Bovine Erythrocyte Superoxide Dismutase

SUBUNIT STRUCTURE AND SEQUENCE LOCATION OF THE INTRASUBUNIT DISULFIDE BOND*

(Received for publication, February 19, 1974)

JOHN L. ABERNETHY, HOWARD M. STEINMAN, AND ROBERT L. HILL
From the Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710

SUMMARY

The subunit assembly of bovine erythrocyte superoxide dismutase has been studied, through analysis of the effects of heat, removal of metal ion cofactors, and disulfide bond reduction, upon the dissociation of the subunits. Investigations utilizing sodium dodecyl sulfate-acrylamide gel electrophoresis and sedimentation equilibrium analysis demonstrate that the 2 polypeptide subunits of the protein are not covalently joined, but are associated through unusually strong noncovalent interactions. This absence of intersubunit disulfide bonds, in conjunction with the titration of a single free sulfhydryl group per subunit polypeptide chain, necessitates that the 3 half-cystine residues per subunit exist as 1 residue of cystine, forming an intrasubunit disulfide bond, and 1 residue of cysteine.

The specific half-cystine residues participating in the disulfide linkage and contributing the free thiol function were identified. Analysis of the products of cyanogen bromide cleavage, with acrylamide gel electrophoresis, demonstrates that the intrasubunit disulfide links 1 half-cystine residue in the sequence NH2-terminal to Met-115, to another half-cystine residue in the sequence COOH-terminal to Met-115. Through conventional procedures of enzymatic digestion, peptide isolation, and peptide characterization, Cys-55 and Cys-144 are shown to participate in the intrasubunit disulfide linkage, and Cys-6 to exist as free cysteine.

Amino acid analyses of bovine erythrocyte superoxide dismutase have demonstrated that there are 3 half-cystine residues per polypeptide chain of the protein (1, 2). These 3 residues are located at positions 6, 55, and 144 in the primary structure of the reduced and S-carboxymethylated protein (2). Knowledge of the oxidation state of these half-cystine residues and the presence of disulfide bonds, as intra- or intersubunit linkages, is vital to the understanding of the biological assembly, the unusual structural stability (3, 4), and the catalytic function of superoxide dismutase. This information may also be useful in the interpretation of low resolution electron density maps of the crystalline enzyme (5).

In this investigation, the presence of a single free sulfhydryl group per subunit has been demonstrated by titration under denaturing conditions. The absence of intersubunit disulfide bonds has been shown by sedimentation equilibrium studies in the presence of denaturants. Analysis of the protein treated with cyanogen bromide, by Na dodecyl-SO4, polyacrylamide gel electrophoresis, has indicated that the disulfide bond contained within each subunit bridges 2 half-cystine residues on either side of Met-115 (2). Finally, tryptic peptides containing the intact disulfide bond have been isolated and characterized, and the disulfide bridge has been shown to involve exclusively Cys-55 and Cys-144, and the free thiol function to reside solely on Cys-6.

EXPERIMENTAL PROCEDURES

Materials—Sodium dodecyl sulfate and urea (ultrapure grade) were obtained from Schwarz-Mann. Acrylamide and N,N'-methylenbisacrylamide were purchased from Eastman, and recrystallized from chloroform and from acetone, respectively. Guanidine hydrochloride (ultrapure) was obtained from Heico, N-ethylmaleimide from Sigma, N-ethyl[2,3,4-C]maleimide from Amersham-Searle; N-ethylmorpholine (Sequanal Grade) and 5,5'-dithiobis(2-nitrobenzoic acid) were from Pierce Chemical Co. The proteins which served as molecular weight standards in Na dodecyl-SO4 acrylamide electrophoresis were products of the following suppliers: phosphorylase a, ovalbumin (grade II), bovine a-chymotrypsinogen A (type II), and horse heart cytochrome c (type III), Sigma Chemical; catalase and lysozyme, Worthington Biochemical; bovine serum albumin (grade A), Calbiochem; human transferrin, Pentex. Trypsin, TPCK-treated, was from Worthington. Sephadexes for chromatography (G-75, SE C-25, and QAE A-25) were products of Pharmacia Fine Chemicals.

