Bovine Erythrocyte Superoxide Dismutase

COMPLETE AMINO ACID SEQUENCE*

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SUMMARY

The complete primary structure of reduced and S-carboxymethylated bovine erythrocyte superoxide dismutase has been derived through analysis of peptides from peptic, plasmin, and hydroxylamine digests of the intact polypeptide chain, and from chymotryptic, subtilisin, and dilute acid digests of derived peptides. From these data, the following unique amino acid sequence of 151 residues was deduced, corresponding to a molecular weight of 15,600, in each of the 2 apparently identical subunits of the protein molecule. (Cmc denotes S-carboxymethylcysteine.)

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  1  Ac-Ala-Thr-Lys-Ala-Val-Cmc-Val-Leu-Lys-Gly-Asp-20
  Gly-Pro-Val-Gln-Gly-Thr-Ile-His-Phe-Glu-Ala-Lys-50
  Gly-Asp-Thr-Val-Val-Val-Thr-Gly-Ser-Ile-Thr-Gly-40
  Leu-Thr-Glu-Gly-Asp-His-Gly-Phe-His-Ile-Gln-50
  Phe-Gly-Asp-Asn-Thr-Gln-Gly-Cmc-Thr-Ser-Ala-Gly-70
  Pro-His-Phe-Asn-Pro-Leu-Ser-Lys-His-Gly-Gly-100
  Pro-Lys-Asp-Glu-Glu-Arg-Ile-Gly-Lys-Ala-Leu-50
  Asn-Val-Thr-Ala-Asp-Lys-Asn-Gly-Val-Ala-Ile-Val-100
  Asp-Ile-Val-Asp-Leu-Ile-Ser-Leu-Gly-Glu-110
  Tyr-Ser-Ile-Asp-Gly-Arg-Thr-Met-Val-Val-His-120
  Gln-Pro-Leu-Gly-Arg-Gly-Gly-Leu-Glu-Val-Asn-Ala-Gly-140
  Ser-Thr-Lys-Thr-Gly-Arg-Asn-Ala-Ser-Arg-Leu-Ala-Cmc-Gly-150
  Val-Ile-Gly-Ile-Ala-Lys
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cyanogen bromide digests of the reduced and S-carboxymethylated protein, and from individual tryptic digests of the two cyanogen bromide peptides, after maleylation (1). Approximately 60% of the amino acid residues were placed in sequence within these various peptides, by manual or automated sequencing procedures, and about one-half of the tryptic peptides were ordered through direct analysis of overlapping peptides, through compositional analysis of overlap peptides, or through end group analysis (Ref. 1, Fig. 6). Determination of the total primary structure reported here required characterization of the peptides derived from several other types of digests, including cleavage with pepsin, plasmin, chymotrypsin, subtilisin, hydroxylamine, and dilute acid. The assignment of the single cysteine and cystine functions to the 3 half-cystine residues per subunit, identified here as S-carboxymethylcysteine, is reported in the following article (2).

EXPERIMENTAL PROCEDURES

Enzyme Preparation

Bovine erythrocyte superoxide dismutase was purchased from Truett Laboratories (Dallas, Texas) and purified further by chromatography on DEAE-cellulose (3) or by passage through a sulfanilamide affinity column (4) for removal of the carbonic anhydrase. All dismutase preparations showed a single protein band when subjected to electrophoresis on acrylamide gels (5) and had specific activities between 3500 and 4000 (AA280 = 0.0125 per min) units per mg in the xanthine oxidase-cytochrome c assay system (3). The reduced and S-carboxymethylated derivative was prepared as described earlier (1). However, the apoenzyme was not isolated, but was prepared in situ, by including EDTA (7 to 8 mM) in the 6 M guanidine hydrochloride-Tris (0.5 to 1.0 M) buffer solution used as the denaturing solvent for the reduction and subsequent alkylation reactions.

Materials

In addition to those cited in the preceding paper (1), the following enzyme and chemical reagents were employed. Pepsin and α-chymotrypsin (code CDS) were from Worthington Biochemical, subtilisin from Schwartz-Mann, and prolidase (imidodipeptidase) from Miles Laboratories, Research Division. Human urokinase was obtained from Leo Pharmaceuticals, Copenhagen. Dimethylallylamine and N-ethylmorpholine were “Sequanal Grade” (organic bases) from Pierce Chemical Company, and both SE-Sephadex C-25, and QAE-Sephadex A-25 from Pharmacia Fine Chemicals. Urea for column chromatography was deionized, as a 10 M solution, and diluted into the buffer solution to make the desired final concentration. The DEAE-cellulose was a Whatman product (TFF32).
Preparation of Cyanogen Bromide and tryptic Peptides from 
RCM1 Superoxide Dismutase for Subfragmentation

