hydrodynamic volume form, respectively, as expected from the reported high degree of generation of enzymic activity by oxidation in the presence of β-mercaptoethanol of reduced lysozyme (Epstein & Goldberger, 1963).

Denaturation and Subsequent Renaturation by Sulfhydryl-Disulfide Interchange of Sample LH_1. Sample LH_1 was incubated with 1 mM β-mercaptoethanol in the presence of 8 M urea at pH 7.5, 23 °C, for 16 h. The reaction mixture was desalted and lyophilized. Upon gel filtration of this radioactive sample mixed with denatured (nonradioactive) lysozyme, all the radioactivity eluted at the position of higher hydrodynamic volume form (Figure 2B) in contrast to original sample LH_1. This radioactive higher hydrodynamic volume form species (0.025 mg/mL) was incubated at 37 °C, pH 8.0, with 1.5 mM β-mercaptoethanol. After 16-h incubation, the protein sample was isolated by dialysis and lyophilization. Upon gel filtration of the sample, approximately 55 and 45% of the radioactivity eluted at the positions of the lower and the higher hydrodynamic volume form, respectively. By ion-exchange chromatography this lower hydrodynamic volume form sample eluted at the position expected for original sample LH_1.

Stability of Sample LH_1 to Thiol Catalyzed Sulfhydryl-Disulfide Interchange. The elution position by gel filtration of intact lysozyme did not change after incubation with 1.5 mM β-mercaptoethanol at pH 8.0, 37 °C, for 16 h. However, when sample LH_1 (0.025 mg/mL) was incubated under the same conditions for 1, 5, and 16 h (in three different experiments), the distributions of the lower and the higher hydrodynamic volume forms were 65 and 35%, 58 and 42%, and 60 and 40%, respectively. When a mixture of equimolar amounts of sample LH_1 (radioactive) and reduced lysozyme was in-

TABLE I: Distributions of Sample LH_1 as the Lower (LH) and Higher (HH) Hydrodynamic Volume Forms after Treatment by Various Procedures.

procedure[a]	distribution HH form (%)	distribution LH form (%)
reoxidation of reduced LH_1 in presence of β-mercaptoethanol for 24 h	40	60
LH_1 incubated with β-mercaptoethanol for 1 h	35	65
LH_1 incubated with β-mercaptoethanol for 5 h	42	58
LH_1 incubated with β-mercaptoethanol for 16 h	40	60
LH_1 incubated with an equimolar amount of reduced lysozyme for 16 h	55	45
the higher hydrodynamic volume form of LH_1 incubated with β-mercaptoethanol for 16 h	45	55

[a] All the incubations were carried out at pH 8.0, 37 °C, and the concentration of β-mercaptoethanol was 1.5 mM (see text).

cubated at pH 8.0, 37 °C, for 16 h, approximately 45 and 55% of the radioactive sample converted to the lower and the higher hydrodynamic volume form, respectively. Incubation of sample LH_1 at pH 8.0, 37 °C, for 16 h in the absence of free thiol groups resulted in no changes in its hydrodynamic volume character.

In an attempt to assess the changes in the redox potential of this system, the concentration of sulfhydryl groups of β-mercaptoethanol at various intervals was determined using Ellman's reagent (Ellman, 1959) in a control experiment in which the protein component is omitted in the incubation mixture. No significant changes in the concentration (1.5 mM) of sulfhydryl groups were detected at least up to 6 h; however, after 24-h incubation the concentration of sulfhydryl groups decreased by 10 to 15%, presumably due to air-oxidation.

Examination of Free Sulfhydryl Groups. The lower hydrodynamic volume form sample isolated from reduced-reoxidized sample LH_1 did not contain any detectable amounts of free sulfhydryl groups, when analyzed with the Ellman's reagent. The higher hydrodynamic volume form sample contained about 0.2 mol of free sulfhydryl groups. In order to test whether the partially reduced species had been reoxidized during the purification, cold sample LH_1 (prepared by the methods described under Materials and Methods using cold iodoacetic acid instead of the [^{14}C]iodoacetic acid) was incubated at a concentration of 0.025 mg/mL with 1.5 mM β-mercaptoethanol at pH 8.0, 37 °C, for 16 h. Then, the mixture was reacted with threefold molar excess (to the amount of β-mercaptoethanol) of [1-^{14}C]iodoacetic acid at 23 °C for 1 h,[1] and the protein was isolated by dialysis, lyophilization, and desalting. The material thus isolated contained approximately 0.85 mol of [^{14}C]carboxymethyl groups. Upon gel filtration approximately 55 and 45% of the material eluted in the positions of the lower and the higher hydrodynamic volume forms, respectively. The samples of former and the latter forms contained approximately 0.8 and 1.2 mol of [^{14}C]carboxymethyl groups, respectively. On the basis of these values, a calculation would show that at least approximately 60% of the lower hydrodynamic volume form (approximately 36% of the total population) and maximum approximately 80% of the higher hydrodynamic volume form (approximately 32% of the total population) could be three disulfide bonded (no free sulfhydryl groups) species.