Preparation of Superoxide Dismutase—The enzyme used as starting material for the isolation of the sulfhydryl- and the disulfide-containing tryptic peptides (vide infra) was a product of Truett Laboratories (Dallas, Texas), which was further purified to remove carbonic anhydrase contamination (9). The enzyme used for the analytical scale studies, sulfhydryl titration, the molecular weight studies by ultracentrifugation, and Na dodecyl-SO4 acrylamide electrophoresis, was purified directly from bovine blood, obtained at a local abattoir, by procedures previously described (7). Chromatography on DE52 cellulose (Whatman) was performed at pH 7.8, rather than at pH 7.4 (7), to facilitate the initial adsorption of the enzyme. Only the fractions with highest specific activities, in the xanthine oxidase-cytochrome c assay sys-

* Financial support for this investigation was received from Research Grant HE-06400 from the National Heart and Lung Institute, National Institutes of Health, and Research Grant GB-29334X from the National Science Foundation.

† Predoctoral Fellow, Medical Scientist Training Program, National Institute of General Medical Sciences (GM-01678).
5.69 f .09 mg per ml, in 0.03 M potassium phosphate, pH 7.8) was assayed by reaction with 5,5'-dithiobis(2-nitrobenzine Dersulfate in the polymerization solution was 0.017%, and gel solutions were adjusted to pH 7.1. These gels contained 8% acrylamide and 1.25% bisacrylamide concentrations were 12.5 and 1.25%, respectively. These gels were stained and destained in acetic acid analysis, and for NH₂-terminal analysis by the dansylation method (11). The N-ethylmaleimide was present to react with the sulfhydryl groups of the enzyme, and thus prevent their participation in thiol-disulfide interchange (19). The solutions were centrifuged at 25,000 rpm, using a Spinco rotor, in the characterization of subunit proteins is ascertaining whether the constituent polypeptides are bonded through disulfide linkages, or solely by noncovalent interactions. This question has been of considerable interest in the present study in light of the well documented stability of superoxide dismutase (4), and its ability to withstand rather severe denaturing stress in its conventional purification procedure (7). A combination of electrophoretic analyses on Na dodecyl-SO₄ polyacrylamide gels and ultracentrifugation was used to investigate the nature of subunit interactions in bovine dismutase.

Isolation and Characterization of Sulfhydryl- and Disulfide-Containing Tryptic Peptides—The cysteine side chains of bovine dismutase were first converted to mixed disulfide derivatives, by reaction at pH 6.5 with carboxyethyl disulfide monosulfoxide (26, 27). Monosulfides have previously been utilized to modify protein sulfhydryl groups. The preparation of the monosulfoxide (28), the partial specific volume of the native protein (0.725 ml per gm) was estimated (22, 23), and the apparent partial specific volume in 6 M guanidine hydrochloride (0.715 ml per gm) obtained by subtracting 0.01 ml per gm from this value (24, 25).

RESULTS

Electrophoretic and Ultracentrifugal Analyses of Subunit Composition of Bovine Superoxide Dismutase—An essential aspect of the characterization of subunit proteins is ascertaining whether the constituent polypeptides are bonded through disulfide linkages, or solely by noncovalent interactions. This question has been of considerable interest in the present study in light of the well documented stability of superoxide dismutase (4), and its ability to withstand rather severe denaturing stress in its conventional purification procedure (7). A combination of electrophoretic analyses on Na dodecyl-SO₄ polyacrylamide gels and ultracentrifugation was used to investigate the nature of subunit interactions in bovine dismutase.

An essentially unmodified samples of the holoenzyme (free amino acid, 1 mm concentration) with carboxyethyl-disulfide monosulfoxide (4 mM) was chosen as a model. Monitored by paper electrophoresis, the reaction appeared to be complete in 1 hour at 20-24°C. Superoxide dismutase was allowed to react in a similar fashion with the monosulfide. The holoenzyme (36 mg, 3.59 amoles of subunit) was mixed with 3.8 mg of carboxyethyl disulfide monosulfoxide (17 amoles), and dissolved at 23-24°C in 3.4 ml of 0.1 M N-ethylmorpholine, 0.01 M EDTA, and 6 M guanidine hydrochloride, pH 6.5. After 20 min, an additional 3.8 mg of monosulfide was added. After a total reaction time of 2 hours, 0.3 ml of glacial acetic acid was added, and the solution was dialyzed against 5% acetic acid, then water. The slightly turbid solution was removed from the dialysis bag, mixed with 0.2 ml of 2 M N-ethylmorpholine acetate, pH 6.5, and adjusted to pH 6.5 with neat N-ethylmorpholine.