The NH2-terminal cyanogen bromide peptide, B1, was isolated by G-75 Sephadex gel filtration as described previously (1), except that the column was eluted with 30% (v/v) acetic acid. Tryptic peptides T4 and T7 were isolated as an “insoluble” fraction. For example, the tryptic digest from 12.6 μmoles of RCM superoxide dismutase (subunit) was freeze-dried, and the resultant solid was extracted once with 6 ml 0.1 M acetic acid and twice with 4 ml of water. The residual precipitate was dissolved in 50% (v/v) acetic acid and chromatographed on Sephadex G-75 in 30% acetic acid to effect purification of T4 (1). In these studies, the tryptic digest was not extracted with 0.02 M pyridine-acetate, pH 2.20, as described previously (1).

Selective Enzymatic and Chemical Cleavage of Superoxide Dismutase and Dismutase Peptides

In the following sections, the preparations and conditions of the digests are described. The purification schemes are only briefly alluded to; further details and all of the chromatogram profiles are given in the supplementary data section, which appears in miniprint at the end of this article. The purity of small peptides was assessed by their amino acid composition and the purity of large peptides by their composition in conjunction with the results of sequencer analysis.

Pepdic Digest of Dismutase—The single cysteine residue per subunit was alkylated with N-ethylmaleimide prior to peptic digestion. Salt-free protein (57 mg, 5.57 μmoles of subunits) was dissolved in 10 ml of 6 M guanidine hydrochloride, 0.01 M EDTA, pH 6.85, to which was added 120 μmoles of N-ethyl-(2,3,4-3H)-maleimide (specific activity, 0.41 mCi per μmole). After 24 hours at 24°, the solution was dialyzed against water. The protein recovered after freeze-drying was suspended in 10 ml of 5 M HCl, and 2 mg of pepsin were added. After 22 hours at 37°, the suspension had clarified. It was freeze-dried, extracted with 2 ml of 1% acetic acid, and centrifuged to remove a small amount of insoluble material. The precipitate was washed once with 2 ml of 1% acetic acid, and the combined supernatants were chromatographed on Sephadex G-50. Sephadex fractions were further purified by chromatography on SE-Sephadex, then on QAE-Sephadex, if required, as depicted in the “Supplementary Material” section.

Plasmin Digest of Reduced and S-Carboxymethylated Superoxide Dismutase—Plasminogen was purified from human plasma by affinity chromatography (6), and stored as a suspension in 40% ammonium sulfate. Approximately 2 ml of suspension were centrifuged, and the precipitate dissolved in 5 ml of 0.1 M sodium phosphate, pH 7.4, then dialyzed against pH 7.4 phosphate, and finally freeze-dried. A portion of the dried material, containing approximately 1.5 mg of plasminogen (determined by absorbance measurements (7)) was dissolved in 2.0 ml of water. Urokinase (100 μl of 0.1 M sodium phosphate solution, pH 7.4, containing 2500 units of enzyme per ml) was added to this solution, and after 5 min at 37°, the enzyme activation mixture was added to 59 μg (3.78 μmoles of subunit) of S-carboxymethylated superoxide dismutase suspended in 5.0 ml of water. This digestion mixture (resultant pH of 7.8) was incubated at 37° for 6 hours, then acidified to pH 2 with HCl, and rotary evaporated to dryness. The residue was extracted three times with a total of 10 ml of 0.1 M triethylamine-acetate, pH 8.9, and the combined supernatants were chromatographed on Sephadex G-75. Sephadex fractions were further purified by chromatography on Whatman DE52 in 0.5 M urea.

Hydroxylamine Treatment of Reduced and S-Carboxymethylated Superoxide Dismutase—A solution of 4 μl in 5 M urea was prepared immediately before use (8). Five milliliters of water were added to 0.1 μl of hydroxylamine hydrochloride. At 0° with vigorous stirring, 4.0 ml of 12.5 M sodium hydroxide were added, followed by 10 ml of 1 M potassium carbonate, and the resulting solution adjusted to pH 9.0. This hydroxylamine solution (20 ml) was added to a solution of S-carboxymethylated dismutase (115 mg of protein, 7.37 μmoles of subunit, in 20 ml of 8 M urea, pH 6.6). This reaction mixture (pH 9.0), was incubated at 45° for 3 hours, and then desalted by chromatography on Sephadex G-25, equilibrated with 0.1 M triethylamine-acetate, pH 8.9. The mixture was rotary evaporated to dryness and then extracted with approximately 8 ml of 0.02 M ammonium bicarbonate in 0.5 M urea, pH 8.0. A small amount of insoluble material was removed by centrifugation, and the supernatant solution was chromatographed on Whatman DE52, in 0.5 M urea. Fractions were collected and chromatographed on Sephadex G-75, if required, for purification of the hydroxylamine peptides.

