Comparison between the Folding of Reduced Hen Egg White Lysozyme and That of Reduced Human Milk Lysozyme†

Thierry Dubois,* Roger Guillard, Jean-Paul Prieels, and Jean-Paul Perraudin

ABSTRACT: In vitro, renaturation of reduced and unfolded lysozyme is catalyzed by a mixture of reduced and oxidized glutathione. After initiation of disulfide bond formation associated with the folding process of reduced human lysozyme, molecules have been trapped in a stable form with iodoacetic acid (preserving disulfide bonds) at various times of reoxidation. Each population of molecules trapped in this way was then analyzed by acrylamide gel electrophoresis which separates intermediates on the basis of the number of disulfide bonds they contain and the mean volume of the polypeptide chain. Moreover, the rate of reoxidation of the regeneration mixture was monitored by changes in enzymatic activity, fluorescence quantum yield, and global sulfhydryl group titer. Enzymatic activity was observed to appear after an induction period, and no intermediate, except the fully regenerated species, is active. The first two disulfide bonds reoxidize rapidly, and very few intermediates containing one or two disulfide bonds could be trapped. On the other hand, the intermediates containing three and four disulfide bonds are more predominant, and their formation proceeds more slowly. A folding pathway is suggested, based on the kinetic studies of appearance and disappearance of the various observed intermediates. When these results are compared with those obtained for hen egg white lysozyme and with those found in literature, it can be concluded that the reduced human protein recovers its native conformation more progressively and with more difficulty than the hen egg white protein. This difference might be explained by a greater organization and a greater hydrophobicity in the human molecule.

It is now generally well accepted that the three-dimensional structure of a polypeptide is entirely determined by its amino acid sequence and that no additional information is required for protein folding (Anfinsen, 1973). The main goal of folding studies is to predict the tertiary structure of a protein on the basis of its primary structure. Many miscellaneous experiments have been carried out to reach this objective. Two frequently used methods are insertion (or deletion) of amino acid residues (Frensdorff et al., 1967; Lin, 1970; Taniuchi, 1970; Puett, 1972; Creighton, 1978) and chemical (or enzymatic) modification of amino acid side chains (Wetlaufer, 1975; Andria & Taniuchi, 1978; Creighton, 1981). Surprisingly, folding studies of proteins with homologous but distinct sequences have been neglected in favor of more elaborate studies with proteins that have been modified. In homologous proteins, differences in amino acid sequences do not occur randomly: in any case, they have to remain in accordance with a refolded conformation that ensures biological activity (Ptitsyn, 1974). Human milk (HML)[1] and hen egg white lysozymes (HEWL) are two homologous proteins which have such similarities in their primary sequence, if they are of similar molecular size and basicity, they differ significantly in enzymatic activity and amino acid composition and are immunologically distinct (Tischendorf & Osserman, 1969). Determination of their tertiary structures by X-rays (Blake & Swan, 1971) and elucidation of their primary structures (Jollès, 1971; Canfield et al., 1971) clearly indicate that these two proteins are structurally chemically homologous.

Hen egg white lysozyme has 129 amino acid residues and human lysozyme, 130. The extra amino acid residue in HML is a glycine inserted in the region of the hinge of the β-pleated sheet at position 48 (Canfield et al., 1974). When the two sequences are aligned in order to maximize homology, HML is found to contain 53 amino acid substitutions (about 41% of the total); most of them (about 79%) are residues located at the surface of the molecule or protruding into the solvent. Some of the 11 internal substitutions affect residues buried just under the molecule surface; a few of them are found deep in the hydrophobic core. All things considered, the degree of homology between HML and HEWL may be estimated at 52% for surface residues and 74% for internal residues.

It is the purpose of this paper to identify and characterize, using physicochemical methods, the disulfide bond containing intermediates that appear, as the result of thiol–disulfide exchanges (in the course of the refolding HML), and to compare these results with those obtained for hen egg white lysozyme (Perraudin et al., 1979).

Experimental Procedures

Materials

Twice crystallized hen egg white lysozyme was purchased from Worthington Chemical Corp. and further purified by ion-exchange chromatography on a Bio-Rex 70 column (3 × 50 cm) equilibrated and eluted with a 0.2 M potassium phosphate buffer at pH 7.14. Human lysozyme was purified according to a previously described procedure (Barel et al., 1972).

Tris base (Trizma quality), dithiothreitol, iodoacetic acid, and an acetone powder of *Micrococcus luteus* cells were purchased from Sigma Chemical Co. Oxidized and reduced glutathiones were provided by Boehringer Mannheim, and Coomassie brilliant blue was from Merck Darmstadt. All other chemicals were of the best grade available.

Methods

Reduction. Human lysozyme (50 mg) and dithiothreitol (50 mg) were dissolved in 5 mL of 1 mM Tris-HCl buffer

† From the Laboratoire de Chimie Générale I, Université Libre de Bruxelles, 1050 Brussels, Belgium. *Received April 28, 1982.*

[1] Abbreviations: HML, human milk lysozyme; HEWL, hen egg white lysozyme; GSH, reduced glutathione; GSSG, oxidized glutathione; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); Tris, tris(hydroxymethyl)-aminomethane; EDTA, ethylenediaminetetraacetic acid.

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containing 10 M urea and 1 mM EDTA, pH 8.6. The reduction was allowed to proceed at room temperature for 2 h, under nitrogen bubbling. The reaction was stopped by lowering the pH to 3 with glacial acetic acid. The reduced enzyme was isolated by gel filtration with Sephadex G-25 (2 × 50 cm) equilibrated and eluted with 0.1 M acetic acid.