The mixed disulfide derivative, thus obtained, was incubated with trypsin (0.55 mg) for 24 hours at 23-24°C. The reaction mixture was then freeze-dried, then redissolved in 2 ml of 50% acetic acid, 0.3 M Na dodecyl sulfate, pH 7.1, and dialyzed against 5% acetic acid, then water. The slightly turbid solution was removed from the dialysis bag, mixed with 0.2 ml of 2 M N-ethylmorpholine acetate, pH 6.5, and adjusted to pH 6.5 with neat-N-ethylmorpholine.

The mixed disulfide derivative, thus obtained, was monitored by electrophoresis (20-24°C) for 24 hours at 23-24°C. The reaction mixture was then freeze-dried, then redissolved in 2 ml of 50% acetic acid, 0.3 M Na dodecyl sulfate, pH 7.1, and dialyzed against 5% acetic acid, then water. The slightly turbid solution was removed from the dialysis bag, mixed with 0.2 ml of 2 M N-ethylmorpholine acetate, pH 6.5, and adjusted to pH 6.5 with neat N-ethylmorpholine.
Holosuperoxide dismutase, examined within several weeks after preparation, is remarkably resistant to dissociation in Na dodecyl-So (Fig. 1). After 4 hours of incubation at 37° in 1% Na dodecyl-So, and 6 mM urea, electrophoresis in Na dodecyl-So, polyacrylamide gels shows no evidence of dissociation of this material to the 16,000 molecular weight subunit (Fig. 1, Gel A). Introduction of additional denaturing stresses prior to electrophoresis, such as 1% 2-mercaptoethanol, or 0.01 mM EDTA, or both, or heating with either 2-mercaptoethanol or EDTA, produces varying degrees of dissociation, but never complete conversion to subunits (Fig. 1, Gels B to E). The apparent molecular weight of the undissociated protein (55,000 to 58,000) is considerably larger than that calculated from the amino acid sequence, 31,200 (2) or that determined through conventional physical chemical procedures, 33,000 to 34,000 (7, 30). However, proteins which are incompletely denatured, because of disulfide bonds or other structural features, may exhibit anomalous Na dodecyl-So binding and thus anomalous molecular weight values on Na dodecyl-So, polyacrylamide gel electrophoresis (31, 32).

A small but reproducible difference is seen between the apparent molecular weight of the subunit produced in the absence of mercaptoethanol, by EDTA alone (15,000, Fig. 1, Gel D), and that observed in the presence of mercaptoethanol (16,000, Fig. 1, Gels B, C, and E). This observation may be construed as provisional evidence for an intrasubunit disulfide bond, whose reductive cleavage alters the subunit conformation and Na dodecyl-So, binding, and thus alters its electrophoretic mobility.

Dissociation of both holo- and apoenzyme into subunits can be forced to completion under certain conditions, as judged by Na dodecyl-So, gels (Fig. 2). Preincubation of the holoenzyme, for 5 hours at 37°, in 1% Na dodecyl-So, 6 mM urea, and 2% 2-mercaptoethanol is sufficient for total conversion to the 16,000 molecular weight species (Fig. 2, Gel A). An identical treatment with only 1% mercaptoethanol leaves considerable material in the undissociated state (Fig. 1, Gel B). When the metal cofactors are removed, the protein is much more susceptible to dissociation in 1% Na dodecyl-So. The apoenzyme, incubated 5 hours at 37° in 1% Na dodecyl-So, and 6 mM urea, with or without 1% mercaptoethanol, is completely resolved into subunits (Fig. 2, Gels B, C, D, and E). A more convincing visualization of the difference between the mobility of the apoenzyme subunit in the presence of mercaptoethanol, and its mobility with disulfide bonds intact, is possible with the highly cross-linked 12.5% acrylamide gels. With mercaptoethanol treatment (Fig. 2, Gel E), the apparent molecular weight is 16,000 to 17,000; analyzed in the absence of mercaptoethanol (Fig. 2, Gel D), the subunit has an apparent molecular weight of 15,000. The reduced and S-carboxymethylated protein was subjected to electrophoresis in tandem with these samples to provide a marker for the expected mobility of the 16,000 molecular weight subunit (Fig. 2, Gel F).

