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Biochemistry

Formation of the four isomers of hen egg white lysozyme containing three native disulfide bonds and one open disulfide bond

(protein folding/renaturation of reduced proteins/native hydrodynamic volume/intermediates/carboxymethylated lysozyme)

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ABSTRACT **Reduced partially carboxymethylated hen egg white lysozyme (mucopeptide *N*-acetylmuramoylhydrolase; EC 3.2.1.17) (approximately 0.8 mol of [*1*-^{14}C]carboxymethyl groups) was air oxidized at pH 8.0 and 37° in the presence of 1.5 mM 2-mercaptoethanol for 36 hr. Gel filtration of this product gave the lower (native) and higher hydrodynamic volume forms, both containing radioactivity (approximately 35 and 65%, respectively). Ion exchange chromatography of the lower hydrodynamic volume forms yielded renatured lysozyme, two major radioactive samples (LH_C and LH_D) eluting at the positions of monocarboxymethylated lysozyme, and two minor radioactive samples eluting at the positions of dicarboxymethylated lysozyme. Sample LH_C (approximately 23% of the radioactivity) was essentially homogeneous with respect to electrophoretic mobility, exhibited approximately 39% of the enzymic activity of lysozyme, and contained 0.95 mol of [^{14}C]carboxymethyl groups. Sample LH_D (approximately 8% of the radioactivity) was also enzymically active and contained approximately 0.5 mol of [^{14}C]carboxymethyl groups; this low value is apparently due to contamination of noncarboxymethylated species. The radioactive tryptic peptides from samples LH_C and LH_D were characterized. The results indicated that all eight isomers, containing three presumably native disulfide bonds and one free and one carboxymethylated sulfhydryl group, are formed on air oxidation of reduced partially carboxymethylated lysozyme. Since in each of these isomers the formation of one of the four native disulfide bonds is permanently blocked, it would follow that no one of the four disulfide bonds of native lysozyme is obligatory in the formation of the other three native disulfide bonds.**

We have previously shown (1) that during renaturation of reduced hen egg white lysozyme (mucopeptide *N*-acetylmuramoylhydrolase; EC 3.2.1.17) by air oxidation, three enzymically active isomers containing three, presumably native, disulfide bonds and one open disulfide bond between Cys 6 and Cys 127 or between Cys 64 and Cys 80 or between Cys 76 and 94 (structures I, III, and IV, Fig. 1) are formed. However, the fourth isomer, having the open disulfide bond between Cys 30 and Cys 115 (structure II, Fig. 1), was not found. Interestingly, in the native three-dimensional structure the Cys 30—Cys 115 disulfide bond is the only disulfide bridge having a helix on both sides (3).

This failure to detect the fourth isomer raises the question whether it is because the disulfide bond between Cys 30 and 115 is "obligatory" (4) for the formation of other native disulfide bonds or because of the limitations of the methods, in which only the species having "native"-like hydrodynamic volume were examined (1). It is also possible that pairing of Cys 30 and Cys 115 by oxidation is extremely fast, so that it is hard to trap the fourth isomer. The results described in this communication indicate that all the four isomers (Fig. 1), containing three native disulfide bonds and one open disulfide bond, are formed on renaturation of reduced lysozyme that has one of the eight cysteinyl residues, presumably, randomly [*1*-^{14}C]carboxymethylated.

Abbreviation: LH forms, lower hydrodynamic volume forms.

MATERIALS AND METHODS

The procedures for preparation of reduced hen egg white lysozyme, gel filtration of the air-oxidation products on a BioGel P-30 column (2 × 200 cm), ion exchange chromatography of lysozyme and its derivatives on a Biorex-70 column, ion-exchange chromatography of tryptic peptides of carboxymethylated lysozyme, measurement of radioactivity, and assay of enzymic activity (5) have been described (1). Electrophoretic analysis of lysozyme and its derivatives was carried out using the urea/polyacrylamide gel system (8 M urea, pH 4.8/7.5% gel) (6).