Discussion

In the present studies three different disulfide bonded forms of sample LH_1 have been exposed to the same conditions, which permits the disulfide interchange, and then the populations of the higher and the lower hydrodynamic volume forms have been determined (Table I). First, reduced sample LH_1 was air-oxidized in the presence of 1.5 mM β-mercaptoethanol at pH 8.0, 37 °C, for 24 h, and approximately 60 and 40% of the reoxidized sample distributed in the lower and higher hydrodynamic volume forms, respectively. Second, when denatured sample LH_1 containing, presumably, nonnative sets of disulfide bonds was incubated with 1.5 mM β-mercaptoethanol at pH 8.0, 37 °C, for 16 h, the lower and the higher hydrodynamic volume forms were generated in 55 and 45% yields, respectively. Third, sample LH_1 (containing three, presumably, native disulfide bonds) was incubated with 1.5 mM β-mercaptoethanol at pH 8.0, 37 °C, for 1, 5, and 16 h, resulting in the formation of the lower and higher hydrodynamic volume forms in approximately 65 and 35%, 58 and 42%, and 60 and 40% yields, respectively. This redistribution of sample LH_1 into the lower and the higher hydrodynamic volume forms does not occur in the absence of free thiol compounds.

Therefore, irrespective of the fact whether the system is started from reduced sample LH_1 or denatured sample LH_1 or original sample LH_1, the final distribution of the lower and higher hydrodynamic volume forms in each of these cases is nearly the same (Table I). A control experiment had indicated that the concentration of sulfhydryl groups of β-mercaptoethanol remains constant during the initial 6-h incubation and decreases by 10 to 15% after the 24-h incubation. Thus, these systems containing approximately 60 and 40% of the lower (native) and higher hydrodynamic volume forms, respectively, (in the presence of 1.5 mM β-mercaptoethanol at pH 8.0 at 37°) appear to be in a quasi-equilibrium state in terms of the protein species present.

Creighton (1977a) has observed that oxidation of reduced pancreatic trypsin inhibitor by oxidized glutathione or hydroxyethyl disulfide (disulfide groups (S–S) excess over sulfhydryl groups (SH) in the system) yields small populations of the species containing mixed disulfide formed between cysteine residues and glutathione or mercaptoethanol in addition to the species containing no mixed disulfide. Therefore, it could be possible that sample LH_1 (S–S) forms a mixed disulfide with β-mercaptoethanol (SH). However, Hantgen et al. (1974) have shown that when reduced RNase A was subjected to the reaction with the mixture of equal moles of reduced (SH) and oxidized (S–S) glutathione, no appreciable mixed disulfide was detected. Price et al. (1969) have also observed that air-oxidation of reduced pancreatic DNase (containing four sulfhydryl groups) in the presence of calcium ion and β-mercaptoethanol (SH excess over S–S) results in the formation of the enzymically active species containing one disulfide bond and two sulfhydryl groups. In fact, Bradshaw et al. (1967) have reported that when a mixed disulfide of reduced lysozyme and cysteine is incubated at pH 8.0 with β-mercaptoethanol (SH excess over S–S), all the mixed disulfide bonds get rearranged to the protein disulfide bonds within a minute. On the basis of these considerations, we assume that the species containing mixed disulfide bonds, if any, is negligible in the present system (SH excess over S–S). This hypothesis is supported by the

[1] In a control experiment when 1.5 mM β-mercaptoethanol was reacted with a threefold molar excess of iodoacetate, the half-time of reaction is about 2 min. This value is in agreement with the studies by Barron (1951). A preliminary experiment has indicated that the reaction of lysozyme derivative with tenfold molar excess over 1.5 mM β-mercaptoethanol present for 1 h at pH 8.0 23 °C does not result in detectable carboxymethylation of lysine and histidine residues by amino acid analysis and that methionine residues also appear not to be affected by this reaction.

observation that the incubation of sample LH_1 (radioactive) with reduced lysozyme (instead of β-mercaptoethanol) resulted in approximately equal distributions of the radioactive material into the higher and the lower hydrodynamic volume forms (Table I). In this case, if the formation of the mixed disulfide was appreciable, a dimeric form of lysozyme–sample LH_1 species should have been formed and eluted earlier than the higher hydrodynamic volume form by gel filtration (Acharya & Taniuchi, 1976).