Upon prolonged cold storage (approximately 1 year at -20°, as a freeze-dried solid or frozen solution), holosuperoxide dismu-
erythrocyte superoxide dismutase (33). A sample of the reduced prolonged frozen storage was not investigated. Protein bands then analyzed by electrophoresis.

in the legend to Fig. 1, with the additional treatments noted, and then analyzed by electrophoresis. A, holoenzyme, none; B, holoenzyme, 1% 2-mercaptoethanol; C, holoenzyme, 0.05 M EDTA; D, holoenzyme, 0.05 EDTA, 50° for 15 min (then the 5-hour incubation at 37°); E, holoenzyme, 1% 2-mercaptoethanol, 0.05 M EDTA; F, reduced and S-carboxymethylated protein, none.

tase becomes more susceptible to dissociation. This decreased strength of the subunit association is readily evident on comparison of Na dodecyl-SO₄, acrylamide gel studies (Fig. 3) with those performed upon freshly prepared holoenzyme (Fig. 1). Under every set of conditions used, a larger extent of dissociation is observed in the holoenzyme which was kept in frozen storage. A second observation correlated with this prolonged storage is the appearance of bands migrating at apparent molecular weights of 28,000 to 31,000 in the unreduced samples (Fig. 3, Gels A and D), but not in the reduced protein samples (Fig. 3, Gels B and E). The physical chemical basis of this effect of prolonged frozen storage was not investigated. Protein bands of these intermediate molecular weights have been observed, also as minor components, in electrophoretic studies of human erythrocyte superoxide dismutase (33). A sample of the reduced and S-carboxymethylated protein is shown (Fig. 3, Gel F) to provide a marker for the expected mobility of the 16,000 molecular weight subunit.

These gel electrophoresis experiments demonstrate that the 2 subunits of bovine superoxide dismutase are held together with remarkable tenacity, and they also leave little doubt that disulfide bonds are more stable (neutral pH, 37° for several hours, in the absence of mercaptan reducing agents). As further proof of the absence of intersubunit disulfide bonds in bovine dismutase, the dissociation into subunits occurs under conditions where disulfide cross-links are present (31, 32).

Sulphydryl Titration of Holoenzyme—The results of such a gel electrophoretic analysis are shown in Fig. 2. Cyanogen bromide-treated apoenzyme, incubated in 1% Na dodecyl-SO₄ without mercaptoethanol, shows one major band migrating at the subunit molecular weight of 15,000 (Fig. 2, Gel C). Preincubation of cyanogen bromide-treated apoenzyme in 1% Na dodecyl-SO₄ containing 1% mercaptoethanol results in appearance of two bands of higher mobility (Fig. 2, Gel H). The apparent molecular weights of these two peptides (12,000 and 4,700) approximate those of the two cyanogen bromide fragments, B1 and B2 (2), and their sum accounts for the total molecular weight of the subunit. It is evident from these observations that the intrasubunit disulfide bond spans Met-115, and involves Cys-144 and either Cys-6 or Cys-55. The residual material at the 16,000 molecular weight position in gel H indicates that, under the conditions employed, only partial reaction occurred, in either the cyanogen bromide cleavage or the reduction of the intrasubunit disulfide linking the two peptides.

