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Superoxide Dismutase from *Bacillus stearothermophilus*. Complete Amino Acid Sequence of a Manganese Enzyme†

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ABSTRACT: Superoxide dismutase from the thermophilic bacterium *Bacillus stearothermophilus* is dimeric with a molecular weight of 45 487. It contains one atom of manganese(III) per dimeric molecule [Brock, C. J., Harris, J. I., & Sato, S. (1976) *J. Mol. Biol. 107*, 175–178]. The subunits are identical, and the following primary structure comprising 203 amino acids has been determined:

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          10        20        30        40
PFELPALPYPYDALEPHIDKETMNIHHTKHHNTYVTNLNAALE-
      50        60        70        80
GHPDLQNKSLEELLSNLEALPESIRTAVRNNGGGHANHSLFWTI-
  90        100       110       120       130
LSPNGGGEPTGELADAINKKFGSFTAFKDEFSKAAAGRFGSGW-
          140       150       160       170
AWLVVNNGELEITSTPNQDSPIMEGKTPILGLDVWEHAYYLKY-
                180       190       200
QNRRPEYIAAFWNVVNWDEVAKRYSEAKAK
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The enzyme is identical in 60% of its residues with the *Escherichia coli* B manganese enzyme [Steinman, H. M. (1978) *J. Biol. Chem. 253*, 8708–8720]. Neither manganese enzyme has significant homology with the Cu/Zn superoxide dismutase from bovine erythrocytes. The secondary structures of the manganese enzymes predicted by McLachlan's method [McLachlan, A. D. (1977) *Int. J. Quantum Chem. 12*, 371–385] indicate that the eight-stranded β barrel of the Cu/Zn enzyme [Richardson, J. S., Thomas, K. A., Byron, H. R., & Richardson, D. C. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 1349–1353] is absent from the manganese enzymes.

Superoxide dismutase catalyzes the dismutation of the $O_2^{-}\cdot$ radical to dioxygen and hydrogen peroxide. The enzyme is found in all organisms which metabolize oxygen. Its role may be to act as a defense against oxygen and superoxide toxicity (Fridovich, 1975).

The enzymes isolated from prokaryotes and mitochondria contain either manganese or iron. Both manganese and iron enzymes have been detected in *Escherichia coli* B (Dougherty et al., 1978). Only a manganese enzyme has been found in *Bacillus stearothermophilus*, but the existence of an iron enzyme cannot be excluded. The amino-terminal sequences of these enzymes are related to each other but not to the more widely studied enzymes found in the cytoplasms of eukaryotic cells (Harris & Steinman, 1977; Harris et al., 1980a; Walker et al., 1980a). These latter enzymes are dimeric and contain one Cu and one Zn per subunit (Fridovich, 1974). The dominant structural feature of the bovine erythrocyte Cu/Zn enzyme is an eight-stranded barrel of antiparallel β-pleated sheet (Richardson et al., 1975).

The manganoenzyme from the moderate thermophile *B. stearothermophilus* is dimeric and comprises two identical subunits of apparent molecular weight of ~20000 as estimated by gel electrophoresis in dodecyl sulfate and gel filtration in

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guanidine. It contains one manganese per dimer (Brock et al., 1976). The enzyme from this source is very stable to heat and chemical denaturants and, in common with other enzymes from this source, can be readily crystallized (Bridgen et al., 1976; Walker, 1979).

As part of a collaborative study of its structure with crystallographers (Smit et al., 1977), we have determined the amino acid sequence of this enzyme. It is highly homologeous with the sequence of the *E. coli* B manganese enzyme independently determined by Steinman (1978).

Materials and Methods

Materials. All chemicals were of Analar grade from B.D.H., Ltd., Poole, England, unless otherwise specified. Ferricytochrome *c*, xanthine, xanthine oxidase from raw cream, nitro blue tetrazolium, dianisidine, hemoglobin, and soybean trypsin inhibitor were all products of Sigma London Chemical Co., Ltd., Poole, England, TPCK-treated trypsin and chymotrypsin were from Worthington Biochemical Corp., *N*,*N*,*N*′,*N*′-tetramethylethylenediamine was from Koch-Light Laboratories, fluorescamine was from Roche Biochemicals, ninhydrin was from Merck, and Polybrene was from Aldrich Chemical Co., Ltd.

Aldolase, D-glyceraldehyde-3-phosphate dehydrogenase (Harris et al., 1980a,b) and triosephosphate isomerase, all from *B. stearothermophilus*, were kind gifts from colleagues. Potassium superoxide was donated by Dr. B. Halliwell, Department of Biochemistry, King's College, London.

Sephadex gel filtration media were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden, and DEAE-cellulose Whatman DE-52 was from W. & R. Balston, Maidstone, Kent, England.

Purification of Superoxide Dismutase from B. stearothermophilus. Superoxide dismutase was purified from *B. stearothermophilus* (strain NCA 1503) as part of a large-scale multienzyme isolation procedure. Proteins were purified from 5 kg of cells (Atkinson et al., 1972) or by a similar procedure from 70 kg of cells (Atkinson et al., 1979).

The early stages of the purification, which involved cell growth, cell breakage, deoxyribonuclease treatment, removal of cell debris by differential centrifugation, batchwise elution from DEAE-cellulose, and chromatography on DEAE-Sephadex, were carried out at either the Microbiological Research Establishment, Porton, Wiltshire, or the Imperial College of Science and Technology, London.