The fractions containing the protein were pooled. An aliquot was taken and analyzed by the slightly modified Ellman's method (1959). So that complete reduction of the enzyme could be checked for, a buffer containing 10 M urea was used to avoid enzyme precipitation and to allow complete exposure of free thiol groups. A standardization with cysteine was carried out under the same conditions to determine the new molar extinction coefficient (11 600 ± 200 mol^{-1} cm^{-1}).

Protein Concentration Measurement. Lysozyme concentration was determined spectrophotometrically with a Zeiss PMQII spectrophotometer; a $A_{280}^{1\%}$ of 25.5 for native HML and a $A_{280}^{1\%}$ of 22.4 for reduced HML were used according to Wetlaufer et al. (1977).

Regeneration Procedure. In the system described by Saxena & Wetlaufer (1970), the regeneration mixture contained 1 mM reduced glutathione, 0.1 mM oxidized glutathione, and 1 mM EDTA in a 0.1 M Tris–acetate buffer at pH 8.0. Regeneration was initiated by mixing 1 volume of reduced lysozyme in 0.1 M acetic acid with 1000 volumes of regeneration mixture. Lysozyme concentration in the regeneration mixture was 2.4×10^{-6} M, and reoxidation was allowed to proceed at 37 °C.

Stopping Regeneration and Blocking Thiols. The regeneration was stopped by converting the remaining free thiols into *S*-(carboxymethyl)cysteine. To the regeneration mixture were added solid urea and iodoacetic acid until final concentrations of 8 and 0.1 M, respectively, were reached. The pH dropped to 4 following the addition of iodoacetic acid and was immediately readjusted to 7 by adding 6 M NaOH. The S-carboxymethylation reaction was allowed to proceed at this pH for 15 min at room temperature. Glacial acetic acid was then added to decrease the pH to 3.

Concentration of the Protein. Partially reoxidized, S-carboxymethylated lysozyme was concentrated by subjecting the regeneration mixture to chromatography on a SP-Sephadex G-25 column (0.5 × 6 cm) previously equilibrated with 0.02 M sodium citrate containing 4 M urea, pH 4.0. The column was then washed with the conditioning buffer. Lysozyme was eluted with 0.02 M sodium citrate containing 4 M urea and 0.3 M NaCl at pH 5.5. The enzyme was desalted with a Sephadex G-25 column (1 × 14 cm) equilibrated and eluted with 0.1 M acetic acid.

S-(Carboxymethyl)cysteine Concentration. The *S*-(carboxymethyl)cysteine content of each sample was determined by amino acid analysis after acid hydrolysis in 5.6 N HCl at 105 °C for 24 h.

Lysozyme Assay. For each sample, a 0.05 mg/mL lysozyme solution was prepared in 0.066 M phosphate buffer at pH 6.2 containing 0.9% NaCl. A suspension (13 mg/100 mL) of *Micrococcus luteus* dried cells was prepared in the same buffer. To 2.5 mL of cell suspension was added 100 μL of the protein solution and mixed well in a 1.00-cm path length cuvette. The decrease of the turbidity was followed at 450 nm with a Zeiss PMQII spectrophotometer.

Fluorescence Measurements. Fluorescence was measured at 25 °C with a Hitachi Perkin-Elmer Model MPF-2A spectrofluorometer equipped with an Osram WBO 150-W xenon lamp and an RCA 1 P-28 photomultiplier. An aqueous solution of L-tryptophan served as a standard. The values obtained for the standard solution were compared before and after assays of the protein solution in order to correct for small mechanical instrument fluctuations. Both excitation and emission bandwidths were 5 nm. The lysozyme concentration never exceeded 3 μM. Under these conditions, fluorescence intensity could be linearly related to protein concentration. Quantum yields were estimated by using a neutral, aqueous tryptophan solution, with a yield of 0.13 as a reference (Chen, 1967).

Polyacrylamide Gel Electrophoresis. Electrophoresis was performed according to the technique outline by Paugin & Chulkley (1969). The protein was stained with Coomassie brilliant blue.

Resolution of Densitometer Tracings and Determination of Percentages of Intermediates for Each Reoxidation Time. The electrophoresis gels were scanned spectrophotometrically with a Beckman G2400 densitometer equipped with a tungsten lamp and a Gilford 2000 scanner. The scanning showed poorly resolved band structure for most of the densitometer tracings, and a numerical analysis of data was performed.

Gauss–Newton's method was chosen based on the fact that the shape of the bands can be evaluated coarsely from the densitometer tracings (Fraser & Suzuki, 1973). The parameters describing the bands were modified between experimental and computed points, assuming that each band is well described by the function

$$A(X,A_0,X_0,\Delta X_{1/2}) = fA_0 \exp\left(-\ln 2 \left[\frac{2(X-X_0)}{\Delta X_{1/2}}\right]^2\right) + \frac{(1-f)A_0}{1+\left[2\frac{X-X_0}{\Delta X_{1/2}}\right]^2} \quad (1)$$

in which the first term corresponds to Gauss's function and the second term to Cauchy's function where A is the abcissa, f is the proportion of each constituent ($0 \le f \le 1$), X is the coordinate, A_0 is the peak height, X_0 is the X coordinate of the peak, and $\Delta X_{1/2}$ is the bandwidth at half-height. The mean square deviation is minimized by annulling its partial derivative with respect to each parameter and is given by

$$S = \sum_{i+1}^{h} Y_i - F_i \quad (2)$$

where h is the number of experimental points and

$$F_i = \sum_{j=1}^{m} A_j(X_i,A_0^j,X_0^j,X_{1/2}^j) \quad (3)$$

where m is the number of peaks.

The initial program was modified by introducing a constraint on the ratio between height and bandwidth at half-height. In fact, those parameters, a priori independent, are linked as demonstrated by the analyses of well-resolved peaks.

