The Disulfide Bonds of Egg White Lysozyme (Muramidase)*

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The availability of the amino acid sequence of egg white lysozyme makes it possible to identify the pairing of the 8 half-cystine residues that give rise to the four disulfide bonds present in the native protein (1, 2). In establishing the positions of the disulfide bonds in native lysozyme in the earlier sequence studies (7) there was shown that disulfide interchange, which occurs under certain experimental conditions, may lead to factitious pairing of half-cystine residues. The studies on lysozyme reported here were designed to minimize this hazard, particularly since data from the peptide digestion of native lysozyme published earlier (7) were in direct conflict with those of Jolles, who reported that a disulfide bond exists between half-cystines I and II (8, 9). In view of this disagreement, and in view of other conflicting data concerning the amino acid sequence of this protein (1, 2), all of the peptides resulting from the peptic digestion of native lysozyme have been examined, and, where pertinent, either the disulfide bonds have been identified or further sequence studies have been performed.

During the course of these studies, a new method of establishing the positions of disulfide bonds in proteins has been applied to lysozyme by Brown (10), and his results are in complete agreement with those reported here. In their most recent publication, Jolles, Jauregui-Adell, and Jolles (11) give a pairing of half-cystine residues which is different from that reported earlier (8, 9) and is the same as those described in this communication.

EXPERIMENTAL PROCEDURE

Egg white lysozyme was obtained from the Worthington Biochemical Corporation (Batch No. 590). This was the same lot of enzyme that was employed in the earlier sequence studies (12), and it has been in continuous storage at -20°C. Twice recrystallized pepsin (Batch No. 635), leucine aminopeptidase (Batch No. 5971), and twice recrystallized trypsin (Batch TRSF 717) were also obtained from the Worthington Biochemical Corporation. Subtilisin (Novo crystalline bacterial proteinase, Batch 56-2) was purchased from the Enzyme Development Corporation. The manufacturer states that this bacterial proteinase achieves 95% activity against denatured hemoglobin at pH 6.0 with optimal activity at approximately pH 6.5. Sephadex G-25 was of the medium particle size, not the bead form.

Enzymic Digestion—Lysozyme (800 mg) was dissolved in 80 ml of 5% aqueous formic acid (v/v) and 12 mg of pepsin were added. The solution was kept at 37°C for 16 hours and an additional 12 mg of pepsin were added at the end of the first 6 hours. The peptic digestion was terminated by lyophilization.

Peptide Separation—The peptides resulting from peptic digestion were dissolved in 20 ml of 1% formic acid, and placed on a 3.0- × 330-cm column of Sephadex G-25. The peptides were eluted with 1% formic acid at a rate of 75 ml per hour and collected in 10-ml fractions. Phenol red was added to the original peptide mixture to serve as a marker during elution. The effluent was divided into 13 fractions (A to M) on the basis of the optical density at 280 nm (see Fig. 1), and these were lyophilized. Each peptide fraction was then dissolved in 4 ml of 1% formic acid, and aliquots were subjected to electrophoresis in pyridinium acetate buffer, pH 3.6, with the aid of a Gilson model D electrophorator. Whatman filter paper (No. 3MM) measuring 47 × 110 cm was employed and, after drying, the paper was placed on the nylon-tricine gel plate. Amino acid analyses of these oxidized peptides contained in any given fraction could be separated in this manner, preparative paper electrophoresis, followed by elution of the peptide material, was utilized to prepare large quantities of individual fragments. Each peptide, following acid hydrolysis, was subjected to quantitative amino acid analysis using a Spinco model 120B amino acid analyzer.

Disulfide Identification—Aliquots of approximately 2 µmoles of the peptides that were found to contain cystine were subjected to performic acid oxidation by the method of Hirs (14), and the products were separated by either preparative electrophoresis or chromatography on 0.9- × 50-cm columns of Dowex 50-X4. Ammonium acetate buffer of increasing salt concentration and pH was used as eluent. Amino acid analyses of these oxidized fragments identified their site of origin in the native lysozyme molecule.

Sequence Studies—Individual peptides were subjected to Edman degradation and serial leucine aminopeptidase digestion as described earlier (1). Selected details are noted in the text.