Fractions from the DEAE-Sephadex column were assayed for superoxide dismutase activity by the method of Henry et al. (1976) and found to contain ~26 g of active enzyme. Those fractions containing the enzyme were pooled in two batches according to whether or not they also contained phosphofructokinase activity. Each batch from a preparation from 70 kg of cells was received at this stage for final purification, having been concentrated to ~4 L final volume by ultrafiltration. Each was concentrated further to ~500 mL in an Amicon ultrafiltration cell containing a PM-10 membrane.

The phosphofructokinase-positive fraction was dialyzed against 50 mM Tris-HCl, pH 7.5, containing 1 mM $MgSO_4$, 2 mM 2-mercaptoethanol, 0.1 mM ethylenediaminetetraacetic acid, and 0.25 M KCl. It was then split up into 10 aliquots (50 mg of phosphofructokinase per aliquot), each of which was passed through a column of AMP–Sepharose with a bed volume of 40 mL in the same buffer. Under these conditions only the phosphofructokinase was bound (Hengartner & Harris, 1975). The material which did not bind to AMP–Sepharose from the entire phosphofructokinase-positive fraction was pooled and again concentrated to a final volume of ~500 mL by ultrafiltration. Thereafter, this material was treated in exactly the same way as the other superoxide dismutase containing fraction from the DEAE-Sephadex column.

If the enzyme was to be stored for an extended period of time, an equal volume of glycerol was added at this stage and it was then kept at −20 °C. Under these conditions partially purified superoxide dismutase could be stored for at least 3 years without significant loss of activity.

A portion of this partially purified enzyme was further purified as follows. From the superoxide dismutase positive, phosphofructokinase-negative fraction from 70 kg of cells (total volume 900 mL in 50% glycerol) was taken a 200-mL portion which contained approximately 45 mg/mL total protein and 3.75×10^6 units of superoxide dismutase activity by the method of McCord & Fridovich (1969) (equivalent to ~3 g of fully active enzyme). To this was added 1500 mL of 50 mM Tris-HCl, pH 7.5, and this solution was concentrated to 100 mL by ultrafiltration. A further 1500 mL of the same buffer was added, and the concentration step was repeated, this time to a final volume of 20 mL of highly concentrated protein (450 mg/mL) containing less than 0.4% glycerol. This material was split into four aliquots of 5 mL, each of which was applied to a 180 × 2 cm column of Sephadex G-75 superfine in 50 mM Tris-HCl, pH 7.5. Fractions (4 mL) from this column were assayed for superoxide dismutase activity by the method of McCord & Fridovich (1969), but it was found that this was coincident with absorbance of the column effluent at 480 nm and the distinctive purple–red color of the pure enzyme visible in the fractions. Consequently, on subsequent occasions it was localized on the basis of these last two properties alone. The purified material from the four aliquots applied to this column was pooled and yielded 150 mL of purified enzyme at a concentration of 10 mg/mL with a specific activity of 1250 units/mg by the method of McCord & Fridovich (1969) and with a second-order rate of 3.9×10^8 M^{-1} s^{-1} as measured by pulse radiolysis (Rotillo et al., 1972). This specific activity is less than half that reported for most other purified superoxide dismutases. This is probably because the assay is performed at a temperature considerably lower than that at which the enzyme operates under physiological conditions, although the optimum temperature has not been determined. A similar effect was noted with another thermophile enzyme (Harris et al., 1980b). The 200-mL portion taken yielded 1.5 g of pure enzyme. The 900-mL phosphofructokinase-negative fraction yielded a further 6.75 g. The overall yield from 70 kg of *B. stearothermophilus* cells was ~3 g of pure superoxide dismutase.

The purity of the enzyme was established by two criteria. Only a single amino-terminal residue, proline, was observed by the dansyl procedure, and there was a single band after polyacrylamide gel electrophoresis in a 12.5% gel containing 0.1% sodium dodecyl sulfate. The material used in this study was prepared independently of the enzyme used in a mass spectrometric study of its sequence (A. D. Auffret, T. J. A. Blake, and D. H. Williams, unpublished results).

Polyacrylamide Gel Electrophoresis. This was carried out in 20 × 20 cm slab gels according to either Davis (1964) or Laemmli (1970).

Determination of Protein Concentration. Protein concentrations of purified solutions of enzyme were determined from their optical densities at 280 nm. Calculations were based upon a value for $A_{1\%}^{280}$ of 14.1. This figure was derived from measurements on a solution of known concentration as determined by the method of Lowry et al. (1951) using bovine

serum albumin as a calibration standard.

Amino Acid Composition. Amino acid analyses were carried out with the aid of a Durrum D-500 amino acid analyzer.

Peptides were hydrolyzed for 24 h in vacuo at 105 °C in 6 N HCl containing 0.1% phenol (Thompson & Sanger, 1963).

The amino acid composition of superoxide dismutase was determined by hydrolysis of samples of protein for 24, 48, and 72 h in vacuo at 105 °C in 6 N HCl containing 0.1% phenol. Serine and threonine were extrapolated to zero time. For valine and isoleucine the values at 72 h were used.