For introduction of this constraint, a linear relation between the experimentally determined height and bandwidth at half-height was supposed. Peak adjustment necessitated this relation. After optimization, one is thus able to calculate each peak weight from the equation

$$W_j = \frac{(A_0^j \Delta X_{1/2}^j/2)[F_j(\pi/\ln 2)^{1/2} + (1-f)\pi]}{\sum_{i=1}^{m} W_i} \quad (4)$$

It can be observed that the adopted values for the variables did not correspond to an optimum minimized mean square deviation when this optimization would have disturbed the

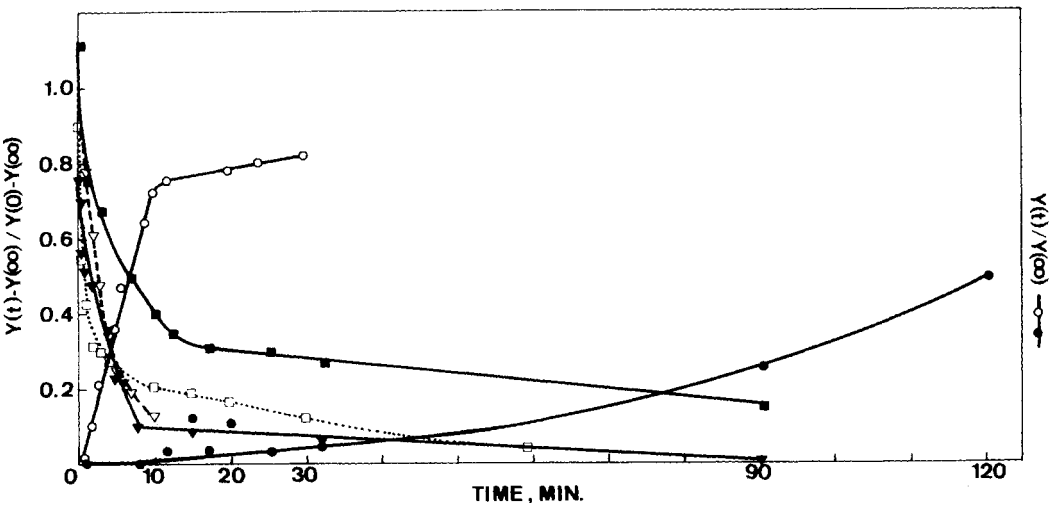


FIGURE 1: Changes in enzymatic activity, fluorescence emission quantum yield, and sulfhydryl group titer as a function of regeneration time. $Y(0)$, $Y(t)$, and $Y(\infty)$ are the values of the parameter Y at zero, t, and infinite time (corresponding to the fully renatured protein), respectively, of reoxidation (fully renatured enzyme and native enzyme have the same fluorescence emission quantum yield). The experimental conditions were as follows: a 2.5 μM solution of reduced lysozyme was added to a mixture of 1 mM reduced glutathione and 0.1 mM oxidized glutathione in a 0.1 M Tris–acetate buffer at pH 8. Regeneration was allowed to proceed at 37 °C. Human lysozyme: activity (●), fluorescence (■), and free SH group (▼). Hen egg white lysozyme: activity (○), fluorescence (□), and free SH group (▽) (from Perraudin et al., 1979).

peaks' natural order. This would have been equivalent to imposing a constraint on parameter X_0's limits. Mean square deviation minimization algorithm and equations are fully described by Fraser & Suzuki (1973).

The procedure has been tested on an arbitrary set of data generated by using eq 1 for each peak and by the addition of a random noise to the overall signal. The characteristic parameters of each peak (see eq 1) were recovered by the procedure described above when test values are not too different from actual ones. For wrong initial test values the minimization diverged.

Even though data fitting with an experimental standard shape for each peak gives a better mean square deviation for a single peak, it could not be used for a combination of peaks. As in the present case, the height–width ratio is not constant; the empirical relation between them could only be introduced in the combination of Cauchy and Gauss peaks characterized by three parameters.

Results

SH in Function of the Regeneration Time. Iodoacetic acid was used to block free thiol groups during renaturation. *S*-(Carboxymethyl)cysteine content, as determined by amino acid analysis, allowed us to estimate free SH groups in the regeneration mixture. This estimation proved to be accurate and efficient since completely reduced lysozyme contained 8.3 mol of cysteine residues.

Nevertheless, an error of ±0.5 SH (±6% with respect to the reduced protein) in the estimation of the free thiol groups is not at all negligible when the titer decreases below 1 SH (after 10 min of regeneration). As the initial free thiol titer is half after 2-min regeneration for both proteins (Figure 1), this indicates that cysteine oxidation leading to disulfide bond formation occurs rapidly. The decrease in the sulfhydryl content could appear to be biphasic, but since the second phase appears after 10 min at which point experimental accuracy is inadequate, thus the biphasic character of the curve cannot be clearly established with certainty.

Enzymatic Activity in Function of the Regeneration Time. As shown in Figure 1, reduced human lysozyme appears to be devoid of any enzymatic activity. The activity starts to slowly increase after a 10-min induction period, whereas after

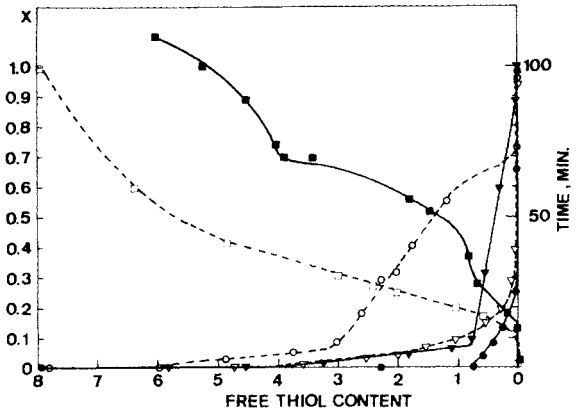


FIGURE 2: Changes in enzymatic activity, fluorescence emission, and regeneration time as a function of decreasing sulfhydryl group titer. The left axis is the fraction of activity when X is equal to $Y(t)/Y(\infty)$ and the fraction of fluorescence when X is equal to $[Y(t) - Y(\infty)]/[Y(0) - Y(\infty)]$. The right axis is the reoxidation time in minutes. Human lysozyme: activity (●), fluorescence (■), and free SH group (▼). Hen lysozyme: activity (○), fluorescence (□), and free SH group (▽) (from Perraudin et al., 1979).

the same period of time almost 90% of the activity has been regained for HEWL.