RESULTS

Disulfide Bonds—Fig. 1 illustrates the elution from the Sephadex column of peptides resulting from peptic digestion of native lysozyme. Only those peptides containing tryptophan or tyrosine were located by measurement of the optical density at 280 nm, and preliminary experiments with 35S-lysozyme have indicated that the cystine-containing peptides appear coincident with the first two peaks (7). In any case, all of the material was collected so every peptide had to be present in at least one of the fractions. A tracing of the peptide spots located by filter paper electrophoresis is shown in Fig. 2. Thirty-two major
polypeptide chain. Thus, I represents the half-cystine at positions beginning at the N-terminal end of the lysozyme. Peptides were isolated and analyzed and, of these, only Peptides 1, 4 and 8 (which were identical), and 9 contained cystine. Their compositions are listed in Table I. In the text, each half-cystine residue is referred to by a roman numeral in the order of its occurrence beginning at the NH-terminal end of the lysozyme polypeptide chain. Thus, I represents the half-cystine at position 6, II represents the half-cystine at position 20, etc.

Peptide 1—This peptide contained 4 half-cystine residues. The presence of both proline residues of lysozyme indicates that this fragment must have included positions 70 and 79. The low values for isoleucine and valine are a result of incomplete hydrolysis between positions 98 and 99 (15). The same is true for Peptide 1oxB below. After performic acid oxidation, two major peptides were isolated by chromatography on a Dowex 50-X4 column and the compositions of these peptides are shown in Table I as 1oxA and 1oxB. The first (1oxA) fits the composition for the region from position 64 to position 83 and contains half-cystines III, IV, and V. The composition of the second oxidation product (1oxB) fits the region from residues 91 to 108 and contains half-cystine VI. The variations in the aspartic acid and alanine values suggest that the cleavage by pepsin was not entirely specific, so the COOH terminus of 1oxB may have been at position 105 in some of the molecules and at 108 in the rest. These results are generally consistent with the specificity observed for pepsin when much shorter periods were employed for the digestion of reduced, carboxymethylated lysozyme (12).

A large aliquot (approximately 15 μmoles) of Peptide 1 was digested with trypsin at pH 6.5 at 23° for 20 minutes in pyridinium acetate buffer, and the products were separated on the column of Sephadex G-25. Four different products were obtained: (a) free lysine derived from position 97; (b) a peptide with the electrophoretic mobility and composition of the trypsin peptide derived from positions 69 to 73; (c) a peptide the composition of which accounted for residues 98 to 108; and (d) a fragment the composition of which accounted for the rest of Peptide 1 (see 1-Tryp in Table I). Although there is some minor contamination present, the composition of 1-Tryp fits well with the concept that it is composed of fragments derived from positions 63 to 68, 75 to 83, and 91 to 96 or 97, all held together by disulfide bonds; this is exactly what is expected when the composition of the other recovered fragments is subtracted from that of Peptide 1. The high lysine content of 1-Tryp may be accounted for by the anticipated incomplete tryptic cleavage between 06 and 07. If half-cystine III had been linked to half-cystine VI in the native protein, tryptic hydrolysis at arginines 68 and 73 would have created two different cystine-containing peptides. Since this was not the case, half-cystine III and half-cystine VI must be linked to half-cystines IV and V, and in order to establish how they are paired, it is necessary to hydrolyze the peptide in the neighborhood of residues 77 to 79. Dilute acid hydrolysis to induce cleavage at the arginine in position 77 was not successful. Pursuing the observation of Brown (10), an exhaustive digestion of 1-Tryp at 37° with bacterial proteinase in 0.05 M pyridinium acetate at pH 6.0 was carried out for 40 hours. The peptide (5 μmoles) was dissolved in 5 ml of buffer, and aliquots (1 mg) of the proteinase were added at 0, 16, and 24 hours. The products were separated into nine regions by preparative electrophoresis at pH 6.5 and eluted from the paper. Each fraction so obtained was oxidized with performic acid and subjected to electrophoresis in a second direction at pH 6.5 in a manner similar to that used by Brown (10). One region yielded 0.1 μmole each of new peptides of altered electrophoretic mobility; their compositions were (CySO₂,H, Ala) and (CySO₂,H, Leu). This matches half-cystine III with half-cystine V. By difference, one may infer that half-
cystine IV is paired with half-cystine VI, but no peptides accounting for this combination were isolated. Since this procedure required two consecutive elutions from paper, one might have expected a maximum yield of only 10%. Nevertheless, the recovery noted above, which corresponds to 2%, is somewhat low.

Peptides 4 and 8—These two spots represent a single peptide that was present in highest yield in Fraction C but was also found in Fraction D. Its oxidation products were easily purified by paper electrophoresis at pH 3.6 and, as listed in Table I, 4oxA fits exactly the composition for the NH2-terminal positions 1 to 4 and 9. Similarly, the recovery of Peptide 4 after elution from paper was found to be 18%. When one considers that the latter two peptides were subjected to peptic digestion. The calculated recovery of Peptide 9, following preparative paper electrophoresis, was 18%. Similarly, the recovery of Peptide 4 after elution from paper was 12%. When one considers that the latter two peptides were distributed between Fractions C and D, and that large losses are incurred due to incomplete elution of peptides from paper, it is likely that both Peptides 4 and 9 were present in the original digestion mixture in excess of 50 or 60% of the total lysozyme employed.