Tryptophan was estimated by the method of Edelhoch (1967). A known quantity of freeze-dried protein was dissolved in 6 M guanidine hydrochloride and 50 mM sodium phosphate, pH 6.5. After 4 h the absorbance of the solution was measured at 280 and 288 nm.

Cleavage with Cyanogen Bromide. Freeze-dried protein (2 μmol) was dissolved in 70% formic acid (2 mL), and solid cyanogen bromide was added to give a final concentration of ~10 M. Digestion was complete after 4 h.

Succinylation and Cleavage with Hydroxylamine. Superoxide dismutase was succinylated primarily to block the amino terminus of protein. This has the advantage that the fragment containing the known amino-terminal sequence is masked in subsequent sequencing procedures. The succinyl protein was cleaved with hydroxylamine as described by Walker et al. (1980b). The cleavage procedure was based upon that of Bornstein (1970). The protein (20 mg) was dissolved in 5 mL of 6 M guanidine hydrochloride, and the pH was adjusted to 9.0 with 4 M NaOH. Succinic anhydride (50 mg) was dissolved in 1,4-dioxane and added to the protein solution over a period of 15 min; the pH was maintained at 9.0 by the addition of 4 M NaOH. This solution was then dialyzed overnight against 0.5% acetic acid and lyophilized.

Hydroxylamine hydrochloride (2.8 g) and sodium hydroxide (1.6 g) were dissolved in water (6 mL) in an ice bath to give a solution of pH 8.3. Potassium carbonate (0.6 g) was added which raised the pH to 9.8. This solution was added to the succinylated protein followed by 6 g of guanidine hydrochloride. The pH of this solution was adjusted to 9.5, after which it was incubated at 45 °C for 90 min. A precipitate which had formed upon addition of the guanidine hydrochloride was removed by centrifugation. The supernatant solution was desalted, and the peptides were partially purified on a 150 × 2 cm column of Sephadex G-25 superfine in 50 mM ammonium bicarbonate.

Digestion with Trypsin, Chymotrypsin, Thermolysin, and Protease from Staphylococcus aureus. Peptides were digested in 50 mM ammonium bicarbonate with trypsin (1:100 by weight) for 4 h, with chymotrypsin (1:50 by weight and 1:200 by weight soybean trypsin inhibitor) for 3 h, with thermolysin (1:50 by weight) for 24 h, or with protease from *S. aureus* (1:30 by weight) for 48 h. All digestions were carried out at 37 °C except for that with thermolysin which was at 45 °C.

These conditions were found to be unsuitable for the digestion of native superoxide dismutase. This could only be achieved by first denaturing the protein as follows. Superoxide dismutase was dialyzed against water and freeze-dried. It was then dissolved in a known volume of 8 M urea to a final concentration of ~40 mg/mL, and glacial acetic acid was added (final concentration 1 to 2%) until the distinctive purple–red color of the native enzyme had disappeared. The proteolytic enzyme was added in sufficient 50 mM ammonium bicarbonate to give a final solution containing 2 M urea and ~10 mg/mL superoxide dismutase. The pH of this solution was checked and adjusted if necessary with ammonia until it was within the range pH 7 to 8. The times and temperatures of digestion and enzyme/substrate ratios were the same as those used for the digestion of peptides.

Digestion with Carboxypeptidase Y. Digestion of peptides was carried out in a buffer containing 0.1 M sodium citrate, pH 5.3, 1 mM EDTA, and norleucine (0.1 mM) at room temperature and using an enzyme/substrate ratio of 1:200 (Hayashi, 1977). Samples were taken at intervals, and the reaction was stopped by placing them in a boiling water bath for 5 min. Samples were analyzed for free amino acids with the aid of a Durrum D-500 amino acid analyzer.

Native superoxide dismutase was denatured prior to digestion by dissolving it in a buffer containing 0.5% sodium dodecyl sulfate, 1 mM EDTA, and either 0.1 M sodium citrate, pH 5.3, or 0.1 M sodium citrate, pH 6.3 (Martin et al., 1977), and heating in a boiling water bath for 5 min. Norleucine (0.1 mM) was then added as an internal standard, and digestion was carried out as described for peptides.

Peptide Purification. Peptides from preparative scale digests were first subjected to gel filtration on Sephadex G-50 superfine or Sephadex G-25 superfine in 50 mM ammonium bicarbonate for enzymic digests or 1% formic acid for cyanogen bromide cleaved protein.

Pooled fractions were freeze-dried and were purified further by one or more of several techniques. Some larger peptides were subjected to ion-exchange chromatography on DEAE-cellulose (Whatman DE-52) using gradients of ammonium bicarbonate (5–200 mM or 5–500 mM).

On occasion such peptides were purified by cation-exchange chromatography using a method similar to that described by Walker et al. (1976). A 35.0 × 1.5 cm column of SP-Sephadex was used. A linear pH gradient of pyridine acetate buffer was generated in a Buchler gradient maker. Buffer was pumped at 10 mL/h.

Mixtures of smaller peptides were purified by using the same technique except that sulfonated polystyrene (Locarte LA-49 amino acid analyzer resin) was used in a 50.0 × 0.9 cm thermostated column maintained at 50 °C with a flow rate of 15 mL/h.

After cation-exchange chromatography in pyridine acetate, peptides were located by high-voltage electrophoresis (25 V/cm) at pH 6.5 on 0.1-mm cellulose thin layers precoated onto polyamide sheets (Macherey Nagel Polygram Cel-300, 20 × 20 cm).

