The Folding Pathway of Reduced Lysozyme*

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The present work is an investigation of the identities of early disulfides in lysozyme regenerations. A description of a folding pathway for a disulfide protein can begin with answers to the question, what are the early disulfides? Are they a collection of small amounts of all the native disulfides? Are some non-native disulfides present in appreciable amount? Are all the early disulfides found in one part of the protein? Clearly each of these possibilities would put different constraints on the folding mechanism. This approach to the problem was introduced by Ristow and Wetlaufer (10), who were able to estimate the number, but not unproven, that formation of disulfide I-VIII completes the cross-linking of lysozyme.

Studies of acquisition of three-dimensional structure by proteins have attracted continued interest since the early demonstration of reversible denaturation of hemoglobin (2). The topic has recently been reviewed (3, 4), and several experimental studies have since appeared (5-8). An earlier study from this laboratory has demonstrated a physiologically feasible glutathione system for the regeneration of activity in reduced denatured lysozyme (9).

A description of a folding pathway for a disulfide protein can begin with answers to the question, what are the early disulfides? Are they a collection of small amounts of all the native disulfides? Are some non-native disulfides present in appreciable amount? Are all the early disulfides found in one part of the protein? Clearly each of these possibilities would put different constraints on the folding mechanism. This approach to the problem was introduced by Ristow and Wetlaufer (10), who were able to estimate the number, but not the identities, of early disulfides in lysozyme regenerations. Their results were taken as evidence for a limited-search folding pathway for the Cu²⁺-catalyzed regeneration of hen egg lysozyme. More recently this approach to the folding problem has been employed in investigation of the folding of ribonuclease A (8) and of pancreatic trypsin inhibitor (5-7).

The present work is an investigation of the identities of early disulfides and cysteinyl residues in lysozyme early in its regeneration by glutathione. The results are interpreted in terms of their implications for folding mechanisms.

MATERIALS AND METHODS

Methods for the purification, reduction, and assay of lysozyme have been described previously (10).

Regeneration

Reduced lysozyme at 10⁻⁶ M was regenerated using the non-enzymic glutathione buffer system of Saxena and Wetlaufer (9). This regeneration system is composed of 1 x 10⁻⁴ M GSH and 1 x 10⁻⁴ M GSSG in a 0.10 N Tris acetate buffer, pH 8.0, at 97°. In order to inhibit the copper-catalyzed air oxidation of thiols during the regeneration, all glassware was rinsed in 6.0 M HCl to remove copper ion, all solutions were deoxygenated with nitrogen, and an atmosphere of nitrogen was maintained over the regeneration solution. For regenerations longer than 3.0 min, 1 x 10⁻³ M EDTA was included in the regeneration mixture.

Stopping Regeneration and Blocking Thiols

The regeneration was stopped and the thiols were blocked by a modification of the method of Ristow and Wetlaufer (10). The pH of the regeneration solution was first reduced to 5.5 with acetic acid. Samples were removed for activity measurements. Unreacted thiols were then blocked by the addition of a 10-fold molar excess of N-ethylmaleimide (1 x 10⁻² M) after area was added to a final concentration of 4 M to facilitate complete alkylation. The alkylation was allowed to proceed for 30 min, after which the pH was lowered to 7.0 by the addition of concentrated HCl. The alkylated, partially oxidized protein was isolated from this solution.

Isolation of Partly Regenerated Product

The product was concentrated from the alkylation solution by ultrafiltration with an Amicon PM 10 membrane. The concentrated protein was then separated from regeneration and blocking reagents by gel filtration on a column (2.5 x 100 cm) of Sephadex G-25 (coarse) equilibrated and eluted with 0.10 N acetic acid. This protein was typhosphinated and stored dry at 0-5°C.
The partly regenerated protein was dissolved in 5% formic acid to a concentration of 10 mg/ml. Pepsin was added at an enzyme to substrate ratio of 1:100 by weight. After 18 hours a second aliquot of pepsin was added. The digestion was stopped after 24 hours by freezing and lyophilization.