Sequence Studies—As noted earlier, certain other peptides isolated in this study were of interest because they provided an opportunity to re-examine regions of the amino acid sequence of lysozyme that were in conflict with the data of Jollès et al. (2, 17). The results for selected peptides are noted below.

The composition of Peptide 6 is listed in Table I and indicates that this fragment originated from the region of residues 39 to 52. After tryptic digestion, two components were isolated by column chromatography on Dowex 50-X4. The first peak had the composition in micromoles: 1 Asp, 0.56; Thr, 0.36; Ser, 0.18.

1 The compositions reported in the text are expressed in micro-

### Table I

<table>
<thead>
<tr>
<th>Peptide No.</th>
<th>1oxA</th>
<th>1oxB</th>
<th>1-Tryp</th>
<th>4oxA</th>
<th>4oxB</th>
<th>9oxA</th>
<th>9oxB</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptide 1</td>
<td>0.39 (2)</td>
<td>0.25 (2)</td>
<td>0.68 (1-2)</td>
<td>0.11 (1)</td>
<td>0.40 (1)</td>
<td>0.18 (1)</td>
<td>0.17 (1)</td>
<td>0.40 (1)</td>
</tr>
<tr>
<td>Peptide 2</td>
<td>0.46 (2)</td>
<td>0.31 (2)</td>
<td>0.64 (1-2)</td>
<td>0.36 (3)</td>
<td>0.44 (1)</td>
<td>0.29 (2)</td>
<td>0.41 (2)</td>
<td>0.33 (2)</td>
</tr>
<tr>
<td>Peptide 3</td>
<td>0.46 (3)</td>
<td>0.14 (1)</td>
<td>0.44 (1)</td>
<td>0.17 (1)</td>
<td>0.15 (1)</td>
<td>0.21 (1)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The values are not corrected for destruction during acid hydrolysis. The assumed number of residues is given in parentheses.
Composition of peptides from this region reported earlier (1), the only arrangement that is consistent with all the data is a sequence Thr-GluNHz-Ala for positions 40 to 42. Following a single Edman degradation, the composition of the residual peptide was: Asp, 0.40 (2); Thr, 0.37 (2); Ser, 0.16 (1); and Gly, 0.19 (1). The phenylthiohydantoin derivative of the NH2-terminal amino acid, corresponding to position 46, was identified as asparagine in the solvent system of Edman and Sjoquist (18). In addition, this peptide was subjected to serial digestions with leucine aminopeptidase. The analysis of an early aliquot was as follows: Asp, 0.06; Thr, 0.27; Ser or color equivalent, 0.25; and Gly, 0.03. The small quantity of glycine liberated from position 49 defines the limiting amount of serine that could have been derived from position 50. Thus, threonine and asparagine are present in excess of aspartic acid, confirming the assignment of asparagine to position 46. An analysis of the amino acids liberated following a longer period of digestion was: Asp, 0.33; Thr, 0.36; Ser or color equivalent, 0.36; and Gly, 0.11. The increase in aspartic acid is consistent with the original assignment of aspartic acid to position 48.

The composition of the second peptide that emerged from the Dowex column noted above was: Arg, 0.19 (1); Asp, 0.38 (2); Thr, 0.36 (2); Glu, 0.18 (1); and Ala, 0.19 (1). This fragment was clearly derived from positions 39 to 45 of the lysozyme molecule. The analysis of the neutral and acidic amino acids of the residual peptide following the first cycle of the Edman degradation was: Asp, 0.19 (1); Thr, 0.34 (2); Glu, 0.16 (1); and Ala, 0.17 (1). Following the second cycle it was: Asp, 0.16 (1); Thr, 0.15 (1); Glu, 0.14 (1); and Ala, 0.14 (1). After the third cycle it was: Asp, 0.07 (1); Thr, 0.07 (1); Glu, 0.04 (0.6); and Ala, 0.07 (1). Thus, the NH2-terminal amino acid of this peptide is asparagine, followed by threonine, and probably then by glutamine. The incomplete removal of glutamine by the first cycle of the Edman degradation is probably due to the tendency for the NH2-terminal glutamine to form a residue of pyrrolidone carboxylic acid (19), and this would explain the limited but definite fall in isoleucine following the second cycle. Coincidentally, it is of interest that Peptide 26 in Fraction H did not stain with ninhydrin but did produce intense color with the Ehrlich stain for tryptophan. The composition of Peptide 26 following acid hydrolysis is identical to that of 24, but the electrophoretic mobility was quite different and this probably was due to the formation of a residue of pyrrolidone carboxylic acid during the course of the peptic digestion and subsequent elution from the Sephadex column.