Preparation of Reduced, Partially [*1*-^{14}C]Carboxymethylated, Oxidized Lysozyme. Reduced lysozyme (4.0 mg/ml) (1) was reacted with approximately 0.8 mol equivalent of iodo[*1*-^{14}C]acetic acid (New England Nuclear) in 0.1 M Tris/acetate, pH 8.0, containing 6 M guanidine·HCl at 23° for 90 min. The reaction mixture was desalted on a Sephadex G-25 column (1). The desalted solution containing reduced, partially carboxymethylated lysozyme was incubated at 37° in 0.1 M Tris/acetate, pH 8.0, containing 1.5 mM 2-mercaptoethanol at a protein concentration of 0.025–0.04 mg/ml for 36 hr for air oxidation (1). The reoxidation mixture was then adjusted to pH 4.0, dialyzed against 0.1 M acetic acid, lyophilized, and desalted by gel filtration. This sample is referred to as reduced, partially [*1*-^{14}C]carboxymethylated, oxidized lysozyme. A control experiment showed that under these conditions unmodified reduced lysozyme was completely renatured, as judged by gel filtration analysis (1) and measurement of enzymic activity (5).

RESULTS

On gel filtration of reduced, partially [*1*-^{14}C]carboxymethylated, oxidized lysozyme, approximately 65 and 35% of the radioactivity eluted as the higher and the lower (native) hydrodynamic volume forms (LH forms), respectively (Fig. 2*A*) (1). The higher hydrodynamic volume forms contained 1.25 ± 0.05 mol of [*1*-^{14}C]carboxymethyl groups whereas the LH forms had 0.6 ± 0.05 mol of [*1*-^{14}C]carboxymethyl-groups. Repeated gel filtration of the LH forms resulted in the elution of all the radioactivity at the lower hydrodynamic volume position (Fig. 2*B*). However, the peak position of absorbance at 280 nm did not exactly correspond with that of radioactivity (Fig. 2*B*).

Ion-exchange chromatography of the LH forms obtained after repeated gel filtration yielded five samples (Fig. 3). One of these eluted at the position expected for native lysozyme, had