The partially regenerated thiol-blocked protein or its pepsin digest was dissolved at 10 mg/ml in 0.10 N acetic acid that was also 2.0 M in Ultrapure urea (Mann). The pH of this mixture was adjusted to 6.0 with ammonium hydroxide, and diphenylcarbamyl chloride-treated trypsin was then added at an enzyme to substrate ratio of 1:100 by weight. After 18 hours of digestion at 25°, another equal aliquot of trypsin was added. The digestion was stopped after 30 hours by acidification to pH 3.0.

The enzymically digested regenerated material was initially fractionated by gel filtration on a column (2.5 x 150 cm) of Sephadex G-25 (fine) equilibrated and eluted with 0.10 N acetic acid. The absorbance of the effluent was monitored at 280 nm, and disulfides and alkylated cysteines were detected by the method of Maron et al. (11). The fractions containing disulfide and/or alkylated cysteine were lyophilized. The dry peptides were stored at 0-5° until further purification by ion exchange chromatography.

Chromatography was carried out using 1-butanol:acetic acid:water (4:1:5) upper layer. Peptides were detected with ninhydrin, and filtration clearly separates the peptides containing disulfide from the rest. This material was digested with trypsin, and the digest was initially fractionated on a Sephadex G-25 column. The gel filtration fraction (Fraction A) is shown in Fig. 4. All components except 6 and 8 contained either disulfide or alkylated cysteine. Peptides were detected with ninhydrin, and cystines were detected by the method of Karush et al. (16).

RESULTS

In order for inferences based on the identification of disulfide intermediates in the folding of lysozyme to be meaningful, it is essential that the method used to stop the refolding be rapid and efficient and that it not alter the disulfides that have been formed. Reducing the pH of the regeneration mixture from 8.0 to 5.5 prevents shuffling involving enzymically active species for at least 9 hours, as evidenced by the time-stability of the enzymic activity of a sample quenched to pH 5.5 at any point during the course of the regeneration. Following acidification, the unreacted thiols are rapidly blocked with N-ethylmaleimide.

Independent tests of the effectiveness of these blocking conditions were carried out. In the first control, native lysozyme was added to the regeneration mixture, and standard blocking and isolation operations were carried out. As can be seen in Table I, these conditions do not decrease the number of disulfide bonds (4.0) in native lysozyme. In the second control experiment, the pH of the regeneration mixture was reduced to 5.5 before the addition of reduced lysozyme. From this point, standard blocking and isolation procedures were carried out. In the second control no disulfides were formed. These results indicate that these methods used to stop the regeneration, block the thiols, and isolate the product are effective both in preventing further disulfide formation and in preventing the destruction of existing disulfide bonds. The blocking and isolation procedures are thus valid for isolation of covalent intermediates in the folding process.

Figs. 1 and 2 compare the rate of disulfide formation with the rate of regain of enzymic activity. There is a rapid formation of two disulfide bonds followed by a slower formation of the remaining two disulfides in the molecule. Two first-order kinetic processes are required to fit the disulfide rate data, one with a kinetic constant of 0.6 min⁻¹ and one with a kinetic constant of 0.98 min⁻¹. The initial rapid disulfide formation is completely lost when either 3.0 or 6.0 M GdmCl is included in the regeneration buffer. From Fig. 2 it is evident that no enzymic activity appears during the initial rapid formation of disulfide; significant activity is not observed until more than (on the average) two disulfide bonds are formed. Due to the existence of the lag period (confirmed in dozens of experiments) and the poor precision of the lysozyme assay, we have not attempted to fit the activity data to simple kinetic models.

Thin layer mapping on digests (pepsin followed by trypsin) of partially regenerated material was carried out in order to extend the observations of Ristow and Wetlaufer (10) to the glutathione regeneration system. Only six disulfide peptides could be observed in maps of material regenerated to an average of 0.8 disulfides/molecule. No disulfide peptides were observed in maps of material regenerated to the same extent of disulfide formation in 3.0 M GdmCl. These results extend the findings of Ristow and Wetlaufer to the glutathione system: only a few disulfides are formed early in the regeneration.

One-half-minute Regeneration—To identify the first, rapidly formed disulfides, we regenerated a 40-mg batch of lysozyme for 1/2 min under optimal conditions as outlined above. This material was digested with trypsin, and the digest was initially fractionated on a Sephadex G-25 column. The gel filtration clearly separates the peptides containing disulfide and alkylated cysteine into three distinct major fractions, as shown in Fig. 3.