Some other peptides were purified by high-voltage electrophoresis (60 V/cm) on Whatman 3MM or No. 1 paper at pH 6.5, 2.1, 3.5, and 8.9 (Ambler, 1963; Milstein, 1966). Whatman 3MM paper was loaded with 50–500 nmol of peptide per cm, whereas Whatman No. 1 paper was loaded with 5–100 nmol of peptide per cm.

Procedures for location of peptides on paper or thin layers and subsequent elution are described elsewhere (Walker et al., 1980b). Further details of peptide purifications are given in the supplementary material (see paragraph at end of paper regarding supplementary material).

Automated Edman Degradation. Intact superoxide dismutase and many of the peptides purified from it were subjected to automated Edman degradation in an updated Beckman 890B spinning cup sequencer. Three programs were used at various times. For peptides of more than 12 amino acids one of two programs was employed. The first was Beckman Program DMAA 111374 A, which employs a buffer containing 0.4 M *N,N*-dimethyl-*N*-allylamine–trifluoroacetic acid in pyridine–water (3:2 v/v), pH 9.5, and benzene as

solvent 1. The second was a 0.1 Quadrol [*N,N,N',N'*-tetrakis(2-hydroxypropyl)ethylenediamine] program (Brauer et al., 1975). Many peptides containing up to 12 amino acids were degraded by using a rapid 40-min DMAA program with Polybrene as a carrier (Tarr et al., 1978; Klapper et al., 1978).

The amino acid thiazolinones were converted manually to phenylthiohydantoin amino acids as described elsewhere (Walker et al., 1980b).

Identification of Phenylthiohydantoin (PTH) Amino Acids. Phenylthiohydantoin amino acids were identified by a number of techniques. These were gas chromatography, high-pressure liquid chromatography, thin-layer chromatography, and back hydrolysis to the original amino acid followed by amino acid analysis. [For details, see Walker et al. (1980b).]

Reverse-phase high-pressure liquid chromatography of phenylthiohydantoins was carried out on a Waters high-pressure liquid chromatograph equipped with a solvent programmer (Model 660) and a absorbance detector (Model 440) and fitted with a 25 cm × 4.6 mm Zorbax-ODS column (Du Pont Instruments) maintained at 60 °C. Complete resolution of all phenylthiohydantoin amino acids was achieved with gradients of acetonitrile and 0.1 M sodium acetate (pH 5.0).

Regeneration of the corresponding amino acid was accomplished by hydrolysis at 150 °C for 4 h in vacuo in 6 N HCl and 0.1% stannous chloride (Mendez & Lai, 1975). PTH-serine was found to generate not only alanine as previously reported but also two other unidentified products which elute from the analyzer between alanine and valine. These peaks have been found to be diagnostic for serine in a number of studies from this laboratory (J. E. Walker and F. D. Northrop, unpublished observations).

The Dansyl–Edman Method. Some peptides were sequenced manually by the dansyl–Edman technique (Gray, 1972; Woods & Wang, 1967).

Amide Assignments. Identification of asparagine and glutamine was accomplished directly in peptides subjected to automated Edman degradation by high-pressure liquid chromatography or by thin-layer chromatography. In some small peptides assignments were confirmed on the basis of the mobility of the intact peptide at pH 6.5 (Offord, 1966). Mobilities were calculated as the distance migrated from the neutral band relative to aspartic acid; migration in the same direction as aspartic acid was assigned as negative.

Peptide Nomenclature. Peptides are prefixed CB, T, C, Hy, or E according to whether they came from cyanogen bromide, tryptic, chymotryptic, hydroxylamine, or staphylococcal protease cleavage of the whole protein. Therefore, they are numbered according to their position in the sequence starting from the amino terminus.

Similarly, secondary digestion products are named according to their position in the primary fragment from which they were derived. Thus, C4-T1 is the first tryptic peptide in chymotryptic peptide C4.

Results

Amino Acid Composition. Table I shows the amino acid composition of a subunit of superoxide dismutase from *B. stearothermophilus* determined by amino acid analysis compared with the composition derived from the complete amino acid sequence.

Strategy of Amino Acid Sequence Analysis. The amino acid sequence of the protein shown in Figure 1 was derived principally by automated sequence analysis of fragments resulting from chemical cleavage of the protein with cyanogen bromide and hydroxylamine. Smaller peptides derived by proteolytic digestion of the intact protein were used to establish the overlaps between these fragments and to provide sequence of regions not readily accessible from the intact cyanogen bromide and hydroxylamine fragments.

Table I: Amino Acid Composition of Superoxide Dismutase from *B. stearothermophilus*[a]

amino acid	analysis	sequence
Asx	24.5	25
Thr	11.7	11
Ser	11.1	11
Glx	22.4	21
Pro	13.2	13
Gly	14.6	15
Ala	19.9	20
Cys	0.0	0
Val	8.1	8
Met	2.2	2
Ile	8.9	9
Leu	19.1	19
Tyr	7.8	8
Phe	7.7	8
His	8.5	9
Lys	12.5	12
Arg	5.9	6
Trp	>4.35[b] 5.0[c]	6

[a] For hydrolysis conditions, see Materials and Methods. [b] Method of Edelhoch (1967). [c] Method of Liu & Chang (1971).