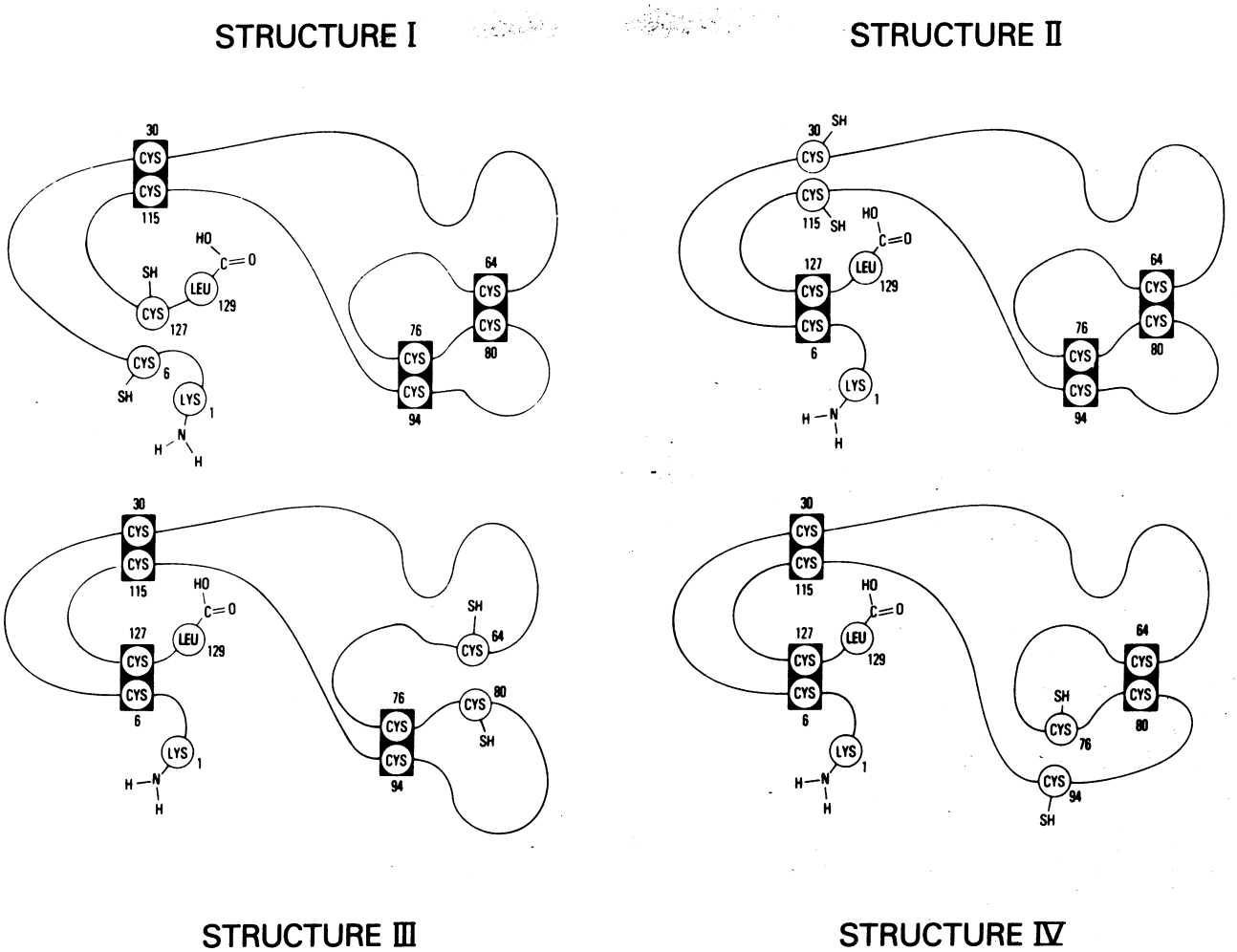

FIG. 1. Diagrammatic representation of the four isomeric structures of lysozyme containing three native disulfide bonds and one open disulfide bond on the basis of Canfield and Liu's (2) presentation of the amino acid sequence.

the same specific enzymic activity as that of native lysozyme, and did not contain any radioactivity. The four other samples, eluting earlier than this completely renatured lysozyme, were radioactive. These are designated in the order of their elutions as LH_A, LH_B, LH_C, and LH_D (Fig. 3) and contained 5, 8, 65, and 23%, respectively, of the radioactivity of the LH forms.

Samples LH_A and LH_B eluted at the positions corresponding to those of the previously isolated dicarboxymethylated species LH_1 (carboxymethylation at Cys 6 and 127) and LH_{2B} (carboxymethylation at Cys 76 and 94), respectively (1). However, the samples LH_A and LH_B have not been further characterized. The chromatographic positions of samples LH_C and LH_D corresponded to those of monocarboxymethylated species LH_{2C} (carboxymethylation at Cys 76) and LH_3 (carboxymethylation at Cys 80), respectively (1).

Sample LH_C had approximately 39% of the enzymatic activity of lysozyme. It exhibited a tryptophanyl fluorescence emission spectrum similar to that of lysozyme with an emission maximum at 338 nm (1) and contained 0.95 ± 0.05 mol of [1-^{14}C]carboxymethyl groups. This sample was essentially

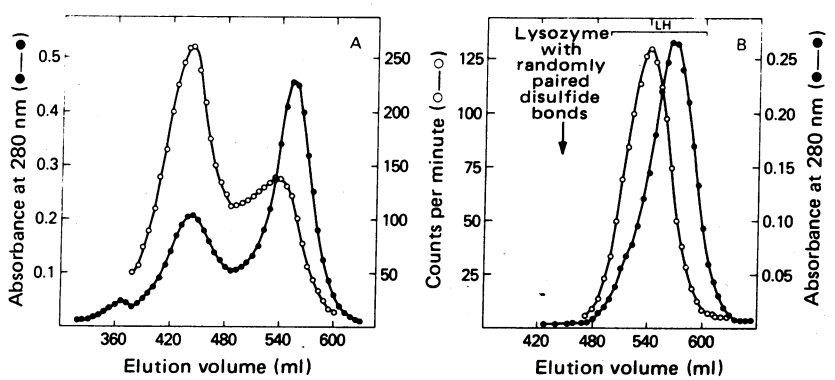

FIG. 2. Gel filtration of reduced, partially [1-^{14}C]carboxymethylated, oxidized lysozyme on a BioGel P-30 column at 23°. (*A*) Reduced, partially [1-^{14}C]carboxymethylated, oxidized lysozyme. (*B*) Repeat gel filtration of the lower hydrodynamic volume forms (LH forms). The LH form fractions obtained from *A* were pooled and lyophilized. The dried material was dissolved in approximately 30 ml of 0.1 M acetic acid and relyophilized. This sample was dissolved in 2 ml of 0.01 M acetic acid/0.1% ammonium acetate (pH 4.8) and applied to the column.