CM-cellulose chromatography of peptides in the largest gel filtration fraction (Fraction A) is shown in Fig. 4. All components except 6 and 8 contained either disulfide or alkylated cysteine residues. Component 1 was further purified by ion exchange chromatography on Aminex 5OW-X4 with pyridine acetate gradients. Components 4 and 5 were further separated by rechromatography on CM-cellulose with a shallower gradient. All other peptides were shown to be pure or to contain only trace contaminants by thin layer chromatography in the three solvent systems described under “Materials and Methods.”

1 The abbreviations used are: SP-Sephadex, sulfopropyl-Sephadex; GdmCl, guanidine chloride; RNase A, ribonuclease A from the bovine pancreas.

2 Experimental details can be found in the thesis of W. L. A. (17).
The SP-Sephadex ion exchange purification of peptides in Fig. 9 shows the ion exchange chromatogram of gel filtration Fraction B. Only component 19 contains analyzable amounts of disulfide or alkylated cysteine, while peaks 6, 7, 13, 14, 15, 16, and 18 contained only traces thereof. The amino acid composition of peptide 19, given in Table III, suggests the existence of a disulfide between cysteines II and VII. This component 19 accounted for 5% of the total disulfide.

Disulfide heterogeneity, or both. The amino acid analyses of these fractions are given in Table III. Because amino acid analyses were performed on very small amounts of material isolated from thin layer chromatographic purification was required for all peptides. Fig. 7 shows the initial gel filtration of this digest. Here also three gel filtration fractions containing either disulfide or alkylated cysteine were obtained. All three fractions were further purified by ion exchange chromatography on Aminex 50. The ion exchange chromatogram of Fraction A is shown in Fig. 8. Components 2, 3, 9, 10, and 12 all contained significant amounts of material detectable with the assay for disulfide and alkylated cysteine. Their amino acid compositions are given in Table II. As can be seen, no cystine was observed in any fraction. These peptides contained only alkylated cysteines, corresponding to cysteines II, VII and VIII. No alkylated cysteines corresponding to half-cystines III, IV, V or VI were found in any of the chromatographic fractions containing cystine and/or N-ethylmaleimide-alkylated cysteinyl residues.

One-minute regeneration—A large scale batch (40 mg) of lysozyme regenerated for 10 min was prepared. Pilot digests with trypsin at pH 6.0 (which was successful with lysozyme that had been regenerated for ½ min) showed incomplete digestion into peptides soluble in our chromatographic systems. From the results of additional pilot digests, we adopted a peptic digestion followed by digestion with trypsin at pH 6.0 (both digestions are described under "Materials and Methods"). Disulfide-containing peptides from this 1-min regeneration were isolated and purified by the methods described for the ½-min regeneration. In addition a preparative thin layer chromatographic purification was required for all peptides. Fig. 7 shows the initial gel filtration of this digest. Here also three gel filtration fractions containing either disulfide or alkylated cysteine were obtained. All three fractions were further purified by ion exchange chromatography on Aminex 50. The ion exchange chromatogram of Fraction A is shown in Fig. 8. Components 2, 3, 9, 10, and 12 all contained either disulfide or alkylated cysteine. Only peaks 2 and 12 could be isolated in yields high enough to allow amino acid analysis; the amino acid compositions of these fractions are given in Table III. Because amino acid analyses were performed on very small amounts of material isolated from thin layer chromatograms, the correspondence between analytical and theoretical compositions is not as good as for peptides from a ½-min regeneration. Component 2, corresponding to half-cystines IV, V, and VI, contains one disulfide and one alkylated cysteine. Component 12 contains three peptide chains linked by two disulfide bonds involving half-cystines III, IV, V, and VI. Peptide 2 accounted for 11%, and peptide 12 accounted for 5% of the total disulfide.

Fig. 9 shows the ion exchange chromatogram of gel filtration Fraction B. Only component 19 contained analyzable amounts of disulfide or alkylated cysteine, while peaks 6, 7, 13, 14, 15, 16, and 18 contained only traces thereof. The amino acid composition of peptide 19, given in Table III, suggests the existence of a disulfide between cysteines II and VII. This component 19 accounted for 5% of the total disulfide.