Primary Structural Location of Free Cysteinyl and Disulfide-linked Half-Cystine Residues—In the initial approach toward assigning the free and the disulfide-linked half-cystine residues, superoxide dismutase was allowed to react with radiolabeled N-ε-yllmaleimide, then digested extensively with pepsin (2). A cysteine-containing fraction was isolated from this digest in 42% yield, and, after reduction and S-alkylation, two peptide fractions were obtained from it by gel filtration, each containing S-carboxymethylcysteine (1 mole per mole). The smaller of the two S-Cm-cysteine peptides had an amino acid composition and an NH-terminal sequence in accord with the known sequence surrounding Cys-144 (2). The composition of the larger peptide fraction was in good agreement with the known sequence surrounding Cys-55. These characterizations unambiguously
established that the intrasubunit disulfide bond linked Cys-55 with Cys-144. Being the only unassigned half-cystine residue, Cys-6 was thus established as free cysteine. The large peptide fraction (representing Cys-55) was found to be heterogeneous through sequenator analysis, and by further fractionation several large peptides were purified, but in quite low yield (1 to 4%). Consequently, a second approach was undertaken to isolate peptides from the cysteine residue and the cystine bridge in higher yields, with the use of trypsin rather than pepsin as the protease.

The strategy utilized the specificity of trypsin action, the large size of the disulfide-linked fragment, T4-S-S-T11, the high recoveries usually associated with gel filtration, and the improved yields during chromatography on cation exchangers, when half-cystine residues are oxidized to cysteic acid. For the most part, the anticipated tryptic specificity and peptide behavior were realized, although several half-cystine sequences occurred in more than one peptide because of incomplete tryptic cleavage.

Fig. 4 (left). Separation of the tryptic peptides from superoxide dismutase, with cysteinyl residues converted to mixed disulfide derivatives. A Sephadex G-75 column (2.2 X 106 cm) was eluted with 20% (v/v) acetic acid at 20 ml per hour, and fractions of 1.8 ml collected at 6-min intervals.

Fig. 5 (center). Separation of the peptides produced by performic acid oxidation of Fraction 1, in Fig. 4. A Sephadex G-75 column (2.2 X 164 cm) was eluted with 20% acetic acid at 30 ml per hour, and fractions of 2.9 ml collected at 6-min intervals.

Fig. 6 (right). Separation of the peptides produced by performic acid oxidation of Fraction 2, in Fig. 4. A Sephadex G-75 column (2.2 X 163 cm) was eluted with 20% acetic acid at 28 ml per hour, and fractions of 2.4 ml collected at 6-min intervals.

Table I

Amino acid compositions of disulfide-containing tryptic peptides and their oxidized fragments

Fractions 1 and 2 are the original disulfide-containing peptides (cf. Fig. 4). The half-cysteine content of Fractions 1 and 2 was determined after performic acid oxidation of analytical scale samples. Performic acid oxidation of preparative quantities of Fraction 1 yielded two peptides, 1-A and 1-B (Fig. 5), while oxidation of Fraction 2 correspondingly yielded peptides 2-A and 2-B (Fig. 6). The values listed are the number of residues per molecule. Amino acids present in amounts less than 0.1 residue per molecule are omitted.

<table>
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<tr>
<th>Amino acid</th>
<th>Fraction 1</th>
<th>Fraction 2</th>
<th>Oxidized 1-A</th>
<th>Oxidized 2-A</th>
<th>T4</th>
<th>Oxidized 1-B</th>
<th>Oxidized 2-B</th>
<th>T11</th>
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<td>2.29 (2)</td>
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<td>1</td>
<td>1.00 (1)</td>
<td>0.96 (1)</td>
<td>1</td>
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<td>3.44 (4)</td>
<td>3.06 (3)</td>
<td>3.44 (4)</td>
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<tr>
<td>CySO₂H</td>
<td>2.04 (2)</td>
<td>2.02 (2)</td>
<td>1.29 (1)</td>
<td>1.00 (1)</td>
<td>1</td>
<td>1.05 (2)</td>
<td>1.05 (1)</td>
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<td>2.94 (3)</td>
<td>3</td>
<td>0</td>
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<tr>
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<td>2.03 (3)</td>
<td>2.18 (2)</td>
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<td>3.05 (3)</td>
<td>1.92 (3)</td>
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<td>2</td>
<td>0.96 (1)</td>
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<tr>
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<td>22</td>
<td>29</td>
<td>15</td>
<td>31</td>
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</table>

* Composition calculated from known amino acid sequence (1, 2).
† Assumed integral number of residues per molecule.
‡ Identified as S-carboxymethylcysteine in the original set of tryptic peptides (1, 2).
§ Amino acids incompletely liberated in 24-hour hydrolysis.
Further proof of the Cys-55-Cys-114 disulfide linkage was obtained through performic acid oxidation of preparative quantities of Fractions 1 and 2, and separation of the two cysteic acid-containing peptides, thus generated from each, by Sephadex G-75 gel filtration (Fraction 1, Fig. 5; Fraction 2, Fig. 6). The amino acid compositions of the larger peptides isolated from Fraction 1 (oxidized 1-A) and Fraction 2 (oxidized 2-A, Table I) were in agreement with that of T4 (2), and, as expected, glycine was shown to be NH-terminal, in oxidized 2-A (dansyl method).