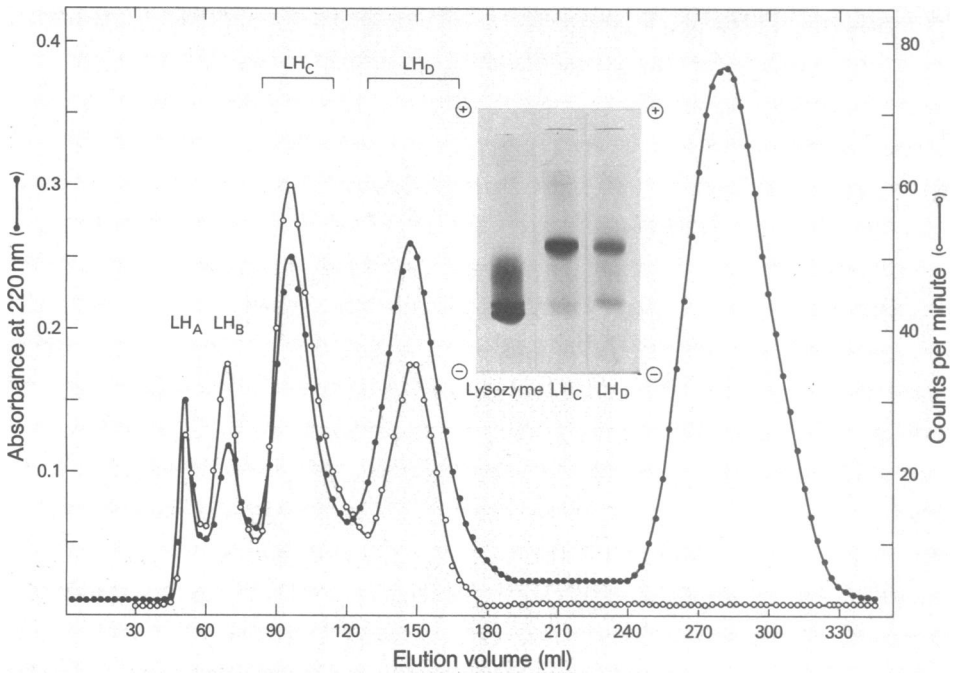

FIG. 3. Ion-exchange chromatography of the LH forms of reduced, partially [1-^{14}C]carboxymethylated, oxidized lysozyme. The sample obtained from the repeated gel filtration (Fig. 2*B*) was dissolved in 1 ml of 0.01 M acetic acid/0.1% ammonium acetate and applied to a Biorex-70 column (12.2 × 36 cm) equilibrated with 0.2 M sodium phosphate, pH 7.05 (1). The radioactive fractions designated as LH_A, LH_B, LH_C, and LH_D were separately pooled, dialyzed against 0.1 M acetic acid, and lyophilized. (*Inset*) The polyacrylamide gel electrophoresis of lysozyme and samples LH_C and LH_D. Approximately 20 μg of each sample was used for electrophoresis (6) at 3 mA per tube for 2.5 hr at 23°. The proteins were stained with Coomassie blue. The disc electrophoretic pattern of lysozyme shows the presence of a small amount of more acidic material as a smearing zone. The ion-exchange chromatography of lysozyme on a Biorex column (1) also showed approximately 6% of the contaminants eluting earlier than lysozyme (7, 8). It has been suggested that the minor contaminants could be deamidation products of lysozyme (9). The presence of these minor contaminants in the lysozyme sample would not affect the conclusion of the present studies.

homogeneous on urea/polyacrylamide gel electrophoresis and migrated more slowly toward the cathode than the native lysozyme. A minor component (about 5–8% by visual inspection) of this sample, presumably due to contamination from sample LH_D (see below), had an electrophoretic mobility similar to that of lysozyme (Fig. 3 *inset*). Thus, sample LH_C appears to be monocarboxymethylated lysozyme.

Sample LH_D contained 0.5 ± 0.05 mol of [1-^{14}C]carboxymethyl groups and had approximately 45% enzymatic activity. It gave two bands on urea/gel electrophoresis, one corresponding to the major band of sample LH_C and the other to lysozyme (Fig. 3 *inset*). Thus, it appears that sample LH_D contained both mono- and noncarboxymethylated species. Bradshaw *et al.* (10) have detected, on ion-exchange chromatography of the activation product of mixed-disulfide derivatives of lysozyme, a species eluting earlier than native lysozyme and having approximately 60% the enzyme activity of lysozyme. Since the chromatographic position of sample LH_D approximately corresponds to this species, the noncarboxymethylated species in LH_D could be one such species.