Table II: Compositions of Cyanogen Bromide Peptides[a]

amino acid	CB1	CB2	CB3
Asx	2.0 (2)	16.1 (18)	4.6 (5)
Thr	1.0 (1)	8.2 (9)	1.5 (1)
Ser	0.8 (0)	7.8 (10)	1.2 (1)
Glx	3.1 (3)	13.9 (12)	6.6 (6)
Pro	3.6 (5)	6.2 (6)	2.2 (2)
Gly	1.0 (0)	13.? (13)	3.1 (2)
Ala	2.3 (2)	13.3 (12)	6.7 (6)
Cys			
Val	0.3 (0)	5.7 (4)	3.2 (4)
Met			
Ile	1.0 (1)	6.2 (6)	
Leu	2.7 (3)	12.8 (13)	3.1 (3)
Tyr	2.2 (2)	2.5 (1)	5.4 (5)
Phe	0.9 (1)	5.9 (6)	1.4 (1)
His	1.0 (1)	5.9 (7)	1.0 (1)
Lys	1.2 (1)	7.1 (6)	4.5 (5)
Arg	0.1 (0)	4.8 (3)	3.2 (3)
Trp[b]	− (0)	+ (3)	+ (3)
homoserine/homoserine lactone	+ (1)	+ (1)	− (0)
position in sequence	1–23	24–153	154–203
amino-terminal residue	P	B	Z
yield[c]	1.6	0.8	1.6

[a] Numbers in parentheses are compositions derived from sequence analysis. [b] By the Ehrlich method (Dawson et al., 1959). [c] 2 μmol of protein was digested.

Purification and Characterization of Peptides. (1) Cyanogen Bromide Cleavage. Three peptides produced by cleavage of the intact protein with cyanogen bromide were purified in a single gel filtration step on Sephadex G-50 superfine in 1% formic acid (Figure 2). Their amino acid compositions are given in Table II.

(2) Hydroxylamine Cleavage. Peptide Hy1 was separated from larger peptides and salts in the cleavage mixture by chromatography on Sephadex G-25 (Figure 3). The remaining major cleavage products were purified by fractionation on Sephadex G-50 of the peak totally excluded from Sephadex G-25 (Figure 3).

The salt peak from the initial fractionation contained no peptide material.