Brief Chymotryptic Digest of T4—Peptide T4 (0.63 μmole) was incubated at 24° in 0.1 M ammonium bicarbonate, pH 8.5, at a concentration of 1 μmole per ml, with a chymotrypsin-peptide (molar) ratio of 1:400. After 15 min, 0.1 μmole of DFP was added, and after an additional 1/2 hour of incubation, the mixture was freeze-dried. The digest was chromatographed on Sephadex G-75 in 20% acetic acid.

Chymotryptic Digest of B1—The NH2-terminal cyanogen bromide peptide, B1 (2.4 μmoles), was suspended in 2 ml of 0.1 M ammonium bicarbonate, pH 8.5, and 0.35 μg of D-chymotrypsin added (molar ratio of peptide to enzyme, 1:170). After 5 hours at 37°, 0.5 μmole of DFP was added, and, following an additional 1/2 hour of incubation at 24°, the clear solution was freeze-dried. The digest was chromatographed on Sephadex G-75 in 20% acetic acid, and individual fractions were rechromatographed on Sephadex G-75 or G-50 and SE-Sephadex for purification of the chymotryptic peptides.

Dilute Acid Hydrolysis of T4—Peptide T4 (2 to 3 μmoles) was incubated in vacuo for 15 to 16 hours, at 105° to 107°, in 0.25 N acetic acid (1 to 1.5 μmoles of peptide at 1 μmole per ml concentration in each evacuated ampule). The hydrolysate was evaporated to dryness and chromatographed on Sephadex G-50 in 20% acetic acid.

Subtilisin Digest of T4-A2—Peptide T4-A2 (0.5 to 1.0 μmole) was incubated for 1 hour or 12 hours at 24°, in 0.1 M ammonium bicarbonate, pH 7.5, at a peptide concentration of 1 μmole per ml, with 20 μg of subtilisin. The digestion was terminated by freeze-drying, and the resultant peptides purified by chromatography on SE-Sephadex, followed by QAE-Sephadex, if required. The 12-hour subtilisin digest contained several peptides in low yield (T4-A2-S1a, T4-A2-S1b) which were not isolated from the 1-hour digest. However, the peptides isolated in highest yields from the two digests were identical.

Amino Acid Analysis, Sequential Degradation, and Exopeptidase Digestion

These operations were performed as described in the preceding paper (1), with minor exceptions. Occasionally, alternative protocols for the manual Edman degradation were used (9, 10). In some instances, hydriodic acid hydrolysis of the thiazolone (11) in place of, or in conjunction with, subtractive techniques was used to analyze the progress of the degradation. The presence of prolinal residues in peptide T4-A2 and its subtilisin fragment, S3, rendered aminopeptidase M alone ineffective for complete enzymatic hydrolysis; inclusion of prolidase in the digest mixture (0.3 to 0.5 mg per 1 μmole of peptide) produced virtually complete hydrolysis.

Peptide Nomenclature

Peptides produced by digestion of the intact polypeptide chain are designated by a capital letter, abbreviating the cleavage agent used, followed by a number, representing the relative location of the peptides from that digest in the sequence, from

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1 The abbreviations used are: RCM, reduced and S-carboxymethylated; DFP, diisopropylphosphorofluoridate; PTH, 1-phenyl-2-thiohydantoin; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl chloride.

2 Miniprinted material may be read without strain, using any type of large-field reading glass, available at most opticians.
NH₂ to COOH terminus (e.g. P₁, P₂). Fragments of these peptides, produced by subsequent cleavage by a different agent, are designated by a hyphen and a similar letter and numeral abbreviation, following the designation of the parent peptide (e.g. B₁-C₃). If nonspecific cleavage occurred in these subfragments, a lower case letter distinguishes the secondary peptides which overlap the initial fragment (e.g. T₄-A₂-S₁a, T₄-A₂-S₁b). The abbreviations for the cleavage agents are: A, dilute acid; B, cyanogen bromide; C, chymotrypsin; H, hydroxylamine; L, plasmin; MT, trypsin after maleylation; P, pepsin; S, subtilisin; T, trypsin.

Computer Comparison of Primary Structures

Sequence relatedness was assessed semiquantitatively with a modification of a computer program described previously (12). In this program, the sequence homology between two proteins is measured by the minimum number of base differences between the RNA triplet codons for the amino acids being compared. A comparison length (12) of 20 residues was employed.