The reactions taking place in the quasi-equilibrium state of the protein species would be the equilibrium reaction between the isomeric species (disulfide interchange) and the oxidation–reduction reaction (flow of the protein species between the different oxidation–reduction levels). The latter reaction would also involve air-oxidation of sulfhydryl groups of the protein species and reduction of the disulfide bond thus formed by β-mercaptoethanol (the net result is air-oxidation of β-mercaptoethanol[2]). The content of free sulfhydryl groups has been estimated only in one (the third experiment) of the three incubation experiments described above. Therefore, the oxidation–reduction states in the two other experiments are not exactly known. However, it is unlikely that there is an extreme difference in the oxidation–reduction state of the protein species (at the end of incubation) between these three experiments for the following reasons.

The completely reduced species should elute exclusively in the higher hydrodynamic volume position by gel filtration as previously shown with reduced lysozyme (Acharya & Taniuchi, 1976). Therefore, provided that the population ratio of the native to the nonnative isomers of sample LH_1 species does not increase with the increase in the extent of reduction of disulfide bonds and that the equilibrium reaction of the isomers by disulfide interchange is fast, the content of sulfhydryl groups (average value for mol of sulfhydryl group per mol of protein) of the higher hydrodynamic volume form would tend to be greater than that of the lower hydrodynamic volume form. This hypothesis is consistent with the results of determination of free sulfhydryl groups in the third experiment.

In a preliminary experiment, incubation of lysozyme containing scrambled disulfide bonds with 1.5 mM β-mercaptoethanol at pH 8.0 at 37 °C resulted in approximately 80% renaturation (conversion from the higher to the lower hydrodynamic volume form) in 10 min. Thus, the rate of sulfhydryl-catalyzed equilibration of the isomeric species appears to be faster than that of air-oxidation of reduced lysozyme (see footnote 2). In this experiment, it was found that the lower hydrodynamic volume form contained the three disulfide bonded species (having two sulfhydryl groups), completely renatured species, and a small amount of the two disulfide bonded species (having four sulfhydryl groups). No appreciable population of the one disulfide bonded species (having six sulfhydryl groups) was present in this lower hydrodynamic volume form as is the case with the lower hydrodynamic volume form obtained in the early air-oxidation of reduced lysozyme (in the absence of β-mercaptoethanol) (Acharya & Taniuchi, 1976).

Thus, an increase in the extent of reduction of sample LH_1 species may be assumed to have a tendency to increase the higher hydrodynamic volume form population with a concomitant decrease in the lower hydrodynamic volume form population and an increase in the extent of oxidation to have the reverse effect.

On the basis of these considerations, the similarity of the ratio of the higher to the lower hydrodynamic volume form in the three experiments may be interpreted as indicating that there is no extreme difference in the oxidation–reduction level of the protein species. Then, it would follow that the distribution of sample LH_1 isomers (three disulfide bonded species) in the lower and the higher hydrodynamic volume forms estimated in the third experiment is close to that in the equilibrium state of these isomers and, therefore, sample LH_1 (native) is thermodynamically favorable over its nonnative isomers.

As reported earlier (Acharya & Taniuchi, 1977) all eight isomers containing three, presumably, native disulfide bonds and one free and one carboxymethylated sulfhydryl group, are formed, in comparable yields, on air-oxidation in the presence of 1.5 mM β-mercaptoethanol (pH 8.0, 37 °C, 36 h) from the reduced forms of respective species. The two of these eight isomers should be that containing carboxymethylcysteine-6 and a sulfhydryl group at residue 127 and that containing carboxymethylcysteine-127 and a sulfhydryl group at residue 6. The stability of these two isomers may be assumed to be similar to that of sample LH_1. Then, it would follow that the thermodynamic stabilities of all the eight isomers over their nonnative isomers are comparable to that of sample LH_1. This may imply that each of the four isomers of partially reduced lysozyme containing three native disulfide bonds and two sulfhydryl groups would also be thermodynamically favored over the isomers containing nonnative sets of three disulfide bonds and two same sulfhydryl groups. These considerations support the hypothesis (Acharya & Taniuchi, 1977) that these four native isomers containing two sulfhydryl groups may have a native-like fold of the polypeptide chain and thereby the two sulfhydryl groups of each isomer may be spatially located in proximity. Thus, the fourth disulfide bond in these isomers could be directly formed upon oxidation.