Effectiveness of lysozyme blocking conditions

Protein was added to a regeneration mixture composed of $1 \times 10^{-4} \text{ M GSH} + 10^{-4} \text{ M GSSG}$ in 0.10 m Tris acetate at 37°. The pH of the native lysozyme control was adjusted to 5.5, and the protein thiols were then blocked using conditions described under "Materials and Methods." After isolation of the protein, disulfides were determined by reduction with dithiothreitol, gel filtration, and estimation of the resulting thiols with 5,5-dithiobis(2-nitrobenzoic acid). The values reported are the average of three trials. The uncertainty represents the range of values.

| Protein            | pH of "regeneration" | Experimental | Theoretical |
|--------------------|----------------------|--------------|-------------|
| Native lysozyme    | 8.0                  | 3.9 ± 0.2    | 4.0         |
| Reduced lysozyme   | 5.5                  | 0.2 ± 0.0    | 0.0         |

In the latter case these peaks correspond to large fragments of the molecule containing alkylated cysteine.

Components 1, 2, 3, 4, and 5 were all found to have the same amino acid composition. The reason for multiple peaks is not entirely clear; they could be the result of either amide or disulfide heterogeneity, or both. The amino acid analyses of the two major disulfide-containing components, 1 and 7, are given in Table II. From this table it is clear that component 1 corresponds to peptide 74-96, containing one disulfide and one alkylated cysteine. Component 7 contains two disulfide bonds, one in the 74-97 fragment and the other between the 62-68 fragment and the 74-97 fragment. Components 1 through 5 account for approximately 20% of the starting disulfide. Component 7 accounts for 2% of the starting disulfide. Component 10 in 13 were never isolated in yields high enough to allow amino acid analysis; however, peaks are found in these elution positions when a tryptic digest of native lysozyme is applied to the CM-cellulose column. In the latter case these peaks correspond to large fragments of the molecule, containing at least three disulfides per fragment.

The SP-Sephadex ion exchange purification of peptides in gel filtration Fraction B is shown in Fig. 5. Only component 2 contained significant amounts of material detectable with the assay for disulfides and alkylated cysteines. The amino acid composition of this peptide is given in Table II. Cystine was not evident in the amino acid analysis; only alkylated cysteine was observed. This fragment corresponds to the 6-13 segment of the molecule containing alkylated cysteine I.

The Aminex chromogram of the smallest gel filtration fraction (Fraction C) is shown in Fig. 6. Only components 6, 8, and 14 contained significant amounts of material detectable with the assay for disulfide and alkylated cysteine. Their amino acid compositions are given in Table II. As can be seen, no cystine was observed in any fraction. These peptides contained only alkylated cysteines, corresponding to cysteines II, VII and VIII. No alkylated cysteines corresponding to half-cystines III, IV, V or VI were found in any of the chromatographic fractions containing cystine and/or N-ethylmaleimide-alkylated cysteinyl residues.

**Table I**

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| Protein       | pH of "regeneration" | Experiment | Theoretical |
|---------------|----------------------|------------|-------------|
| Native lysozyme | 8.0                  | 3.9 ± 0.2  | 4.0         |
| Reduced lysozyme | 5.5                  | 0.2 ± 0.0  | 0.0         |
Amino acid analysis of cystine- and alkylated cysteine-containing peptides isolated from a tryptic hydrolysis of 1/2-min regenerated lysozyme

Hydrolysis was in 6.0 N HCl at 110° for 22 hours. No attempt has been made to correct for serine and threonine destruction. Due to the extensive destruction of both cystine and S-succinyl cysteine only their presence or absence is reported.