RESULTS

Purification of Peptides

Combinations of gel filtration on Sephadex G-50 and G-75 and ion exchange chromatography on SE- and QAE-Sephadex were, in general, effective for purification of the peptides encountered. However, certain fractions from complex digests, most notably the peptic digest, remained impure even following successive gel filtration and cation and anion exchange chromatography. The purification of peptides of medium-to-large size is often an obstacle to the progress of sequence analysis. In this study, peptides containing 45 to 60 residues, from plasmin and hydroxylamine digests of RCM superoxide dismutase, were efficiently purified by chromatography on DEAE-cellulose. To minimize adsorptive losses on the ion exchanger, urea was present in all of the elution buffers used with DEAE-cellulose. The concentration employed, 0.5 M urea, appeared to be adequate for this function, while not being so high as to generate technical problems in desalting the chromatographic fractions. Ion exchange chromatography of peptides, utilizing urea as a non-ionic solubilizer, has been often used (13-15), but usually with higher concentrations (6 to 8 M). The tables of amino acid compositions, of peptides from the various digests, are contained in the miniprinted supplement at the end of this article.

Sequence Analysis of Peptides—Purified peptides were submitted to sequence analysis using the Edman degradation, in its manual or automated form, and exopeptidase digestion. Examples of the application of these procedures appeared in the preceding article (1); the data which substantiated the sequence conclusions drawn here are entirely contained in the miniprinted supplement.

Completion of the primary structural analysis required (a) sequence analysis of unknown regions within tryptic peptides, including gaps encountered in sequenator runs, and (b) ordering of the tryptic peptides through the identification of overlapping peptides. In tryptic peptide T₄ (1), the gaps at cycles 24 and 27 (residues 44 and 47) were identified as histidine and glutamine through characterization of peptide P₅. The ability to perform an extended Edman degradation of hydroxylamine peptide, H₁, with the sequenator was of considerable use in elucidating unknown regions of sequence within peptide T₇. Sequenator analyses were also critical in identifying some overlap sequences for tryptic peptide alignment, and very convenient in providing the amide assignments, through direct identification of asparagine- and glutamine-PTH derivatives. The peptic digest provided a number of small peptides which were indispensable in confirming the alignment of the tryptic peptides. Lastly, it may be noted that the COOH-terminal section of T₄ was inaccessible to sequenator degradation (1). The identification of residues 53 to 66 required subtilisin digestion of an acid cleavage fragment of T₄.

Reconstruction of the Amino Acid Sequence—The complete amino acid sequence of bovine erythrocyte superoxide dismutase has been deduced from the data reported in this and in the preceding article (1). The sequence and summaries of the peptide characterizations which uniquely define it are presented in Fig. 1. The 11 tryptic peptides (1) are unambiguously aligned by direct analysis of overlapping sequences and by compositional analysis of overlapping peptides. Peptide T₁ is placed at the NH₂ terminus by virtue of its acetylated α-amino group and is positioned before T₂ by P₁ sequence data and B₁-C₁ composition data. The overlap between T₂ and T₃ is identified by sequence analysis of B₁-C₂ and that between T₃ and T₄ by sequence analysis of P₂ and the composition of B₁-C₃. The T₄-T₅, T₅-T₆, and T₆-T₇ alignments are demonstrated by direct sequencing of B₁-C₄, L₂, and P₆, respectively. Sequence data for B₁-C₆ and the compositions of P₇ and P₈ serve to position T₇ and T₈, and sequence data for B₉ to position T₈ and T₉. The alignment of T₉ and T₁₀ is established by end group analysis and the composition of B₂-MT₂. Sequence analysis of H₂ and the composition of L₄ identify the alignment of T₁₀ before T₁₁. Peptide T₁₁ is placed at the COOH terminus because its COOH-terminal residue is identical with that of B₂ and also that of the intact polypeptide chain. Of these 10 tryptic peptide alignments, only that between T₂ and T₃ is not rigorously proven. The single residue overlap provided by B₁-C₂ and the consistent composition of B₁-MT₁ do not entirely exclude the possibility that between T₂ and T₃ is a peptide not isolated from the tryptic digest. However, the close fit of the 3A electron density map of crystalline bovine superoxide dismutase with the proposed sequence for this region suggests that this unlikely loss of a tryptic peptide has not occurred.

A molecular weight of 15,600 per subunit polypeptide chain, and 31,200 for the bovine apoenzyme, is calculated from this amino acid sequence. These values are in good agreement with molecular weight analyses of holosuperoxide dismutase by standard physical chemical procedures, which determine a value of 32,000 to 34,000 (3, 16, 17).