In order to identify residues 92 and 93, an aliquot of 10xB (see Table I and text) was digested with trypsin and purified by paper electrophoresis. The analysis of one of the resulting peptides was as follows: Lys, 0.82 (2); CySOH, 0.45 (1); Asp, 0.39 (1); Ser, 0.47 (1); Ala, 0.49 (1); Gly, 0.41 (1). The compositions of the nonbasic amino acids following three cycles of Edman degradation were as follows: first cycle: CySOH, 0.48 (1); Asp, 0.60 (1); Ser, 0.11 (1); Ala, 0.47 (1); Val, 0.39 (1); second cycle: CySOH, 0.50 (1); Asp, 0.62 (1); Ser, 0.10 (1); Ala, 0.50 (1); Val, 0.12 (1); and third cycle: CySOH, 0.64 (1); Asp, 0.40 (<1); Ser, 0.08 (1); Ala, 0.58 (1); Val, 0.08 (1). Although aspartic acid is unaccountably high by 20%, the progression of changes in composition indicates that the NH2-terminal sequence must be Ser-Val-AspNHz as reported previously.

The compositions of Peptides 1, 4, 6, and 9 have been listed in Table I. Amino acid analyses, following acid hydrolysis of the other peptides shown in Fig. 2, permitted the identification of their sites of origin. In every case, the composition was compatible with the sequence of lysozyme shown in Fig. 3. Every region of the protein was represented in at least one peptide.

**DISCUSSION**

**Arrangement of Disulfide Bonds**—Although promising studies of the x-ray crystal structure of lysozyme are in progress (20-22), no information has been available to indicate what specific side chain interactions stabilize the three-dimensional "native" conformation of this protein. The only specific intrachain bonds that can profitably be examined by chemical techniques are the disulfide bonds. In previous studies involving insulin and ribonuclease, disulfide interchange among the several cystine residues in these molecules produced certain difficulties (3, 5).
Sanger noted that following acid hydrolysis of insulin a random exchange between the disulfide pairs must have occurred to account for the many cystine-containing peptides that were isolated (3). Later, Ryle and Sanger, working with model compounds, showed that both very acidic and slightly alkaline conditions favored disulfide interchange and that dilute acid solutions appeared to minimize this reaction (4). Spackman, Stein, and Moore amplified these results with studies of a model reaction involving cystine and glutathione and concluded that the disulfide bonds were most stable at pH 2 (5). The studies of disulfide bonds in ribonuclease were conducted by initially digesting the protein with pepsin at pH 2 and then adjusting to pH 6.5 to permit further proteolysis with trypsin and chymotrypsin. Under these conditions no significant disulfide interchange appeared. However, in an earlier study Ryle and Anfinsen, who digested ribonuclease with subtilisin at pH 8, showed that interchange occurred despite the presence of N-ethylmaleimide, which was thought to exert a protective effect by combining with a mercaptide ion that might catalyze the exchange reaction (6).

The studies with egg white lysozyme reported here followed the general pattern of the earlier experiments of Spackman, Stein, and Moore (5). However, it was clearly quite desirable to attempt an initial isolation of the cystine-containing peptides under conditions as close to pH 2 as possible before attempting further proteolysis at neutral pH. The use of a Sephadex column equilibrated with 0.1% formic acid provided an ideal technique for separation under these limiting conditions. After the fragments had been divided according to size on Sephadex, separation based on their electrical charge was carried out by preparative paper electrophoresis in 4% formic acid, or in pyridinium acetate at pH 3.6. The latter system offered better separation with no apparent significant increase in the risk of disulfide interchange. Thus, two of the four pairings of half-cystines were made without exposing any of the peptides to conditions that might have favored disulfide interchange.

The peptide separation achieved with the Sephadex column was predominantly based upon molecular size. However, the fragments appearing after Fraction E contained aromatic amino acids and were probably retarded by adsorption (23).

The demonstration of a disulfide bond between half cystine residues I and VIII, and of one between II and VII seems clear despite the earlier reports to the contrary (8, 9). Jollès had isolated a peptide containing half-cystines I and II that was eluted from a chromatograph column at pH 9.5, conditions which have already been shown to invite disulfide interchange. Furthermore, he was unable to obtain any information concerning the other three pairs of half-cystine residues in this study (9).