The two smaller peptides purified from oxidized Fractions 1 and 2 were shown to be identical with tryptic peptide T11 (2) in their amino acid compositions (oxidized 1-B and oxidized 2-B, Table I) and in their dansyl end group analyses, which showed the expected leucine in both cases. Since Cys-55 and Cys-144 are the only half-cystine residues in T4 and T11, respectively, the above data uniquely identify these two as the participants in the intrasubunit disulfide bond.

Although the composition of oxidized 2-A (Table I) is in exact accord with that of T4, the composition of oxidized 1-A differs in the presence of an additional residue of lysine. From the known sequence near the COOH terminus of T4 (2), it may be surmised that oxidized 1-A terminates in a cystine sequence (Lys-67-Lys-68), while oxidized 2-A terminates in a single lysine (Lys-67).

In addition to Fractions 1 and 2, only Fractions 3 and 4 (Fig. 4) contained any cysteic acid after performic acid oxidation. These two fractions represented the free cysteinyl residue, isolated as a mixed disulfide derivative, because they both lacked cystine and contained sequences surrounding the same half-cystine residue. The possibility of disulfide linkage between two identical sections of sequence in different subunits is eliminated by the demonstrated absence of intersubunit disulfide bonds.

Fraction 3 was performic acid-oxidized and chromatographed on SE-Sephadex C-25 (Fig. 7). A single fraction was isolated, oxidized 3-A, whose amino acid composition (Table II) and dansyl end group (alanine) were consistent with a sequence containing Cys-6 as a cysteinyl residue, but encompassing two tryptic peptides, T2 and T3. Fraction 3-A was evidently a product of incomplete tryptic cleavage. Fraction 4 (in Fig. 4) was performic acid-oxidized and chromatographed on SE-Sephadex C-25 to produce a single cysteic acid-containing fraction (4-A, Fig. 8), which required further chromatography on QAE-Sephadex for purification (Fraction 4-A1, Fig. 9). The amino acid composition of Fraction 4-A1 (Table II) and the dansyl end group (alanine) were in exact agreement with those expected for the single tryptic peptide, T2 (1, 2) containing Cys-6 as its cysteic acid derivative.

**DISCUSSION**

Bovine superoxide dismutase is an unusually stable enzyme; it is active in 10 M urea or 4% Na dodecyl-SO4, and is reversibly inactivated by 6 M guanidine hydrochloride (4). Bovine dismutase withstands prolonged contact with organic solvents at ambient temperatures in the course of its conventional isolation from erythrocytes (7). In the present work, the stability of this dismutase has been assessed by examining two explicit properties of the protein, the dissociability of the subunits, and the reactivity of the sulfhydryl groups.

Employing electrophoretic analysis in Na dodecyl-SO4 polyacrylamide gels as a criterion, it is found that bovine dismutase does not dissociate in 1% Na dodecyl-SO4, but that inclusion of 1% or 2% 2-mercaptoethanol with the Na dodecyl-SO4 effects partial (or complete) conversion to polypeptides of subunit size. Similar observations of the dependence of dissociation upon mercaptoethanol have been interpreted as evidence of disulfide linkages between the 2 subunits (35). However, the various procedures reported here, by which the molecule may be dissociated under conditions where disulfide linkages are stable, attest to the absence of such intersubunit bonds. Incubation of the holoenzyme with Na dodecyl-SO4 and EDTA, or treatment of the apoenzyme with Na dodecyl-SO4 alone, produces dissociation as observed with Na dodecyl-SO4 polyacrylamide gel electrophoresis. Sedimentation equilibrium analysis conclusively demonstrates that bovine superoxide dismutase is completely dissociated in the absence of mercaptoethanol, when 6 M guanidine hydrochloride and 0.01 M EDTA are present.