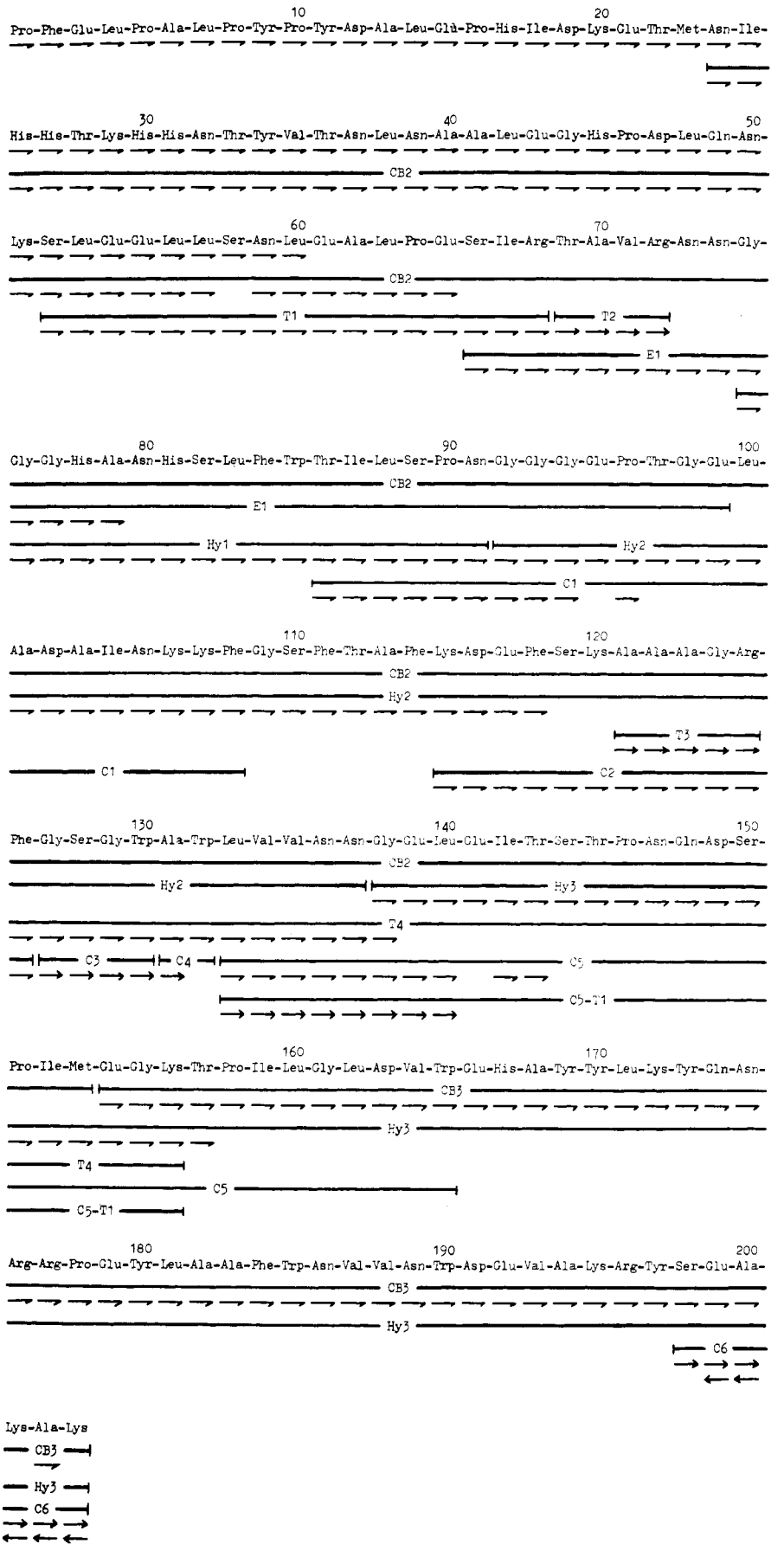

FIGURE 1: Construction of the complete amino acid sequence of *B. stearothermophilus* superoxide dismutase from overlapping peptides. (→) Sequenced by dansyl–Edman procedure; (—) sequenced by automated Edman degradation; (←) sequenced using carboxypeptidase Y. Residues 1–23 are taken from an earlier study (Bridgen et al., 1975).

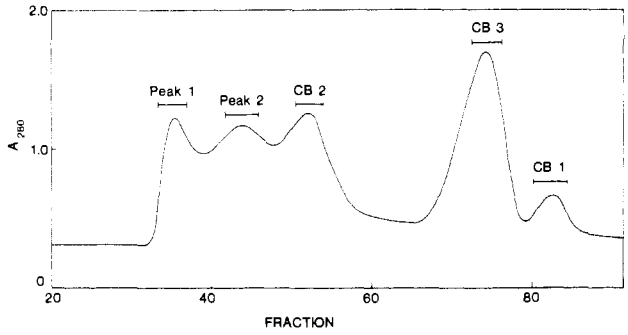

FIGURE 2: Purification of peptides from cyanogen bromide cleavage. 2 μmol of cleaved protein was fractionated on a 150 × 2 cm column of Sephadex G-50 superfine in 1% formic acid. Peak 1 = uncleaved protein; peak 2 = partially cleaved protein.

Table III: Compositions of Hydroxylamine Peptides

	Hy1	Hy2	Hy3
Asx	2.6 (2)	5.2 (5)	7.8 (7)
Thr	1.1 (1)	2.7 (2)	3.9 (3)
Ser	2.0 (2)	2.5 (3)	3.2 (3)
Glx	0.1 (0)	5.1 (3)	7.8 (9)
Pro	0.7 (1)	2.3 (1)	4.3 (4)
Gly	3.5 (3)	5.0 (8)	3.1 (3)
Ala	1.4 (1)	5.4 (7)	5.5 (6)
Cys			
Val	0.2 (0)	2.1 (2)	3.2 (4)
Met			1.0 (1)
Ile	1.1 (1)	1.8 (1)	2.8 (4)
Leu	2.2 (2)	4.0 (2)	7.5 (4)
Tyr		2.0 (0)	4.2 (5)
Phe	0.8 (1)	2.8 (5)	1.3 (1)
His	1.8 (2)	1.0 (0)	2.5 (1)
Lys		2.8 (4)	2.8 (5)
Arg		1.6 (1)	2.1 (3)
Trp	+ (1)	+ (2)	+ (3)
position in sequence	75–91	92–137	138–203
amino-terminal residue	G	G	G
yield[a]	250	200	200

[a] 1 μmol of protein was digested.

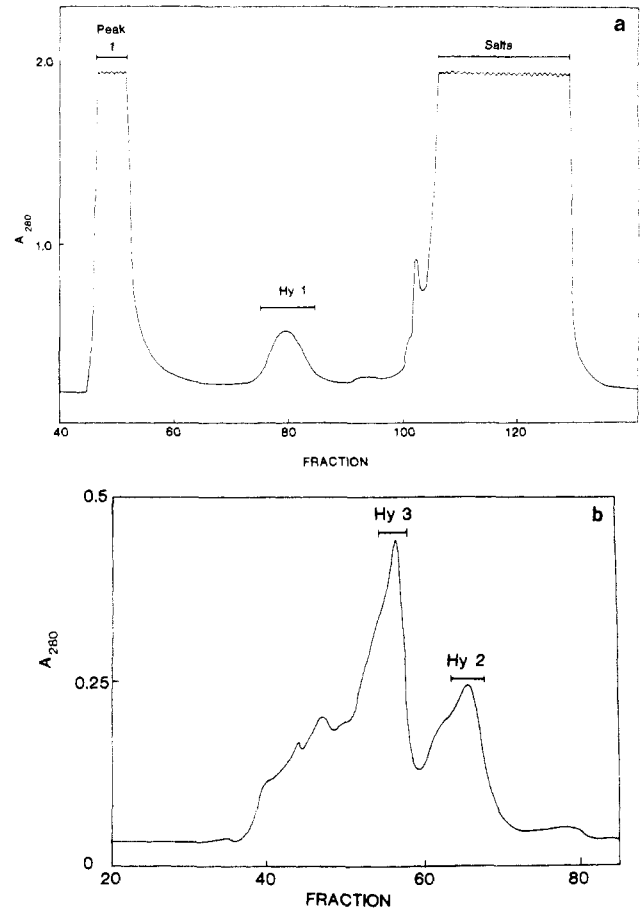

FIGURE 3: Purification of peptides from hydroxylamine cleavage. (a) Gel filtration on a 150 × 2 cm column of Sephadex G-25 superfine in 50 mM ammonium bicarbonate of 1 μmol of cleavage mixture. (b) Gel filtration on a 150 × 2 cm column of Sephadex G-50 superfine in 50 mM ammonium bicarbonate of peak 1 from (a). Peaks containing homogeneous amino-terminal sequences were identified by sequence analysis of a portion of each peak.