Ion-exchange chromatography (11) of the tryptic digest of reduced and carboxymethylated sample prepared from sample LH_C by the method described previously (1) (Fig. 4) indicated that all the tryptic peptides expected to contain carboxymethylcysteines were radioactive (Table 1). Interesting is the presence of radioactivity in peptides T_6, T_9, and T_{15}, containing carboxymethylcysteine 30, 64, and 115, respectively (Table 1). None of the partially oxidized LH forms trapped with [1-^{14}C]iodoacetic acid in the earlier study (1) yielded these three [1-^{14}C]carboxymethylated peptides. For sample LH_D (Fig. 5), almost all the radioactivity was present in peptide T_{11}, as was the case with species LH_3 (1). The small amounts of radioactivity present in T_3 and T_{15} fractions (Fig. 5) are presumably due to the contaminations from sample LH_C.

To show that the monocarboxymethylated species of samples LH_C and LH_D indeed contain three disulfide bonds and one free sulfhydryl group and one [1-^{14}C]carboxymethylcysteine, we reacted the LH forms of reduced, partially [1-^{14}C]carboxymethylated, oxidized lysozyme with a 10-fold molar excess of iodo[1-^{3}H]acetic acid (New England Nuclear) in 6 M guanidine·HCl, pH 8.0, at 23° for 30 min. Indeed, the sample obtained from the reaction mixture after desalting contained 0.8 ± 0.05 mol of [1-^{3}H]carboxymethylcysteine per mol of [1-^{14}C]carboxymethylcysteine. The slightly lower incorporation of tritium label compared to that of ^{14}C label into this sample could be due to the presence of small amounts of presumably dicarboxymethylated derivatives (LH_A and LH_B). Repeated gel filtration showed that the doubly labeled sample retained the lower hydrodynamic volume.

On the basis of quantities of the tryptic peptides containing [1-^{14}C]carboxymethylcysteine of samples LH_C and LH_D (Figs. 4 and 5), the relative quantities of the four isomers of lysozyme containing three native disulfide bonds and one open disulfide bond (structures I, II, III, and IV, Fig. 1) have been estimated (Table 2). The quantities of the four isomers thus determined are qualitatively comparable to each other.

DISCUSSION

The reactivities of all the eight cysteinyl residues of reduced lysozyme in 6 M guanidine·HCl at pH 8.0 with iodoacetic acid are expected to be comparable. Hence it is assumed that the distribution of carboxymethyl groups in the cysteinyl residues of reduced, partially carboxymethylated lysozyme is essentially random.

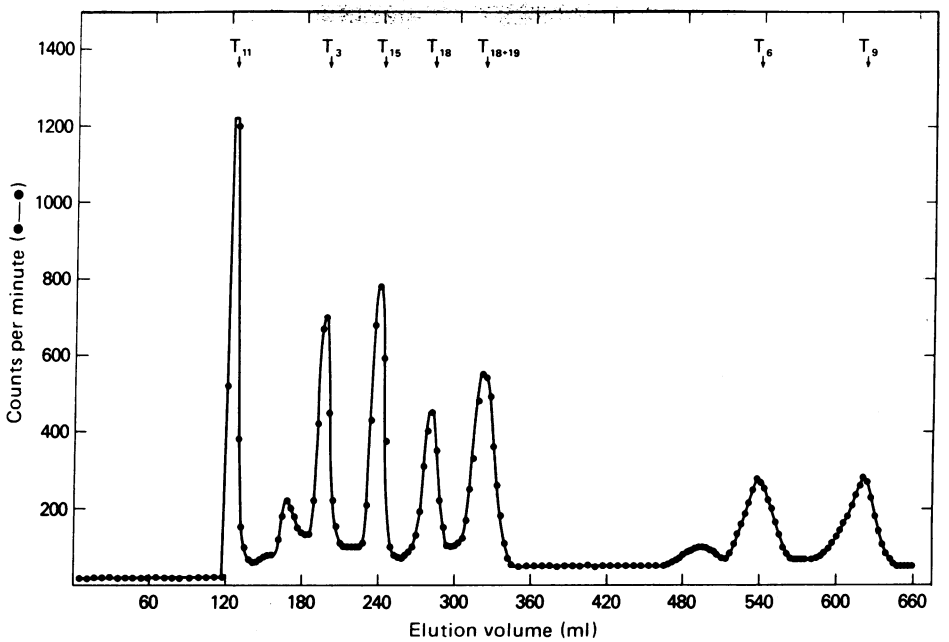

FIG. 4. Ion-exchange chromatography of the tryptic peptides (T) of sample LH_C. The tryptic digest (approximately 15 mg) (see the *text*) was subjected to ion-exchange chromatography according to Canfield's procedure (11), as described earlier (1).