These results further corroborate previous reports of the strength of the subunit interactions of copper- and zinc-containing dismutases from bovine erythrocytes (3), human erythrocytes (33), and wheat germ (36). It is evident that the copper-zinc dismutases are frequently resistant to denaturing procedures which successfully dissociate many other multimeric globular proteins. Dissociation of these dismutases requires additional structural disruption beyond that conventionally used, in the form of heat (3, 36), sulfhydryl group modification (33), or re-
The half-cystine residues, originally present in Fractions 3 and 4 (Fig. 4) as mixed disulfide derivatives, were oxidized to cysteic acid before isolation of 3-A (Fig. 7) and peptide 4-A1 (Fig. 9). The values listed are the number of residues per molecule. Amino acids present in amounts less than 0.1 residue per molecule are omitted.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Oxidized 3-A</th>
<th>T2 + T3</th>
<th>Oxidized 4-A1</th>
<th>T2</th>
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<td>0.98 (1)</td>
<td>1</td>
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<tr>
<td>His</td>
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<td>1</td>
<td>0.99 (1)</td>
<td>1*</td>
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<tr>
<td>CySO₂H</td>
<td>2.00 (2)</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Thr</td>
<td>0.75 (1)</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Glu</td>
<td>0.59 (1)</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Pro</td>
<td>0.89 (1)</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Gly</td>
<td>2.00 (2)</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Ala</td>
<td>1.99 (2)</td>
<td>2</td>
<td>1.03 (1)</td>
<td>1</td>
</tr>
<tr>
<td>Val</td>
<td>3.01 (3)</td>
<td>3</td>
<td>1.97 (2)</td>
<td>2</td>
</tr>
<tr>
<td>Ile</td>
<td>0.78 (1)</td>
<td>0</td>
<td>0.85 (1)</td>
<td>1</td>
</tr>
<tr>
<td>Leu</td>
<td>0.88 (1)</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Phe</td>
<td>1.02 (1)</td>
<td>0</td>
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</tr>
<tr>
<td>No. of residues</td>
<td>20</td>
<td>20</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Yield (%)</td>
<td>7.5</td>
<td>12</td>
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<td></td>
</tr>
</tbody>
</table>

* Composition calculated from the known amino acid sequence (1, 2).

Assumed integral number of residues.

Identified as S-carboxymethylcysteine, in the original set of tryptic peptides (1, 2).

moval of the metal cofactors (33). Consistent with these physical and chemical observations, x-ray crystal studies of the bovine copper-zinc dismutase reveal considerable contact between the 2 subunits of the protein molecule, in the 5.5 Å (5) and 3.0 Å electron density maps.

In accord with previous studies of erythrocyte dismutases (3, 33) the undissociated and unreduced protein is observed to migrate in Na dodecyl-SO₄ polyacrylamide gels with an apparent molecular weight of 55,000 to 60,000. This observation has been interpreted as a dimerization of the 31,200 molecular weight species in Na dodecyl-SO₄ (3, 33). In light of the inapplicability of the Na dodecyl-SO₄ polyacrylamide gel system for accurately estimating molecular weights of proteins which are incompletely denatured, because of covalent cross-links or other structural contraints (31, 32), it is uncertain whether this value of 60,000 represents a numerically fortuitous artifact or a bonafide dimerization of the protein.

Further analysis of three-dimensional structure* and further examination of the procedures used for apoenzyme preparation and sulphydryl titration (37) will be required to resolve the present dilemma concerning bovine dismutase.

From tryptic digests of the sulphydryl-blocked protein, peptides were isolated which contained the disulfide bridge and the blocked cysteine residue. The amino acid compositions and the end group analyses of these peptides are only consistent with a disulfide bond between Cys-55 and Cys-144, and a free sulphydryl group at Cys-6. The yields in which these peptides were isolated further support the contentions that (a) a unique pattern of disulfide pairing exists and (b) the digestion at pH 6.5, although more alkaline than the usual acidic conditions employed (41, 42), was sufficiently acid to minimize alkaline cleavage and inter-change of disulfide bonds (43, 44).