Fluorescence Emission in Function of the Regeneration Time. Fluorescence emission spectra of reduced and regenerated HML are quite different: the reduced HML spectrum is characterized by a quantum yield of 0.77 (with a maximum of intensity at 345 nm) whereas the native protein spectrum is characterized by a quantum yield of 0.028 (with a maximum of intensity at 335 nm).

Variations of enzymatic activity, fluorescence emission, and time as functions of free sulfhydryl groups are presented in Figure 2.

It is apparent that the variation of the quenching of fluorescence of HML as a function of the thiol content occurs in three distinct phases. The two first changes accompany the variation of free thiol content respectively from 8 to 4 and from 4 to less than 1. This second modification appears to be related to the recovery of the enzymatic activity which starts to occur at the same free thiol content.

It is evident that fluorescence emission and enzymatic ac-

tivity continue to vary (the former decreases and the latter increases) after the sulfhydryl group titer reaches zero and exhibits no further change.

Identification and Kinetics of Intermediates. (A) Electrophoretic Separation. In the process of reoxidation blocking, carboxymethylation with iodoacetic acid adds a new acidic group (-SCH_2COOH) to each cysteinyl residue not involved in a disulfide bond. Separation of the different lysozyme species containing different numbers of disulfide bonds should thus be feasible by polyacrylamide gel electrophoresis.

Perraudin et al. (1979) have shown that reduced hen egg white lysozyme treated with iodoacetamide, iodoacetic acid, or *N*-ethylmaleimide migrated considerably more slowly than native protein. Since the best separation was achieved with iodoacetic acid, the same treatment was used with human lysozyme and was found to be as effective as it is with HEWL.

In this electrophoretic system, which provides a way to distinguish between the two extreme conformations of lysozyme (reduced and native), each gel corresponds to a specific regeneration time and thus contains the different species present in a sample. The evolution of the electrophoretic pattern in function of the regeneration time is characterized by the appearance and/or disappearance of a series of resolved bands whose number never exceeds 10. When reduced HML and native HML were used as standards, the first upper band was identified as the completely reduced, carboxymethylated protein (designated R) and the last lower band as the completely renatured protein (designated N). Eight intermediate states were thus detected, designated by Roman numbers I–VIII, according to their order of appearance.

Figure 3 shows several densitometer tracings which represent the changes in the electrophoretic pattern as a function of the reoxidation time. These densitometer tracings were obtained by optical reading of stained proteins present in each gel.

(B) Kinetic Behavior of the Species Trapped during the Regeneration Process. The relative concentrations of the different folding intermediates as a function of regeneration time are shown in Figure 4. These data show that the reduced protein R disappears rapidly, with a half-life of less than 1 min. Simultaneously, species I–V appear rapidly. They accumulate almost to the maximum level in less than 8 min and then disappear progressively (with half-lives ranging from 2 to 17 min). Intermediates VI and VII appear after a short induction period of 20 s. Intermediate VIII and renaturated lysozyme appear slowly after a lag of 10 min.

Table I summarizes, for all the folding intermediates of both HML and HEWL, the values characterizing their kinetics of appearance and disappearance together with their electrophoretic mobility and their disulfide bond content. These last values were calculated from both their total free SH content and the relative concentrations of intermediates for each reoxidation time. These concentrations were derived by solving a system of 10 equations with 10 unknowns by a determinant method. Upon initial examination of results, it was unequivocally concluded that species VI–VIII did not possess free SH residues. In addition, it was already known that R lysozyme had 8 free SH groups and N lysozyme had no free SH group. So, the equation system could be reduced to a system of 5 equations containing 5 unknowns.

Logically, the only expected values would be 8, 6, 4, 2, and 0 (with slight variation due to experimental error) since each disulfide bond formation involves pairing of two free thiol residues. From these values, it was then possible to recalculate the total free thiol content for each regeneration time. These revised values are compared to the experimental ones in Figure

Table I: Parameters Characterizing the Various Species Trapped during the Renaturation Process

	human lysozyme									
	R	I	II	III	IV	V	VI	VII	VIII	N
$t_{1/2}$ (min)[a]		0.1 ± 0.1	0.25 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	1.25 ± 0.2	21 ± 2	21 ± 2		111 ± 1
t_{max} (min)[b]		0.35 ± 0.1	2 ± 0.5	2.5 ± 0.5	5 ± 0.5	8 ± 0.5	15 ± 3	80 ± 5		
%$_{max}$[c]		23 ± 2	16 ± 2	16.5 ± 2	15.5 ± 1	21 ± 2	66 ± 5	59 ± 3		
$t_{-1/2}$ (min)[d]	0.3 ± 0.1	2.7 ± 0.5	6.7 ± 0.5	8 ± 0.5	11.5 ± 1	22.5 ± 5	44 ± 4	113 ± 3		
electrophoretic mobility[e]	0.0	0.071 ± 0.005	0.143 ± 0.005	0.214 ± 0.005	0.314 ± 0.005	0.429 ± 0.005	0.536 ± 0.005	0.643 ± 0.005	0.714 ± 0.005	1
free thiol content[f]	8	6	4	2	2	2	0	0	0	0