The four disulfide bonds of native lysozyme are considered to be buried inside the molecule (Shrake & Rupley, 1973). Therefore the presence of carboxymethyl groups at one or two of the cystine residues of lysozyme may be expected to perturb the interatomic interactions in the three-dimensional structure to at least some extent. Indeed, while the ratio of the "native" population of sample LH_1 to that nonnative in the apparent equilibrium state is measurable, intact lysozyme does not yield any detectable amounts of nonnative species upon incubation with 1.5 mM of β-mercaptoethanol at pH 8.0, in the absence of a denaturant. Sample LH_1 is also susceptible to proteolysis in contrast with intact lysozyme being resistant against proteolysis. A slight increase in the hydrodynamic volume of sample LH_1 as compared with intact lysozyme was also being observed. However, it is not known at the present time whether this destabilization of sample LH_1 is due solely to the stereochemical effect of carboxymethyl groups at residues 6 and 127 on the atomic coordinates of native lysozyme (Blake et al., 1965) or whether the disruption by reduction of any one of the native disulfide bonds results in similar destabilization, even if carboxymethyl groups are not attached to the cysteinyl residues. In any event, it seems that the force maintaining the native three-dimensional structure, though perturbed, is still operative in sample LH_1.

In relation to this point, it should be recalled that, if the native-like conformation is formed in reduced lysozyme in

[2] Approximately 50% of sulfhydryl groups of reduced lysozyme is air-oxidized in about 30 min at 37 °C in the presence of 10^{-6} M Cu^{2+} (catalyzing the oxidation) (Acharya & Taniuchi, 1976). Therefore, if reduced sample LH_1 is air-oxidized at the same rate (even in the absence of Cu^{2+}) (for approximation, assuming the apparent first-order kinetics) and if 0.85 mol of free sulfhydryl group per mol of protein (see Results) is maintained in the incubation mixture in this system of air-oxidation and reduction by β-mercaptoethanol of the protein species, the net result would be oxidation of approximately 3% of the total concentration of β-mercaptoethanol in 24 h.

which no carboxymethyl groups are attached, it would have only a short life (no stabilizing force operative) in that oxidation of reduced lysozyme (without rearrangment of the disulfide bonds formed) does not appear to result in the formation of the native set of disulfide bonds but instead results in the formation of non-native sets of four disulfide bonds (Acharya & Taniuchi, 1976) as is the case with reduced RNase A (Anfinsen et al., 1961; Hantgan et al., 1974). Removal of 4 or 6 carboxy-terminal residues of RNase A also destabilizes the native conformation (Lin, 1970) resulting in lack of renaturation after reduction and oxidation (Taniuchi, 1970; Andria & Taniuchi, 1978). In the case of reduced pancreatic DNase subjected to air-oxidation, no disulfide bond is formed unless calcium ion is added to the system (Price et al., 1969). It would be of interest to examine whether calcium ion binds to reduced pancreatic DNase to induce a conformation in which a pair of sulfhydryl groups are in proximity.

An apparent dispensability of one or more disulfide bonds of many proteins for the maintenance of their biologically active structure has been reported (see Acharya & Taniuchi, 1976). However, studies on the reduction and renaturation of proteins lacking one or more native disulfide bonds are limited except for the studies by Light & Sinha (1976) on the refolding of trypsinogen containing carboxymethylcysteine-179 and -203, and those by Creighton (1977a) on the refolding of derivatives of pancreatic trypsin inhibitor lacking the disulfide bond between residues 14 and 38 either as the dicarboxymethylated derivative or as the monocarboxymethylated species, as well as the one lacking the disulfide bond between residues 5 and 55. The reduced forms of all these derivatives have been found to have the ability to form the "native" species in a significant population.

In summary, it is suggested that the force responsible for the formation of the three-dimensional structure of native lysozyme may be still operative, though it could be perturbed, when any one of the four disulfide bonds is open in the form of two sulfhydryl groups.

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