The results of the tryptic digestion of Peptide 1, which yields Peptide 1-Tryp (Table I), indicate with fair certainty that half-cystines III and VI are bonded to IV and V, but the evidence that these are paired III-V and IV-VI is based on the finding of two peptide fragments, following performic acid oxidation, that are in accord with the pairing IV-VI. These were obtained in low yield after an exhaustive digestion at pH 6 and no trace of the other disulfide-containing peptides was found. The suggested arrangement is in agreement with Brown's study (10), as well as with the newer results of Jollès, Jauregui-Adell, and Jollès (11), and these findings collectively offer strong support for the proposed structure shown in Fig. 3. Nevertheless, none of these studies has produced high yields of cystine-containing peptides to account completely for half-cystines III, IV, V, and VI, and this arrangement must be considered tentative until it can be verified by further degradative studies or by x-ray crystallography.

**Peptide Sequence**—The data that have been published concerning the amino acid sequence of lysozyme contain a number of conflicts, and peptide fractions obtained as an incidental byproduct of this study provided an opportunity to clarify some of these differences. A discussion of some earlier disagreements has already been published (1) and the following relates to more recent data published by Jollès et al. (2).

There appears to be complete agreement on the arrangement of residues 1 to 39. Some initial uncertainty concerning the location of the asparagine and aspartic acid residues in positions 18 and 19 was dispelled when leucine aminopeptidase digestion of Peptide 20, the composition of which uniquely fits the region 19 to 22, confirmed the assignment of an asparagine residue to position 19.

Jollès continues to assign the sequence GluNH₂-Ala-Thr to positions 40 to 42, but results listed here quite clearly indicate a threonine in position 40 and a glutamine in position 41. When these results are added to those obtained earlier for the composition of peptides from this region (1), the proper sequence for positions 40 to 42 must be Thr-GluNH₂-Ala.

The primary structure of positions 47 to 50 in lysozyme has not been published by Jollès, but he has assigned an aspartic acid residue to position 46 and has indicated that an asparagine resides near position 48 (2). Evidence published earlier (1), and the results of both the Edman degradation and serial leucine aminopeptidase digestion of a portion of Peptide 6 described here, confirm the assignment of an asparagine residue to position 46. Thus, there is general agreement concerning the total amide content of this region, and the disagreement appears to be whether the asparagine resides in position 46 or position 48.

Recently, Jollès has proposed for positions 57 to 59 the sequence GluNH₂-AspNH₂-Ileu, and this conflicts with the sequence GluNH₂-Ileu-AspNH₂ shown in Fig. 3 (2). The evidence for the latter arrangement was obtained from repeated Edman degradations which consistently show a fall in the content of isoleucine relative to aspartic acid after the second cyclization.

Although a complete sequence for the ninth tryptic peptide of lysozyme (positions 62 to 68) has not as yet been reported by the Paris group, an aspartic acid residue has been depicted as occupying position 65 (11). An aliquot of this peptide (stored at -20° since the original sequence study) was subjected to serial leucine aminopeptidase digestion and at a point when 0.23 μmole of tryptophan and 0.10 μmole of S-carboxymethyl cysteine had been liberated, 0.09 μmole of asparagine (calculated in serine equivalents) was present with only a slight trace of aspartic acid. Thus, the presence of an asparagine in position 65 is confirmed.

Much of the uncertainty concerning the 11th tryptic peptide (positions 74 to 90) has been resolved. Jollès no longer gives a Cys-Ser-Asp peptide. The sequence Pro--Cys--Ser--Asp is confirmed, and there is now agreement on the assignment of an aspartic acid residue to position 87. Furthermore, the sequence Pro--Cys--Ser for positions 79 to 81 has been confirmed. The only difference that remains concerns positions 82 and 83. Evidence is presented
here indicating, once again, that the sequence is Val–AspNH₂ as reported originally (1).

Finally, Jollès no longer locates a serine in the 13th tryptic peptide, so there is agreement on a total of 129 amino acids in lysozyme.

SUMMARY

1. Evidence has been presented that the 8 half-cystine residues in egg white lysozyme are paired in the arrangement I-VIII, II-VII, III-V, and IV-VI to give the four disulfide bonds present in the native protein.

2. Conflicting results have been reported previously for portions of the primary structure of lysozyme. The structure of these regions has been re-examined, and no evidence was found to alter the amino acid sequence originally proposed.

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REFERENCES