	hen egg white lysozyme									
	R	A	B	C	D	E	G	H	M	N
$t_{1/2}$ (min)		0.25 ± 0.1	0.25 ± 0.1	1.15 ± 0.1	1.5 ± 0.1	3 ± 0.5	5.25 ± 0.5	5.5 ± 0.5	12 ± 1	12.5 ± 1
t_{max} (min)		2.5 ± 0.5	2.5 ± 0.5	5 ± 0.5	5 ± 0.5	11.1 ± 1	7 ± 0.5	7 ± 0.5		
%$_{max}$		21 ± 2	18.4 ± 1	19 ± 1	13 ± 1	10.5 ± 1	45 ± 3	25 ± 1		
$t_{-1/2}$ (min)	0.9 ± 0.1	5 ± 0.5	6 ± 0.5	6.15 ± 0.5			14.5 ± 1	10.5 ± 1		
electrophoretic mobility	0.0	0.055 ± 0.005	0.128 ± 0.005	0.216 ± 0.005	0.304 ± 0.005	0.378 ± 0.005	0.425 ± 0.005	0.513 ± 0.005	0.898 ± 0.005	1
free thiol content	8	6	4	2	2	2	0	0	0	0

[a] Reoxidation time corresponding to the appearance of half the maximum amount of the intermediate. [b] Reoxidation time corresponding to the appearance of the maximum amount of the intermediate. [c] Relative amount expressed as percent of the total amount of reoxidation product found at t_{max}. [d] Reoxidation time corresponding to the disappearance of half the maximum amount of the intermediate. [e] Electrophoretic mobility of the species. Reduced lysozyme was arbitrarily assigned a value of 0.0 and fully renatured (or native) lysozyme was assigned a value of 1.0. [f] Free thiol content of each intermediate [hen egg white lysozyme values from Perraudin et al. (1979)].

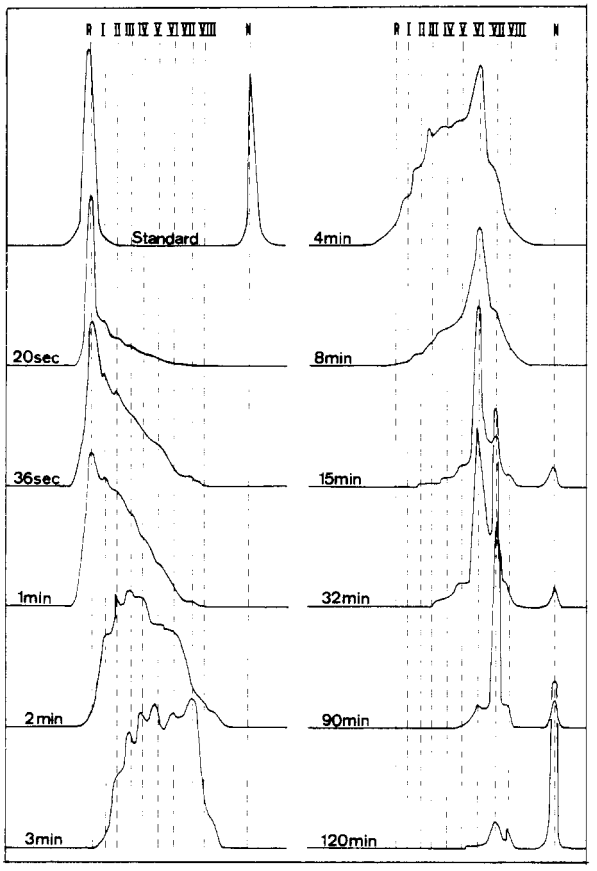


FIGURE 3: Electrophoretic analysis of the species trapped during renaturation of the reduced hyman lysozyme by glutathione, for the indicated time periods.

5. The largest divergence between experimental and theoretical curves appears when total free SH is below 0.75. As mentioned earlier, this difference may be imputed to experimental errors. It should be noted that the better agreement between densitometer tracings and their resolution in terms of Cauchy–Gauss peaks was obtained with an a priori number of intermediate equal to eight. Trials using a greater number of peaks resulted in divergence of the algorithm or appearance of negative peaks which does not make sense.

It is interesting to note how the amount in the different intermediates varies with the decrease, in the regeneration mixture, of total free thiols groups. We can observe three groups coinciding with different zones of free thiol concentrations: first, species R and I predominate in the interval from 8 to 4 SH, then species II–V predominate in the range 4–1 SH, and finally species VI–VIII and N predominate below 1 SH.

Discussion

After disulfide bond formation of reduced human lysozyme was initiated, intermediates were trapped which accumulate during refolding in a stable form (with respect to their disulfide bond) at various regeneration times. Each population of trapped molecules was then analyzed and characterized by measuring changes in fluorescence emission, enzymatic activity, total sulfhydryl group titer, and polyacrylamide gel electrophoresis which separates molecules according to their shapes and the number of their disulfide bonds.

Comparison of the enzymatic activity as a function of the protein regeneration time and the kinetics of appearance of the different intermediates clearly show that the activity is strictly dependent on the concentration of fully renatured lysozyme. No intermediate seems to have any lytic activity against *Micrococcus luteus*.

From the schemes that summarize thiol–disulfide exchanges between the reduced protein and glutathione during refolding more than four intermediates could be expected (Saxena & Wetlaufer, 1970). This was confirmed by gel electrophoresis which allowed discrimination between 10 different species. Among them, three species with three disulfide bonds and four species with four disulfide bonds were formed.