In Table I the amino acid composition of bovine dismutase, determined from the primary structure, is compared with that calculated from amino acid analysis of acid hydrolysates of the RCM protein (1). Duplicate samples were hydrolyzed for 24, 48, and 96 hours. Linear extrapolation to zero hour hydrolysis was used to calculate values for threonine and serine; valine and isoleucine values were taken from analysis of the 96-hour hydrolysates. For all other amino acids the average of the values from the three different times were taken, except for tyrosine, which was assayed spectrophotometrically (18).

Specificity Achieved by Selective Cleavage Agents—The ease with which the primary structure of a protein is reconstructed is related in no small measure to its susceptibility to enzymatic and chemical methods for cleaving peptide bonds in a selective and limited fashion. Scissile peptide bonds in bovine dismutase are identified in Table II, as inferred from these peptic, plasmin, chymotryptic, subtilisin, hydroxylamine, and dilute acid peptides which were isolated. The peptic cleavage sites are in accordance with the known preference of that protease for cleavage on the amino or the carboxyl side of hydrophobic residues (19).

² D. C. Richardson, personal communication.
FIG. 1. The complete amino acid sequence of bovine erythrocyte superoxide dismutase, and the peptides characterized for its derivation. The half-cystine residues were identified as their S-carboxymethyl derivatives. Cleavage agents are abbreviated by T, trypsin; C, chymotrypsin; B, cyanogen bromide; P, pepsin; MT, trypsin following maleylation; A, dilute acid; S, subtilisin; Pl, plasmin; H, hydroxylamine; † designates the site of cleavage by cyanogen bromide. The span of each peptide, which was isolated, is designated by a horizontal line, with arrows on either end; vertical cross-hatching appears beneath the residues which were placed in sequence by manual or automated characterizations of the specific peptide designated.

Pepsin catalyzed cleavage on both the amino and carboxyl side of Leu-36 (producing P3 and P4) and of Tyr-108 (producing P7 and P8). Of the chymotryptic cleavage sites detected, the relative lability of the Leu-Ser bond may be noted. This bond (as Leu-65 to Ser-66) was nearly quantitatively cleaved in the brief chymotryptic digest of T4, and the efficient cleavage of Leu-Ser bonds at both the NH₂ and the COOH terminus of the sequence of B1-C4 (Leu-65 to Ser-66 and Leu-104 to Ser-105) permitted its isolation in high yield. Plasmin, well known for its selective proteolysis in blood-clotting phenomena (20), may find increased use in future protein sequence studies. Although only three plasmin peptides were purified, it is evident from these alone that plasmin is a protease much more selective than trypsin, because each peptide contained one or more bonds which were susceptible to trypsin. Hydroxylamine was initially reported as a highly selective chemical cleavage agent for asparaginyl peptide bonds, showing a strong preference for Asn-Gly (8, 21, 22). In addition to Asn-Gly, cleavage of an Asn-Ala bond has been identified in this study. Hydrolysis with dilute acetic acid has been found to be a selective method for excision of aspartyl residues from peptides (23, 24). Dilute acid cleavage of T4 resulted in the expected excision of Asp-25, and of Asp-50, but showed two instances of lack of strict specificity. (a) There was some evidence for deamidation of Asp-51 and subsequent acid cleavage of the resultant Asp-51 to Thr-52 bond. Edman degradation of peptide T4-A2, obtained by gel filtration, showed Asx NH₂-terminal, and, in addition, a smaller amount of threonine. (b) There was no evidence for excision of Asp-40 between Gly-39 and His-41. An incorrect identification of residue 40 as aspartic acid, rather than asparagine, could account for this observation. However, enzymatic hydrolysis of peptic peptide P4 was in accord with sequenator analyses of T4 in assigning aspartic acid. Ionic interaction with the adjacent imidazole side chain of His-41 may have reduced the participation of the Asp-40 side chain in intramolecular catalysis. The specificity of trypsin was in complete accord with expectations (25). Thus, cleavage was efficiently affected after all lysine and arginine residues except (a) Lys-73, which was followed by the sequence, Asp-Glu-Glu, (b) Lys-120, which was followed by a proline, and (c) Lys-51, which was the COOH terminus of the peptide chain.

DISCUSSION

In recent years, bovine erythrocyte superoxide dismutase has been actively studied in a number of laboratories (e.g. 26–29). This copper- and zinc-containing protein has been found to be suitable for application of highly refined spectroscopic techniques (30–32) and of methods of protein chemical characterization (33, 34). The enzyme has been useful as a biological probe of the involvement of superoxide radicals in biochemical and chemical reactions (20, 35) and has been crystallized in a habit amenable to
x-ray structure analysis (36). Dismutases have been isolated from different biological sources, possessing different metal cofactors (37, 38), and the x-ray crystal analysis has progressed to a 3 A resolution electron density map (39). Amidst the present state of knowledge of bovine superoxide dismutase, the primary structure reported here will not only provide additional insight into the structure and function of this enzyme, but will also serve as a focal point for comparative structural studies among superoxide dismutases (40). The following article (2) describes the assignment of the cysteine and cystine functions to the 3 half-cystine residues in the subunit sequence.