Table III shows the compositions of the three hydroxylamine peptides with free amino terminals. There are discrepancies in the analyses of Hy2 and Hy3 when compared with the compositions expected from their amino acid sequences. This is probably the result of contamination with the blocked amino-terminal peptide and products of partial cleavage.

The yield of Hy1 indicates a 25% cleavage in the reaction with hydroxylamine.

(*3*) *Other.* Peptides obtained from digestion with trypsin, chymotrypsin, and *S. aureus* protease of intact enzyme or of fragments of it were purified and characterized as described in the supplementary material. Their amino acid compositions are given in Table IV.

Amino Acid Sequence. (*1*) *Residues 1–23 (CB1).* A sequence corresponding to residues 1–60 has been published previously (Bridgen et al., 1975). This had been derived by automated sequencer analysis of a sample of the intact protein. With the exception of residue 58, which was originally assigned as proline, this sequence has been fully confirmed. Hence, residues 1–23 corresponding to cyanogen bromide peptide CB1 are not described here.

(*2*) *Residues 24–153 (CB2).* Automated sequencer analysis of CB2 gave complete sequence information on residues 24–54 and partial data on residues 55–65. T1 (CB2-T1) was sequenced to completion and gave all the missing residues from 51 to 68 and showed residue 55 to be glutamic acid and not glutamine. The tetrapeptide T2, sequenced manually, corresponds to residues 69–72 as shown by the overlapping peptide E1. The overlap between T1 and T2 has recently been confirmed independently by sequence analysis from the N terminus of the protein (G. Frank, private communication).

Residues 75–91 were identified by the sequencing of Hy1 to completion. Asparagine-74 can be inferred also from the nature of the hydroxylamine-sensitive bond (N–G). This sequence was overlapped into Hy2 by C1, and Hy2 extended the sequence to residue 124 and partially up to residue 135. The remaining residues in Hy2 were established by two chymotryptic and two tryptic peptides. C2 completed residues 115–126, and T3 (sequenced manually) confirmed residues 121–125. The remaining unknown amino acids up to residue 135 were identified by the sequencing of T4. T4 contained roughly equal amounts of two tryptic peptides, namely, that from the amino terminus of the protein and the sequence of interest, and no other contaminating peptides. This analysis overlapped C2, C3, C4, and C5. C3 and C4 were sequenced manually.

The overlap between the last two hydroxylamine peptides (Hy2 and Hy3) was established by C5. Sequence analysis of Hy3 extended the sequence to residue 153.

(*3*) *Residues 154–203 (CB3).* The overlap between CB2 and CB3 was established by the sequence of Hy3. Moreover, the composition and partial sequence data for C4-T1 ($LVVNNGEL[B_2T_2S_2P_2G_1M_1I_1L_1K_1]$) are consistent with this sequence.

Table IV: Compositions of Peptides from Proteolytic Digestions[a]

	T1[b]	T2	T3	T4[c]	C1	C2	C3	C4	C5	C6	C5-T1	E1
Asx	1.7 (1)			6.1 (6)	3.0 (3)	1.0 (1)			4.7 (5)		3.9 (4)	4.5 (4)
Thr		1.0 (1)		2.6 (2)	1.6 (2)				2.4 (3)		1.9 (2)	2.5 (3)
Ser	2.3 (3)			1.9 (3)	1.3 (1)	0.6 (1)	1.5 (1)		1.8 (2)	0.9 (1)	1.9 (2)	1.7 (3)
Glx	3.6 (4)			6.4 (6)	2.5 (2)	1.0 (1)			4.6 (4)	1.1 (1)	4.2 (4)	2.1 (2)
Pro	1.2 (1)			4.5 (7)	1.9 (2)				2.8 (3)		1.9 (2)	1.8 (2)
Gly	0.9		1.1 (1)	3.8 (4)	3.7 (4)	1.1 (1)	2.0 (2)	0.2	2.6 (3)		2.1 (2)	7.5 (7)
Ala	1.1 (1)	1.1 (1)	3.2 (3)	4.0 (3)	2.0 (2)	2.6 (3)		1.0 (1)	0.2 (0)	2.0 (2)	0.6 (0)	2.0 (2)
Cys												
Val		0.9 (1)		2.9 (2)					2.1 (3)		1.8 (2)	0.6 (1)
Met				1.0 (1)					0.7 (1)		0.8 (1)	
Ile	1.2 (1)			2.6 (3)	1.7 (2)				2.3 (3)		1.9 (2)	1.8 (2)
Leu	4.0 (5)			4.5 (5)	1.9 (2)				4.3 (4)		1.9 (2)	2.1 (2)
Tyr				1.8 (2)					0.1 (0)			
Phe				1.5 (2)	1.3 (1)	1.6 (2)						1.2 (1)
His				1.0 (1)								1.6 (2)
Lys				1.2 (2)	1.5 (2)	1.8 (2)			1.2 (1)	1.9 (2)	0.9 (1)	
Arg	1.0 (1)	1.0 (1)	1.0 (1)			0.9 (1)			0.1 (0)			2.3 (2)
Trp	+ (1)	− (0)	− (0)	+ (2)	− (0)	− (0)	+ (1)	+ (1)	+ (1)	− (0)	− (0)	− (0)
position in sequence	52-68	69-72	121-125	1-20, 126-156	86-108	115-126	127-130	131-132	133-165	198-203	133-156	66-99
amino-terminal residue	S	T	A	F/P	T	K	G	A	L	S	L	S
yield (nmol)	220	500	500	200	300	300	50	40	400	600	180	90
mobility at pH 6.5	ND[d]	+0.46	+0.46	ND	ND	+0.22	0	0	ND	+0.33	ND	ND