The kinetics of apparition of intermediates VI and VII and their enzymatic inactivity suggest that at least one of the two species contains several mispaired disulfide bonds. Indeed, at this level of refolding, correct pairing of all eight cysteine residues is a necessary although not sufficient condition for recovery of the native conformation. Thus, if the polypeptide chain possesses the correct disulfide bonds, its structure will be close to the native one and will probably demonstrate partial enzymatic activity. This assumption is supported by the results obtained by Creighton with reduced bovine pancreatic trypsin inhibitor and bovine ribonuclease, who found that the formation rate of the native disulfide pairs is not limited by the initial disulfide formation rate but rather by the thiol–disulfide exchange rate, which removes nonnative pairings (Creighton, 1978).

The fluorescence emission of reduced lysozyme is mainly due to tryptophan, and when the reduced protein takes on its native conformation, the close interactions between indole aminophores and the polypeptide chain decrease the contribution of tryptophan to fluorescence emission. As it has been shown before (Kronman & Holmes, 1971), variations in fluorescence emission quantum yields can be used to measure local changes in tryptophan microenvironment. Such changes can also indicate some local self-structuration occurring during protein regeneration, and the data suggest that the protein's self-structuration occurs slowly while disulfide bonds are paired rapidly. Indeed the fluorescence emission reaches half of its maximal value while the total sulfhydryl group content is already less than two free thiols. Thus, correct disulfide bond pairing does not seem to be dependent on preliminary spatial folding of the polypeptide chain. Changes in fluorescence quantum yields also indicate that three important stages seem to occur during the folding process. A first decrease in fluorescence quantum efficiency corresponds to the disappearance of reduced lysozyme and accumulation of the first intermediate and, thus, formation of the first disulfide bond. The second phase, during which the total number of free thiol groups varies from four to one residue, is characterized by a second decrease in fluorescence and by the accumulation and subsequent disappearance of species II, III, IV and V which contain respectively two, three, three, and three disulfide bonds. The third phase, which is the rate-limiting step, concerns the accumulation and disappearance of stable species VI and VII, which contain four disulfide bonds, giving rise to the appearance of the renatured protein. The fluorescence emission, which continues to decrease even after no residual free thiol is detected, clearly indicates that conformations VI and VII are distinct from that of fully renatured lysozyme.

Apparently the native conformation of HML is recovered only in species containing four disulfide bonds and involves a relatively compact polypeptide chain in which all the stabilizing interactions of the native conformation are encountered simultaneously. If a kinetically cooperative mechanism of nucleation and a progressive appearance of the native shape must take place, it would seem to occur during the final step when four disulfide bonds containing species appear in the

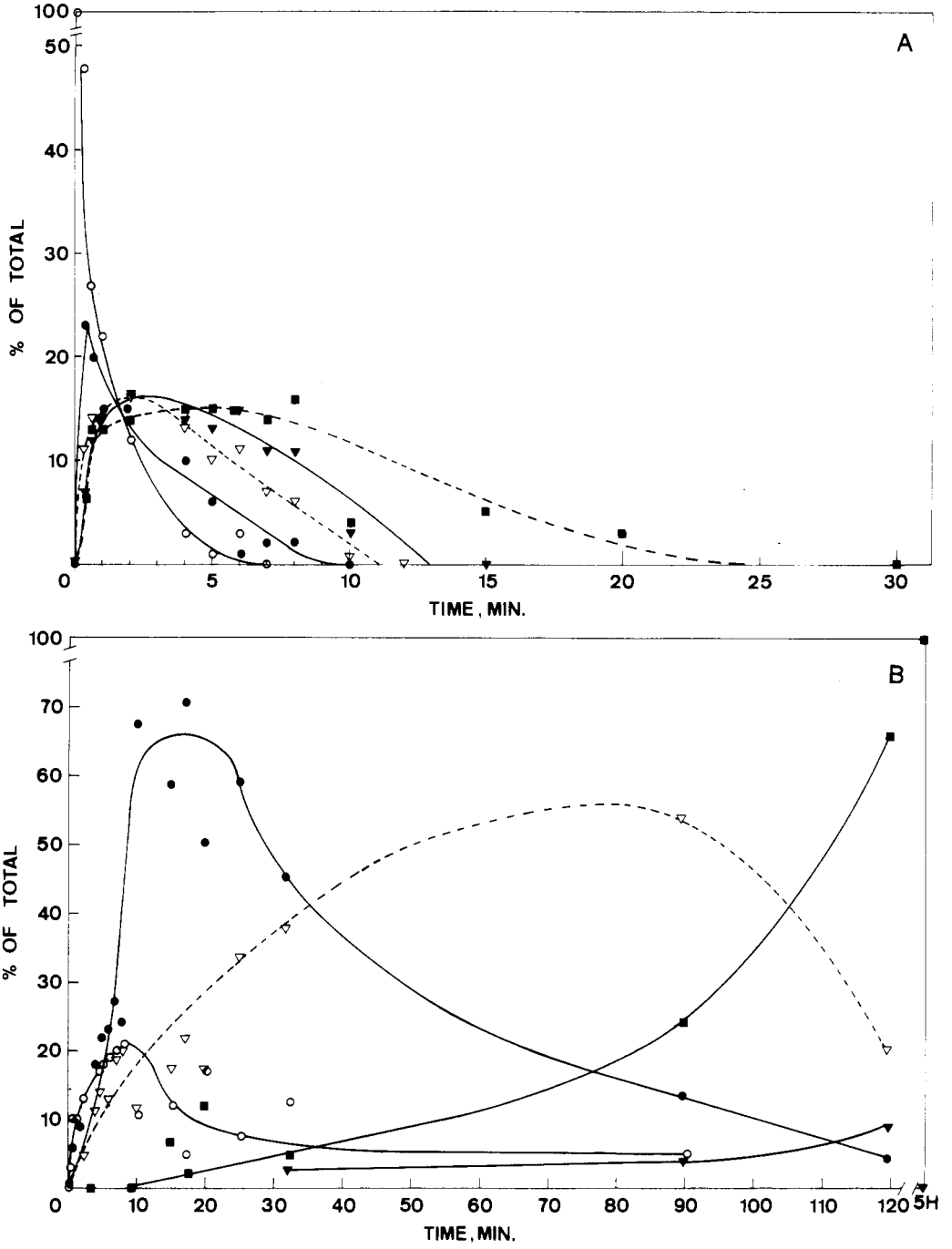
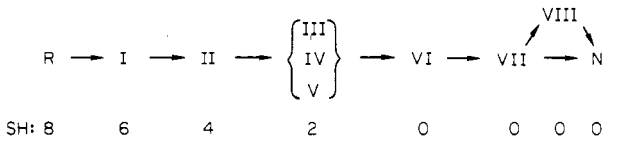