Some comments upon (a) the uniqueness and (b) the accuracy of the reported amino acid sequence are appropriate at this point. Regarding the conflicting reports about the occurrence of tryptophan in bovine dismutase (16, 28, 41), little doubt remains that none is present. In the preceding article, it was noted that no tryptophan was found in hydrolysates of the protein (1). In all of the amino acid analyses, exopeptidase digestions, and Edman degradations of the peptides reported here, there was also no evidence for the presence of tryptophan.

The accumulated evidence from characterization of all of the peptides is summarized in Fig. 1. All of these data are consistent only with the unique amino acid sequence shown. In this primary structure investigation no peptides were isolated from digests by any of the nine different cleavage agents used which could not be placed in this sequence. Since it was not possible to isolate every tryptic peptide in quantitative yield, a definitive statement of the absolute absence of microheterogeneities cannot be made. The reported presence of 1 to 2 residues of hexose per dismutase molecule (16) cannot be excluded, either. However, all of the serine, threonine, and asparagine residues directly identified by sequenator analysis, and 2 of the asparagine residues, whose carboxyl site was cleaved by hydroxylamine, are presumably not glycosylated.

As noted in the previous article (1), several tryptic peptides were isolated in yields exceeding 50%. In addition, there were a number of instances where the same region of sequence was found in peptides isolated from different digests. Furthermore, some of the peptides found to be of homogeneous sequence by sequencor analysis were purified solely by gel filtration procedures which would not be expected to separate microheterogeneous forms containing amino acid substitutions at specific residue loci. Lastly, it may be noted that the 2 subunits of bovine superoxide dismutase appear to have the same over-all conformation in the 5.5 A resolution electron density map (39) and to have the same polypeptide backbone conformation in the 3 A resolution map. All of these observations lend credence to the conclusion drawn here: that the 2 subunits of bovine erythrocyte dismutase possess the same, unique primary structure of 151 residues.

Regarding the accuracy of the sequence reported herein, the data may stand for themselves. Confidence in the accuracy of primary structural information obtained through sequenator analysis has increased as the use of this propitious instrument has increased since it was first reported (42). Although most of the sequenator analyses were performed only once, no errors were ever found when time-proven manual procedures were used to sequence identical regions in overlapping peptides. Furthermore, all of the gaps encountered were subsequently found to correspond to real residues, removed by the Edman degradation but not identified in the course of the sequenator analysis, rather...
than to spurious machine operation. Amide assignments made by direct identification of the amino acid-PTH derivatives were unambiguous. One amide assignment made by exopeptidase digestion is possibly questionable. Residue 47 was assigned as glutamine because (a) only a fractional amount of glutamic acid was liberated by aminopeptidase digestion of P5, while other residues were present in integral amounts, and (b) glutaminyl residues adjacent to histidine are known to be relatively susceptible to deamidation (43).

The agreement is not exact between the composition of bovine dismutase, as determined from the primary structure and as identified from amino acid analysis of acid hydrolysates of the reduced and S-carboxymethylated protein (Table I). The sequence is assuredly a more reliable means to obtain an accurate composition, and some discrepancies merit comment. The low values for valine and isoleucine are the result of incomplete liberation of these amino acid residues, even after 96 hours of hydrolysis. The protein contains: 1 Ile-Ile, 1 Val-Val, 1 Val-Ile, 2 Ile-Val, and 1 Val-Val-Val sequence. The low values found for lysine and histidine are unexpected, but could be attributed to alklyation during the S-carboxymethylation reaction. As noted previously (1), the lysine and histidine contents of unmoldified bovine dismutase (16) are in exact accord with the results of sequence analysis.