Table II

| Fraction A | Fraction B | Fraction C |
|------------|------------|------------|
|            | Peak 1     | Peak 2     | Peak 3     | Peak 4     | Peak 5     | Peak 6     | Peak 7     | Peak 8     | Peak 9     | Peak 10    | Peak 11    | Peak 12    | Peak 13    | Peak 14    |
| Residue    | Residue    | Residue    | Residue    | Residue    | Residue    | Residue    |
| Asp 3.8 (4)| 5.3 (6)    | 0.1 (0)    | 0.7 (0)    | 0.2 (0)    | 1.1 (1)    |
| Thr 0.9 (1)| 1.0 (1)    | -          | -          | -          | -          |
| Ser 3.2 (4)| 3.9 (4)    | 0.1 (0)    | 0.1 (0)    | -          | -          | -          |
| Glu -      | 0.5 (-)    | 1.2 (1)    | -          | -          | -          | -          |
| Pro 1.0 (1)| 0.8 (1)    | -          | -          | -          | -          | -          |
| Gly 0.6 (+)| 1.6 (1)    | 0.2 (0)    | 0.4 (0)    | 1.2 (1)    | 2.2 (2)    |
| Ala 2.9 (3)| 3.8 (3)    | 3.2 (3)    | 0.3 (0)    | 0.1 (0)    | 2.0 (2)    |
| Val 1.2 (1)| 1.2 (1)    | -          | 0.3 (0)    | 0.1 (0)    | 1.0 (1)    |
| Met -      | -          | 0.9 (1)    | -          | -          | 0.1 (0)    |
| Ile 2.0 (2)| 2.0 (2)    | -          | -          | -          | 0.1 (0)    |
| Leu 3.1 (3)| 3.5 (3)    | 1.1 (1)    | 0.3 (0)    | 0.6 (1)    |
| Tyr -      | -          | -          | 0.1 (0)    | 0.7 (1)    |
| Phe -      | -          | -          | -          | -          |
| His -      | -          | -          | -          | -          |
| Lys 1.0 (1)| 1.7 (2)    | 0.9 (1)    | 1.0 (1)    | 0.8 (1)    |
| Arg -      | 0.8 (1)    | -          | -          | 1.0 (1)    |
| LyS +      | +          | +          | +          | +          |
| Suc-C +    | +          | +          | +          | +          | +          |

Discussion

The mechanisms of disulfide formation in the glutathione thiol-disulfide regeneration system have been formally described by Bradshaw et al. (18) and Saxena and Wetlaufer (9). In such a system disulfide bonds are formed by thiol-disulfide exchange reactions. The rates of this reaction have been determined for exchanges between cystamine and glutathione under conditions similar to those used in this communication (19). If the exchange rates between glutathione and protein thiol are similar to the rates described for cystamine and glutathione, then complete equilibration of disulfide with all thiols on lysozyme is possible in less than 1 min (8). This means that there are four types of disulfides to be considered for lysozyme folding in the glutathione regeneration system: native protein disulfides, wrongly paired protein disulfides, mixed disulfides between protein and glutathione, and GSSG. Any proposed folding mechanism should account for all four classes of disulfides.

If the one stochiometric disulfide observed after 1/2-min regeneration were due to the presence of four native disulfides in one quarter of the molecules, we would expect to find approximately 25% of native enzymic activity. That is, if all-or-none folding were taking place, one would expect to
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Presumably, the factor which limits the search of structures during the folding of lysozyme is nucleation. Can we say anything about the location of a nucleation region in lysozyme from these studies? It might be thought that the finding of early disulfides in the linear sequence 62-96 implies that nucleation occurs in that sequence. We believe that while nucleation may occur in that sequence, the evidence of this paper is insufficient to prove it. Suppose the nucleation of native-like three-dimensional structure had occurred in the sequence 1-40. Because there are no native disulfide bonds in sequence 1-40, no native disulfide peptides can form in the event of such a nucleation. Thus, failure to find early disulfides from sequences other than 62-96 cannot be used as evidence against nucleation in other parts of the molecule. However, evidence obtained earlier in this laboratory by Saxena (20) from simultaneous measurement of peptide ellipticity, protein disulfide, and enzymic activity, make a case against independent and simultaneous formation of structure in the NH₂-terminal third of lysozyme while the early disulfide forms in the middle third.

Formation of enzymic activity lags behind formation of protein disulfides, as Fig. 2 clearly shows. Contrary to the interpretations of others (5-8, 21), a lag by itself gives no information about the correctness of the -S-S- intermediates. If activity should be dependent on formation of a protein with at least two (or three or four) correct disulfide bonds, a lag would occur during the oxidation until some of the proteins accumulated the minimum required disulfides, even if only correct disulfide bonds form. For the foregoing to be strictly true, the rate of formation of the first stoichiometric disulfide should not be substantially slower than that of the second. Fig. 1 shows that this condition is met in the present work. One might then ask why GSH is needed at all, if not to allow correction of incorrect -S-S- bonds? It is equally possible that the role of GSH is to reduce what would otherwise become an excessively high concentration of mixed disulfide between protein and GSH (overoxidation). Evidence of the ease with which this can happen was clearly shown in the pioneering investigation of Bradshaw et al. (18). Thus, if we view the role of GSH as that of breaking "wrong" -S-S- bonds, we must remember that mixed disulfides with GSH, as well as improperly paired protein disulfides, are both "wrong" -S-S- bonds. It seems likely that both kinds of "wrong" -S-S- bonds could coexist.