FIGURE 4: (A) Kinetics of appearance and disappearance of the species trapped during the renaturation process and separated by polyacrylamide gel electrophoresis. The relative concentrations of the species R and I–IV are plotted vs. time of isolation. R (○), I (●), II (▽), III (▼), and IV (■). (B) Kinetics of appearance and disappearance of the species trapped during the renaturation process and separated by polyacrylamide gel electrophoresis. The relative concentrations of the species V–VIII and N are plotted vs. time of isolation. V (○), VI (●), VII (▽), VIII (▼), and N (■).

environment.

A simplified basic mechanism of human milk lysozyme regeneration can thus be outlined:



However, comparison of the regeneration pathways of HML and HEWL enables us to refine the last conclusion.

Initial examination of Figures 1 and 2 reveals that HEWL is renatured more rapidly than HML, the average refolding times being respectively less than 60 min and more than 120 min. This difference is probably not intrinsic to the proteins; indeed, according to Wetlaufer et al. (1974), the reduced and oxidized glutathione concentrations which produce optimal regeneration rates of HML and HEWL are not identical, but as the aim of this work was to compare the folding mechanisms of HEWL and HML under identical conditions, the glutathione concentration used in the study of HML folding was the same as the one used with HEWL. Moreover, it was also assumed that concentrations of GSH and GSSG could eventually modify the speed of folding of HML but not its pathway.

HEWL regains enzyme activity following a short 30–60 s lag. This also occurs more slowly than its decrease in thiol

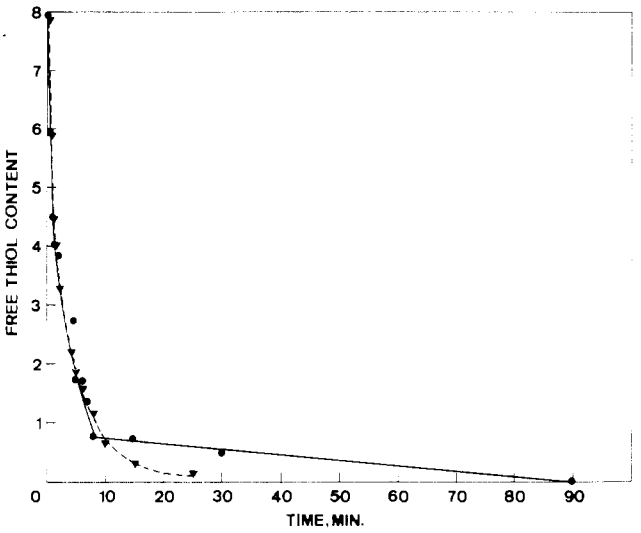


FIGURE 5: Change in the regeneration mixture's total free thiol content as a function of the reoxidation time. (●) Experimental values corresponding to the *S*-carboxymethyl content of reoxidation samples calculated by amino acid analyses. (▼) Theoretical values recalculated from the relative amount at various renaturation times of the various intermediates and their free thiol contents.

grouper titer or the decrease in fluorescence quantum yield. Moreover, the lytic activity of the reoxidation mixture (see Figure 1) and the amount of material migrating electrophoretically as native lysozyme (result not shown) do not evolve the same way as a function of the reoxidation time. All this strongly suggests that in addition to fully renatured HEWL, some intermediates possess some lytic activity against *Micrococcus luteus*. This is one of the main differences between the two lysozymes.

Careful examination of Figure 2 reveals similar decreases in the free thiol content for the two proteins whereas HEWL's fluorescence quantum yield decreases more rapidly. At first glance, self-structuration to its native conformation seems to proceed more quickly in the case of HEWL than HML for the same rate of thiol residues pairing; but comparison of the two lysozyme's primary sequences invalidates this assumption. Indeed, the HEWL fluorescence emission decay corresponds in part to a modification of the environment of Trp-62 and indicates the formation of the disulfide bond 64–80. On the other hand, in HML, Trp-62 is substituted by a tyrosine residue and the contribution of Trp-63 to fluorescence intensity is weak so that a local modification in this region cannot be observed. Thus, the lower change in fluorescence observed in HML as compared to HEWL does not necessarily mean a slower secondary structure formation. As for disulfide bond 76–94 formation, it may also possibly influence Trp-108, but this would affect both lysozymes.

Initially, a certain degree of structure exists in the reduced HEWL (Saxena & Wetlaufr, 1970). After the nucleation step, the polypeptide chain undergoes an important conformational change which is strongly dependent on the presence of structural "nuclei" and exhibits a highly cooperative behavior. As a result of this conformational change in the polypeptide backbone, the appropriate thiol groups come into contact and react to form the first intraprotein disulfide bond. It can thus be concluded that correct HEWL disulfide bond formation is conditioned by a previous spatial folding of the polypeptide chain. As mentioned before, this could not be the case for HML, but the assumption is weak, and additional information obtained from other spectroscopic measurements is needed.