It has been suggested that the amino acid sequences of bovine erythrocyte superoxide dismutase and carbonic anhydrase may be homologous because of the striking similarity in the magnetic circular dichroism spectra of derivatives of these proteins in which the naturally occurring zinc (II) is replaced by cobalt (II) (32). In fact, no sequence homology whatsoever was detected on comparison of the dismutase sequence with that of human carbonic anhydrase B (44) by computer technology (12). The human enzyme sequence was employed because the bovine sequence has not been reported. In view of the striking similarity that is frequently found on comparing sequences of other proteins isolated from bovine and from human sources (45), a similar lack of homology may be anticipated between the sequences of bovine dismutase and bovine carbonic anhydrase. It is possible that the three-dimensional conformations around the zinc binding sites of bovine dismutase and carbonic anhydrase are indeed similar, as suggested by the magnetic circular dichroism studies, while other portions of their structures differ. Such a case of local conformation identity has been observed in the virtually identical three-dimensional structures of the coenzyme binding sites in dehydrogenases of apparently unrelated amino acid sequences (46). An ultimate disposition to these projections is possible now that the crystal structure of bovine dismutase has been solved at a resolution of 3 A. The crystal structure of human carbonic anhydrase C is known at 2 A resolution (47). No search was undertaken for sequence homologies between bovine dismutase and other proteins; however, it may be mentioned that computerized comparison procedures showed no internal homology within the dismutase sequence itself. Two repeating tripeptide sequences are evident on inspection: Lys-Gly-Asp, at residues 8 to 11 and 23 to 25, and Ile Val Asp, at residues 94 to 96 and 97 to 99.
Amino acid composition of S-carboxymethyl bovine dismutases

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Sequence</th>
<th>Acid Hydrolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>10</td>
<td>9.32 ± 0.1</td>
</tr>
<tr>
<td>Histidine</td>
<td>8</td>
<td>6.78 ± 0.1</td>
</tr>
<tr>
<td>Arginine</td>
<td>4</td>
<td>3.85 ± 0.03</td>
</tr>
<tr>
<td>S-Cm Cysteine</td>
<td>3</td>
<td>2.42 ± 0.07</td>
</tr>
<tr>
<td>Aspartic Acid</td>
<td>17</td>
<td>17.1 ± 0.7</td>
</tr>
<tr>
<td>Threonine</td>
<td>12</td>
<td>11.1 ± 0.1</td>
</tr>
<tr>
<td>Serine</td>
<td>8</td>
<td>8.0 ± 0.1</td>
</tr>
<tr>
<td>Glutamic Acid</td>
<td>11</td>
<td>11.7 ± 0.1</td>
</tr>
<tr>
<td>Proline</td>
<td>6</td>
<td>6.60 ± 0.1</td>
</tr>
<tr>
<td>Glycine</td>
<td>25</td>
<td>22.5 ± 0.3</td>
</tr>
<tr>
<td>Alanine</td>
<td>9</td>
<td>8.83 ± 0.06</td>
</tr>
<tr>
<td>Valine</td>
<td>15</td>
<td>13.0 ± 0.1</td>
</tr>
<tr>
<td>Methionine</td>
<td>1</td>
<td>0.97 ± 0.08</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>9</td>
<td>6.32 ± 0.07</td>
</tr>
<tr>
<td>Leucine</td>
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</tr>
<tr>
<td>Tyrosine</td>
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<td>1.30 ± 0.05d</td>
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<tr>
<td>Phenylalanine</td>
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<td>4.10 ± 0.04</td>
</tr>
</tbody>
</table>

- Residues per molecule of subunit as calculated from the sequence or determined from acid hydrolytic procedures, described under "Experimental Procedure."
- Includes glutamine.
- Determined spectrophotometrically (18).

Table II

Documented peptide bond cleavages in bovine dismutase

The peptides from which these cleavages were deduced, are summarized in Fig. 1.

<table>
<thead>
<tr>
<th>Agent</th>
<th>Bond</th>
<th>Agent</th>
<th>Bond</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pepsin</td>
<td>Phe-Glu, Phe-His,</td>
<td>Collagen</td>
<td>Gly-Gly, His-Asp,</td>
</tr>
<tr>
<td></td>
<td>Tyr-Ser, Glu-Tyr,</td>
<td></td>
<td>Ser-Ala, Leu-Ser,</td>
</tr>
<tr>
<td></td>
<td>Leu-W, Leu-Asn,</td>
<td></td>
<td>S-Cm-Cys-Thr,</td>
</tr>
<tr>
<td></td>
<td>Leu-Thr, Asn-Thr,</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Thr-Met, Thr-Val,</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ala-Val, Ala-Ile,</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ala-Asp, Glu-Glu</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chymotrypsin</td>
<td>Phe-Glu, Phe-His,</td>
<td>Dilute Acid</td>
<td>Gly-Asp, Asp-Asn,</td>
</tr>
<tr>
<td></td>
<td>Leu-Ser, Leu-Lys,</td>
<td></td>
<td>Asp-Thr</td>
</tr>
<tr>
<td></td>
<td>Tyr-Ser</td>
<td></td>
<td>Hydroxylamine</td>
</tr>
</tbody>
</table>

The amino acid sequence reported here excluded the possibility that the metal ions are bound via the α-amino nitrogen atoms, for the α-amino groups are acetylated. However, a histidine-rich sequence located within peptide T4 (His-Gly-Phe-His-Val-His, residues 41 to 46) was an obvious candidate for one or more of the metal binding ligands in this enzyme. In fact, His-44 and His-46 (as well as His-61 and His-118) are observed to be ligands of the copper atom in the 3 A resolution electron density map of bovine dismutase.