It might be argued that the rapid early formation of disulfide is due to a higher chemical reactivity of specific thiol, independent of protein conformation. If this were the case, observe enzymic activity increasing linearly from zero to four disulfides. Since this is contrary to our findings, all-or-none folding is ruled out.

The results presented above show that when reduced lysozyme is regenerated, disulfide bonds are formed much more rapidly than enzymic activity. This rapid formation of disulfide can be explained by either a rapid formation of many "wrongly" paired and mixed disulfides which eventually shuffle into the correct pairings, or by a pathway of disulfide formation in which a few specific disulfide form. The former of these alternatives is, in the limiting case, the random search mechanism; the latter is the limited search mechanism. The second alternative was suggested as the mechanism for folding of RNase A by Hantgan et al. (8), and for trypsin inhibitor by Creighton (5-7). The second alternative was suggested by Ristow and Wetlaufer (10) as the mechanism of folding for the Cu²⁺-catalyzed oxidation of reduced lysozyme. The present findings of at most a few early disulfides in the glutathione regeneration adds strong support to the limited search mechanism for lysozyme folding.

### Table III

Amino acid analysis of cystine- and alkylated cysteine-containing peptides isolated from a peptic-tryptic hydrolysis of ½ min regenerated lysozyme

| Amino Acid | Peak 2 residue | Peak 12 residue | Fraction B peak 19 residue |
|------------|----------------|----------------|--------------------------|
| Aspartic acid | 2.0 (2) | 9.4 (3) | 3.3 (3) |
| Threonine | - | - | - |
| Serine | 0.9 (1) | 1.1 (1) | 0.2 (1) |
| Glutamic acid | 0.4 (0) | - | - |
| Proline | 1.0 (1) | 1.0 (1) | - |
| Glycine | 0.2 (0) | 1.0 (1) | 2.4 (3) |
| Alanine | 1.0 (1) | 1.5 (2) | 0.2 (0) |
| Valine | - | - | - |
| Methionine | - | - | - |
| Isoleucine | 1.0 (1) | 1.2 (1) | - |
| Leucine | 2.1 (2) | 2.3 (3) | 1.3 (2) |
| Tyrosine | - | - | 1.0 (2) |
| Phenylalanine | - | - | - |
| Histidine | - | - | 1.0 (1) |
| Lysine | - | 0.8 (1) | - |
| Arginine | - | 0.7 (1) | 1.3 (2) |
| GluCys | + | + | + |
| Succinate C | + | - | + |

Conditions are described in the Table II legend.

### Fig. 8.
Aminex 50W-X4 chromatography of Fraction A from Fig. 7. Conditions are described in the Fig. 6 legend.

### Fig. 9.
Aminex 50W-X4 chromatography of Fraction B from Fig. 7. Conditions are described in the Fig. 6 legend.

1 V. P. Saxena, manuscript in preparation.
The parentheses above indicate ambiguity in a way formally similar to their use to show the composition of a peptide whose sequence is unresolved. Thus, the first disulfide could involve the pairing of Cys residues IV-V, IV-VI, or V-VI. The first and second disulfides as written above are not clearly resolved in their order of appearance. On present evidence their order might equally well be reversed. It is also possible that there is no obligatory order for the first two disulfides formed. The suggested folding pathway is based on the isolation of 25% of the starting disulfide. It could be argued that these yields are the result of an initial rapid formation of many wrongly paired disulfides that shuffle into the correct pairings in the order indicated by the proposed sequence of disulfide formation. The argument of low yield alone, however, is insufficient to prove or disprove such a folding mechanism. In experiments to assign half-cystine pairings in native proteins, disulfide peptides are generally recovered in low yields, when techniques similar to those used in this work are used. Experiments to test for a folding pathway with an initial rapid formation of wrongly paired disulfides shuffling into correct pairings are now under way. Until the results of such experiments can be obtained we are proposing the following model for the folding of lysozyme. (a) Reduced lysozyme initially forms both mixed and “wrongly” and correctly paired intramolecular disulfides. The relative amounts of the various disulfides formed is unknown. The evidence for “wrongly” paired disulfide formation is indirect, inferred from low yields of isolated disulfide peptides and the known rapid rates of thio-disulfide exchange. (b) The initial disulfides formed shuffle into correct pairings, predominantly in the 68-96 sequence of the molecule. (c) After formation of disulfides in the 68-96 sequence of the molecule, disulfides II-VII and I-VIII are more slowly formed to yield native lysozyme.