The only point, common to the two proteins that has been disclosed, is the same number of intermediates containing the same number of disulfide bonds (see Table I). Concomitantly, after a long period of renaturation (and for the same free thiol concentrations), similar electrophoretic densitometer tracings are obtained for both proteins (results not shown), in contrast to Perraudin's results (1979) obtained from comparisons of different HEWL reoxidation systems. It can thus be assumed that under identical experimental conditions, the two renaturation pathways have common features.

According to Looze's results (1973), HML contains a higher percentage of helical structure than HEWL and almost no β-sheet structure, and the fluorescence spectra seems to indicate that the environment of tryptophan residues is less polar on the average in HML and HEWL. Tryptophans are probably buried in the protein's hydrophobic core; the human lysozyme would thus possess a more hydrophobic and more structured surface.

These observations are in accordance with the folding process which has been presented. Indeed, owing to its strong hydrophobic character, the HML polypeptide chain enjoys less flexibility than the HEWL chain when refolding in a polar solvent, which could explain why the fully reduced human protein regains its native conformation more gradually and less easily than hen lysozyme. On the other hand, human lysozyme must have a more organized native conformation, and the last intermediates which appear during refolding and contain four disulfide bonds will consequently have more difficulty in adopting the tridimensional structure of the fully renatured molecule, thus explaining the final rate-limiting step, which is not observed during refolding of hen egg white lysozyme.

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Thermodynamics of Structural Fluctuations in Lysozyme As Revealed by Hydrogen Exchange Kinetics†

Roger B. Gregory,* Donald G. Knox,‡ Amy J. Percy, and Andreas Rosenberg

ABSTRACT: A new method is described that makes use of the empirical enthalpy–entropy compensation behavior of a related series of processes for deriving the activation enthalpy and entropy probability density functions from the corresponding rate constant density function. The method has been applied to data obtained from a study of the temperature dependence of hydrogen–tritium exchange in lysozyme. Analysis of the temperature dependence of t_j, the time required to reach a particular number of hydrogens remaining unexchanged, provides estimates of $\Delta G^{\ddagger}$, $\Delta H^{\ddagger}$, and $\Delta S^{\ddagger}$ for the exchange process. The results are consistent with the notion of two mechanisms of exchange characterized by different activation energies. Increases in $\Delta H^{\ddagger}$ are compensated by corresponding increases in $\Delta S^{\ddagger}$. The compensation plot, however, reveals two distinct apparent compensation temperatures, which reflect the operation of two qualitatively different mechanisms of exchange. The faster hydrogens exchange with $\Delta H^{\ddagger}$ values between 8 and 18 kcal·mol^{-1} and are characterized by a high compensation temperature of 470 K. The slower hydrogens exchange with $\Delta H^{\ddagger}$ values that reach 40 kcal·mol^{-1} and display a compensation temperature of $\simeq$360 K. The latter is associated with a thermal unfolding mechanism of exchange.

There is now considerable evidence for a dynamic view of protein structure. Theoretical and experimental evidence [see the review by Gurd & Rothgeb (1979)] points to a broad distribution of closely related conformational states and to a diverse range of internal motions in proteins. Hydrogen isotope exchange is one of a number of techniques that can provide considerable information about protein conformational dynamics by measuring the accessibility of the amide protons and the interior of the protein to solvent. One pathway of exchange appears to be associated with major cooperative unfolding of the protein (Woodward et al., 1975; Ellis et al., 1975, Hilton & Woodward, 1979; Knox & Rosenberg, 1980). However, under conditions that favor the folded form, most amide protons exchange with solvent from the folded conformation without contributions from the major unfolding process.

There is no general agreement as to the types of internal motion that mediate exchange from the native state. A number of models have been proposed that differ both in the amplitude and degree of cooperativity of motion involved and in the medium (i.e., within the protein matrix or in bulk solvent) in which exchange is assumed to take place. The local unfolding model involves relatively large amplitude motions and cooperative hydrogen bond rupture of regions of protein secondary structure that expose the normally protected amide sites to bulk solvent where exchange takes place (Englander, 1975; Hvidt & Nielson, 1966).

By contrast, the penetration model proposes that the exchange catalyst migrates to buried sites within the protein and that exchange occurs within the protein interior. A description of the penetration process has been given by Lumry & Rosenberg (1975) in the "mobile defect" hypothesis. Fluctuations in the bonding network allow a redistribution of internal protein free volume, which provide the pathways for water and water–ion migration to and from the buried exchange sites. These mechanisms have been reviewed in detail elsewhere (Woodward & Hilton, 1979; Gurd & Rothgeb, 1979; Englander, 1980; Barksdale & Rosenberg, 1982).

It has proved very difficult to identify unambiguously the types of internal motion responsible for hydrogen exchange from the native state. Much of this difficulty can be attributed to the problems of analyzing the simultaneous exchange of large numbers of hydrogens (Hamming, 1962; Barksdale & Rosenberg, 1982). On a more general level, the free energy is known not to be a good source of information for cooperative processes (Lumry, 1980a,b; Benzinger, 1969, 1971). These considerations force us to seek mechanistic information in the temperature and pressure derivatives of the free energy. The

† From the Department of Laboratory Medicine and Pathology (A.R.) and the Laboratory for Biophysical Chemistry, Department of Chemistry (R.B.G., D.G.K., and A.J.P.), University of Minnesota, Minneapolis, Minnesota 55455. *Received March 15, 1982.* This is Laboratory for Biophysical Chemistry Contribution No. 208. This work was supported by Grant No. PCM80-03744 from the National Science Foundation.
‡ Present address: School of Chemical Sciences, University of Port Harcourt, PMB 5323, Port Harcourt, Nigeria.

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