Certain regions of predominantly polar character are evident, and have been helpful in assigning exterior loops in the molecule as current x-ray crystal studies have progressed from 5.5 A (39) to 3 A resolution. The sequence between residues 67 and 78 is strikingly polar, with ionic side chains in 9 of the 12 amino acids. A significant, but somewhat less concentrated polar region, is contained between residues 118 and 134, with 9 ionic side chains among 17 amino acids. In the 3 A resolution electron density map, both of these sequences occur in exterior loops, which are exposed to solvent.

The most outstanding feature of the amino acid composition of bovine erythrocyte dismutase, and of other copper-zinc dismutases as well (48–50), is the predominance of glycine. In the bovine enzyme, nearly one-sixth of the amino acids are glycine, and the 25 residues of glycine per subunit are distributed relatively evenly throughout the amino acid sequence. This feature could permit many bends in the polypeptide backbone, allowing multiple contacts between nonadjacent regions in the primary structure. Such interactions could contribute to the remarkable stability of this dismutase. In the 5.5 A resolution electron density map of the crystalline enzyme, sharp turns in the subunit polypeptide chain are in fact evident in at least four places (38). In the 3 A resolution electron density map, those and other turns have been observed, and glycine residues have been found in most of them.

The most notable difference between the amino acid composition of the copper-zinc bovine dismutase and that of the bacterial manganese- and iron-containing dismutases (38, 51, 52) is the preponderance of alanine and the lesser amount of glycine in the bacterial enzymes. Although dismutases of both classes of metal cofactors have comparable subunit molecular weight and an identical catalytic action (26), the bacterial enzymes do not display the unusual stability typified by the bovine erythrocyte enzyme (26). It may be queried whether the three-dimensional structures of the two classes of dismutase enzymes are distinctly different. To answer these intriguing questions of protein evolution and the chemical basis of dismutase action, comparative studies have been initiated through amino acid sequence analysis (40) and x-ray crystal structure determination.

Acknowledgments—We wish to thank Dr. Irwin Fridovich, Department of Biochemistry, Duke University, and several members of his laboratory, particularly Bill Lipitt, for providing a substantial amount of the purified bovine dismutase used for this investigation. We thank Philip J. Walther for providing human plasma, and for his advice upon the purification and activation of plasminogen. The advice received from Dr. Mark A. Hermodson, Department of Biochemistry, University of Washington, about sequenator methodology, prior to publication, was greatly appreciated. Dr. Charles Cantor, Department of Chemistry, Columbia University, is thanked for providing the original form of the sequence comparison computer program which was used. We thank Mark A. Polokoff for his technical assistance in many different facets of this work.
REFERENCES

The above figure shows the results of an experiment on the growth of bacteria in different conditions. The x-axis represents the time in hours, and the y-axis represents the number of bacterial colonies. The figure shows four different conditions: control, 10% glucose, 20% glucose, and 30% glucose. The control condition shows a steady increase in bacterial colonies over time. The 10% glucose condition shows a moderate increase in bacterial colonies. The 20% glucose condition shows a significant increase in bacterial colonies. The 30% glucose condition shows the most significant increase in bacterial colonies. The figure also shows a control group that received no glucose, which shows a negligible increase in bacterial colonies.

The results of the experiment suggest that glucose can significantly increase the growth rate of bacteria. The optimal concentration of glucose for bacterial growth appears to be 20%, as it results in the highest number of bacterial colonies. The control group and the 10% glucose condition show a lower growth rate, indicating that glucose is necessary for bacterial growth but not at very high concentrations.
The following amino acid sequence was determined directly through their characterization. 1, as one of 518 of the 202 peptide fragments identified in the sequence of 518. 2, as one of 202 of the 202 peptide fragments identified in the sequence of 518. 3, as one of 202 of the 202 peptide fragments identified in the sequence of 518. 4, as one of 202 of the 202 peptide fragments identified in the sequence of 518. 5, as one of 202 of the 202 peptide fragments identified in the sequence of 518. 6, as one of 202 of the 202 peptide fragments identified in the sequence of 518. 7, as one of 202 of the 202 peptide fragments identified in the sequence of 518. 8, as one of 202 of the 202 peptide fragments identified in the sequence of 518.
Bovine Erythrocyte Superoxide Dismutase: COMPLETE AMINO ACID SEQUENCE
Howard M. Steinman, Vishweshwar R. Naik, John L. Abernethy and Robert L. Hill


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