Air oxidation of reduced, partially carboxymethylated lysozyme having approximately 0.8 mol of [1-^{14}C]carboxymethyl groups in the presence of 2-mercaptoethanol yields species containing 1 mol of [1-^{14}C]carboxymethyl groups and having the "native" hydrodynamic volume (samples LH_C and LH_D). Sample LH_C is enzymically active and exhibits the tryptophan fluorescence spectrum similar to that of native lysozyme. The [1-^{14}C]carboxymethyl groups of sample LH_C are distributed, in more or less comparable proportions, in all the cysteinyl residues with the exception that [1-^{14}C]carboxymethylcysteine 80 was present probably not in sample LH_C but in sample LH_D. Considering the molar quantity of [1-^{3}H]carboxymethyl groups incorporated into the [1-^{14}C]carboxymethylated LH forms, it is reasonable to assume that sample LH_C is a mixture of seven of the eight isomers containing three, presumably native, disulfide bonds and one carboxymethylcysteine and one free sulfhydryl group and that sample LH_D contains one remaining isomer. When, on the basis of the sets of three native disulfide bonds these eight isomers are grouped into the four structures (Fig. 1), the calculated yields of the four structures are comparable to each other (Table 2). These four structures must be formed from the respective species of the eight isomers of monocarboxymethylated reduced protein in which the formation of the respective native disulfide bond is permanently blocked. Thus, none of the four native disulfide bonds appears to be obligatory in the formation of the three other native disulfide bonds.

Table 1. Relative amounts of [1-^{14}C]carboxymethylcysteine residues in the tryptic peptides of reduced and carboxymethylated LH_C

Tryptic peptides*	Amino acid sequence†	Cysteine residues	% of the sum of the radioactivity
T_3	6–13	Cys 6	13
T_6	22–23	Cys 30	12
T_9	62–68	Cys 64	12
		Cys 76	
T_{11}	74–96	Cys 80	22
		Cys 94	
T_{15}	115–116	Cys 115	16
T_{17}	126–128	Cys 127	9
T_{17+18}	126–129	Cys 127	17

* From ref. 11.
† From ref. 2.

The previous study (1) has shown that the dicarboxymethylated forms of structures I and IV as well as the monocarboxymethylated forms of structures III and IV possess approximately 35% of the enzymic activity of lysozyme. Therefore it is likely that the individual monocarboxymethylated species contained in sample LH_C would have similar enzymic activity. If the noncarboxymethylated species of sample LH_D possesses only 60% of the enzymic activity of lysozyme, the monocarboxymethylated species of sample LH_D could have approximately 30% of the enzymic activity, if the molar ratio of the noncarboxymethylated to the monocarboxymethylated species would be 2–3 (see Fig. 3). The lowered activity of these species could be due to the slight perturbation in the three-dimensional structure due to the absence of the fourth native disulfide bonds.

On renaturation of reduced, partially carboxymethylated lysozyme only 35% of the material possessed lower (native) hydrodynamic volume structure, whereas the renaturation of unmodified reduced lysozyme under similar conditions yielded only the LH forms (1). When the higher hydrodynamic volume forms were reduced and reoxidized in the presence of 2-mercaptoethanol, again approximately 30% of the material was found in the LH forms. If the reoxidation of reduced, partially carboxymethylated lysozyme is carried out in the absence of 2-mercaptoethanol, only 6–8% of the material is found in the LH forms. Apparently, thiol-catalyzed sulfhydryl–disulfide interchange is operative in this renaturation, as it is in native lysozyme (1). Conceivably, this lower yield of the native hydrodynamic volume form for reduced, partially carboxymethylated lysozyme than for unmodified reduced lysozyme may be due to the smaller difference in free energy between