The tryptic peptides containing the Cys-55-Cys-144 intrasubunit disulfide bond were isolated in a combined yield of 65%, and, following performic acid oxidation, the combined yields of the peptides representing Cys-55 were 51% and of those representing Cys-144, 46% (Table I). The two peptides containing the cysteic acid derivative of Cys-6 were isolated in a combined yield of 20% (Table II). Being less than quantitative, these yields cannot unequivocally exclude the possibility of secondary disulfide bond arrangements. However, yields in excess of 50% do necessitate that the 2 subunits be identical in their predominant pattern of disulfide pairing.

Two examples of anomalous hydrolysis by trypsin were noted on comparing its action upon the disulfide-containing polypeptide chain at the nonoptimal pH of 6.5 with that upon the S-carboxymethylated protein at pH 8.8 (1, 2). Peptide T4 was isolated in...
two forms in the present study (Gly-24 to Lys-67 and Gly-24 to Lys-88), but in exclusively one form (Gly-24 to Lys-67) with the S-carboxymethylated polypeptide chain as substrate (1, 2). Variability of tryptic cleavage in polybasic sequences of lysine and arginine has been documented many times (e.g. 45, 46). This example in superoxide dismutase is an instance in which the same sequence (Ser-66-Lys-67-Lys-68-His-69) undergoes different patterns of tryptic cleavage, depending upon the prior modification of the half-cystine residues and the pH of the digestion. With the S-carboxymethyl derivative, tryptic hydrolysis on the carboxyl side of Lys-67 is complete (1, 2). With the cysteine residue as a mixed disulfide derivative and the disulfide bond intact, hydrolysis after Lys-67 is partial, and thus the dilsine form of T4 (-Lys-67-Lys-68) is isolated.

The second example of anomalous tryptic cleavage is the isolation of oxidized peptide 3-A (Table II) a product of incomplete hydrolysis after lysine, in the sequence Lys-9-Gly-10-Asp-11. Local structural constraints upon trypsin action are implicated by this observation, because an identical sequence of amino acids (as Lys-33-Gly-24-Asp-25) appeared to be quantitatively cleaved in the same digestion. Cleavage after both Lys-9 and Lys-23 appeared to be quantitative with the S-carboxymethylated polypeptide chain as substrate (1, 2).

Bovine erythrocyte dismutase is similar to the human erythrocyte copper-zinc enzyme in molecular weight, in subunit size, and in the absence of intersubunit disulfide bonds (33, 47). In addition, both enzymes contain two titratable sulfhydryl groups per molecule (33).4 Peptides containing 2 different cysteine residues have been isolated from enzymatic digests of human dismutase after S-alkylation with radiolabeled iodoacetic acid (33). The amino acid composition of one of these cysteine peptides (a tryptic peptide, soluble at pH 2, and resistant to cleavage by chymotrypsin) is identical with that of tryptic peptide T2 from bovine dismutase, which contains Cys-6, the single free cysteine in the bovine subunit chain. The composition of the other radiolabeled peptide from human dismutase appears to be corresponding to 1 cysteine and 1 cystine residue, in each of 2 distributed between 2 noncovalently linked subunits (33), which enzyme contains 8 half-cystine residues (33). These 8 residues are distributed in having a larger half-cystine content which is distributed in nonidentical disulfide arrangements in the two subunits. Structural analysis of the human erythrocyte enzyme (33) will establish whether its unusual stability is assisted by a disulfide pairing pattern which is dissimilar or is homologous to that determined here for the bovine enzyme.

Acknowledgments—We wish to thank Mr. Mark A. Polokoff for his technical assistance with the peptide isolation and characterization, and various members of the laboratory of Dr. Irwin Fridovich, Department of Biochemistry, Duke University, for their advice regarding the purification and assistance with the assay of superoxide dismutase. The secretarial assistance of J. M. E., throughout the preparation of these three articles, has been greatly appreciated.

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Bovine Erythrocyte Superoxide Dismutase: SUBUNIT STRUCTURE AND SEQUENCE LOCATION OF THE INTRASUBUNIT DISULFIDE BOND
John L. Abernethy, Howard M. Steinman and Robert L. Hill


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