Early steps of rapid interconversion between “wrongly” and correctly paired disulfides are also a feature of Creighton’s model for trypsin inhibitor folding (5-7). The folding model for RNase A proposed by Hantgan et al. (8) makes even greater use of rapid interconversion, claiming that no native disulfides are formed until all are formed. All these investigators (including the present authors) produce evidence or argument, or both, for at least some rapid interconversion. Rather than trying to answer the question, Does folding go by extensive search or by limited search?, we might better address the question, how extensive is structural search at varying extents of protein —S—S— formation?

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REFERENCES
1. Anderson, W. L., and Wetlaufer, D. B. (1974) Fed. Proc. 33, 488
2. Anson, M. L., and Mirsky, A. E. (1934) J. Gen. Physiol. 17, 399-408
3. Anfinsen, C. B., and Scheraga, H. A. (1975) Adv. Protein Chem. 29, 209-299
4. Wetlaufer, D. B., and Ristow, S. S. (1973) Annu. Rev. Biochem. 42, 135-158
5. Creighton, T. E. (1974) J. Mol. Biol. 87, 563-577
6. Creighton, T. E. (1974) J. Mol. Biol. 87, 579-602
7. Creighton, T. E. (1974) J. Mol. Biol. 87, 693-694
8. Hantgan, R. R., Hammes, G. G., and Scheraga, H. A. (1974) Biochemistry 13, 3421-3431
9. Saxena, V. P., and Wetlaufer, D. B. (1970) Biochemistry 9, 5015-5023
10. Ristow, S. S., and Wetlaufer, D. B. (1973) Biochemistry. Biophys. Res. Commun. 50, 544-550
11. Anderson, W. L., and Wetlaufer, D. B. (1975) Annu. Rev. Biochem. 44, 493-502
12. Maron, E., Shiozawa, C., Anson, R., and Sela, M. (1971) Biochemistry 10, 563-771
13. Schroeder, W. A. (1972) Methods Enzymol. 25, 203-213
14. Catravas, G. N. (1964) Anal. Chem. 36, 1140-1148
15. Burns, D. J. W., and Turner, N. A. (1967) J. Chromatogr. 30, 469-475
16. Karush, F., Khanan, N. R., and Marks, R. (1964) Anal. Biochem. 9, 100-114

*P. W. Pick, K.-J. Oh, and D. Wetlaufer, manuscript in preparation.
Folding Pathway of Reduced Lysozyme

17. Anderson, W. L. (1974) Ph.D. thesis, University of Minnesota, Minneapolis
18. Bradshaw, R. A., Kanarek, L., and Hill, R. L. (1967) J. Biol. Chem. 242, 3789–3798
19. Eldjarn, L., and Pihl, A. (1957) J. Biol. Chem. 225, 499–510
20. Saxena, V. P. (1971) Fed. Proc. 30, 1287
21. Anfinsen, C. B., Jr., Haber, E., Sela, M., and White, I. H. (1961) Proc. Natl. Acad. Sci. U. S. A. 47, 1309–1314
22. Kauzmann, W. (1959) in Sulfur in Proteins (Benesch, R., Benesch, R. E., Boyer, P. D., Klotz, I. M., Middlebrook, W. R., Szent-Gyorgyi, G., and Schwartz, D. R., eds) Academic Press, pp. 93–108, New York
23. Sela, M., and Lifson, S. (1959) Biochim. Biophys. Acta 36, 471–478
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W L Anderson and D B Wetlaufer

*J. Biol. Chem.* 1976, 251:3147-3153.

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