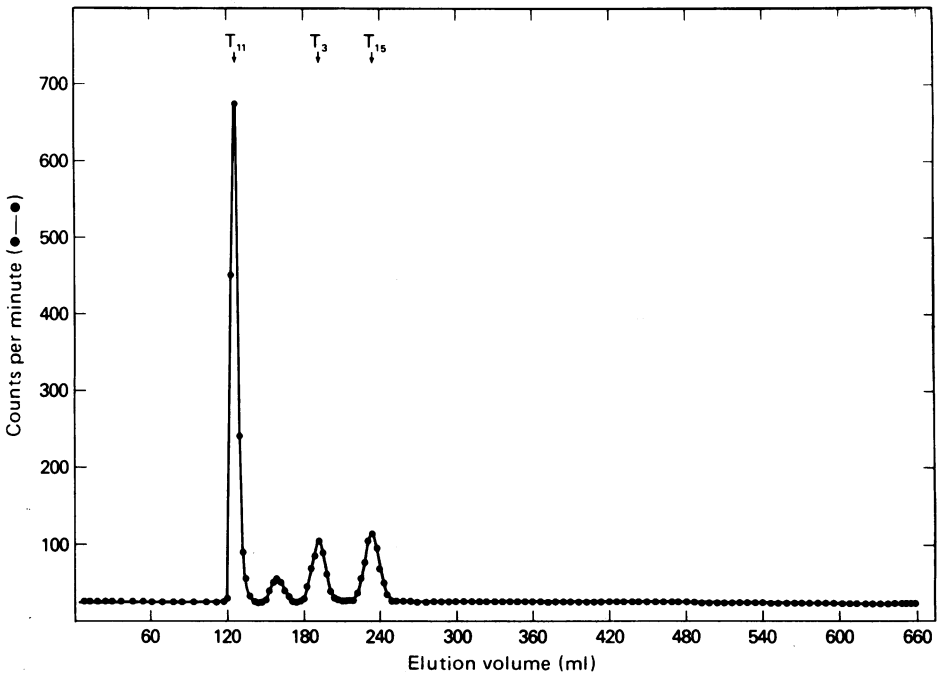

FIG. 5. Ion-exchange chromatography of the tryptic peptides (T) of sample LH_D. The conditions of chromatography were the same as those described for Fig. 4.

the lower and the higher hydrodynamic volume forms in the reduced, partially carboxymethylated lysozyme than in the reduced lysozyme.

In this study we have prepared all of the four forms shown in Fig. 1, corresponding to the eight monocarboxymethylated isomers of lysozyme. Each form contains three of the disulfide links of native lysozyme, the formation of the fourth link being blocked. Presumably, the mode of formation of disulfide bonds reflects the folding process (4, 12, 13). Our data indicate that there is no obligatory disulfide bond, the formation of which would be a necessary prelude to the formation of the others. We do not have evidence on the sequence of events leading to the formation of the four forms; it could involve either a random search, leading eventually to the formation of the three correct linkages, or a more ordered progression of events. It is clear in any case that the pattern of events must be different in each of the four forms. Presumably, the conformation of the polypeptide chain in each of these forms is fairly close to that of native lysozyme (3), so that the fourth disulfide bond would readily form if it were not blocked by carboxymethylation. It remains to be seen whether oxidation of cysteines 30 and 115 is extremely fast, or whether structure II is quickly converted to structure I, III, or IV through sulfydryl–disulfide interchange, so that this form was not trapped in the previous study (1).

Table 2. Relative yields of the four isomers containing three native disulfide bonds and one open disulfide bond

Isomers	% of the sum of the yields
Structure I	32.5
Structure II	23.3
Structure III	26.6
Structure IV	18.5

It is assumed that peptide T_{11} of sample LH_D contained only [*1*-^{14}C]carboxymethylcysteine 80 (see *text*) and that peptide T_{11} of sample LH_C was derived from both the monocarboxymethylated isomers containing [*1*-^{14}C]carboxymethylcysteines 76 and 94, respectively (see Table 1).

Anderson and Wetlaufer (13) have studied the regeneration of native from reduced lysozyme in the presence of glutathione. From their data they formulate a preferred, though not necessarily obligatory, sequence of disulfide bond formation, in which the formation of the link between residues 30 and 115 is one of the later events. Since the design of the present experiments is different from theirs, we cannot at present draw any definite conclusions concerning the apparent discrepancy between the findings of the two laboratories.

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