[a] Amounts of protein digested: trypsin and chymotrypsin, 2 μmol; V8 protease, 1 μmol; trypsin subdigest of C5, 350 nmol. [b] Also isolated from CB2. [c] An equimolar mixture of the amino-terminal peptide (1-20) and residues 126-156. [d] ND = not determined.

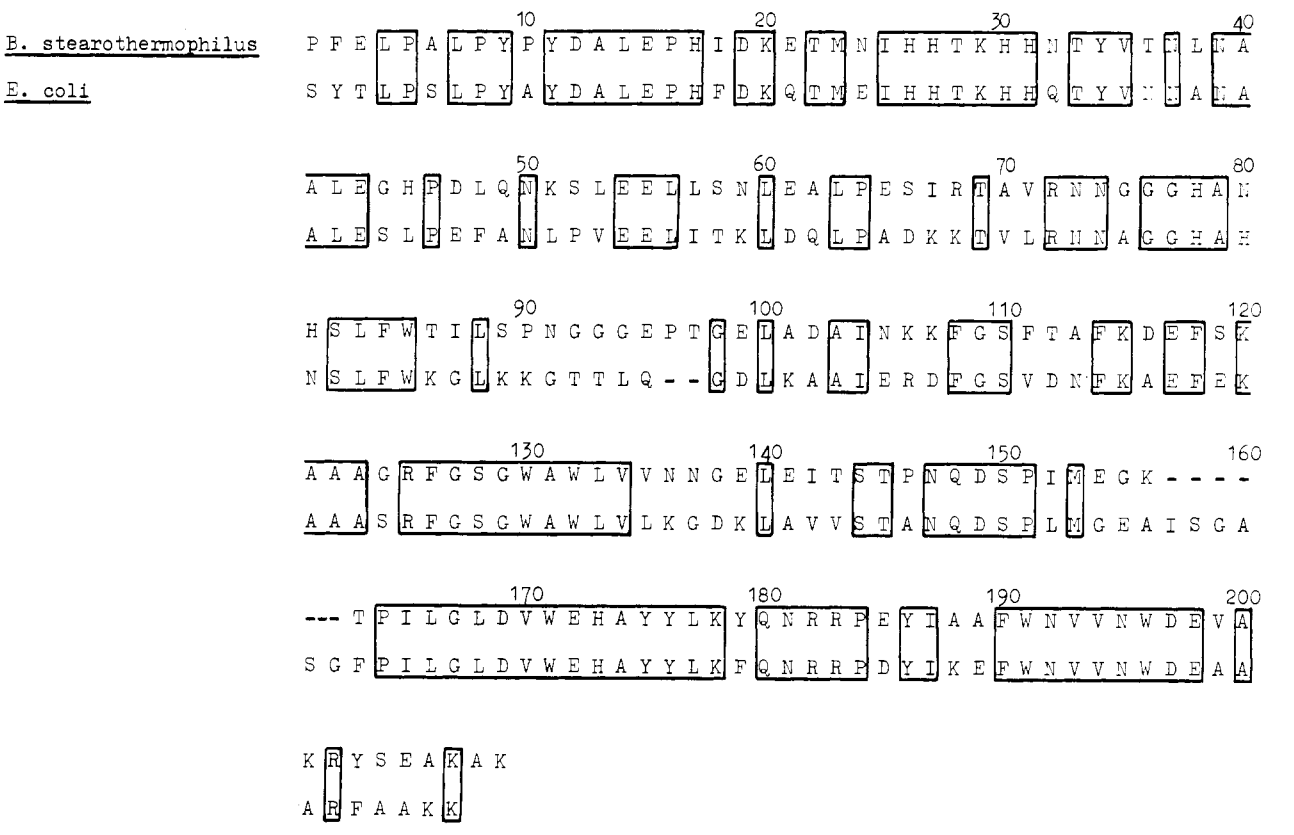

FIGURE 4: Comparison of the sequences of the manganese superoxide dismutases from *B. stearothermophilus* and *E. coli* B. For the purpose of maximizing homologies, a two amino acid deletion has been placed at residues 90 and 91 in the *E. coli* enzyme, a six amino acid insertion at residues 155–160, and a single insertion at residue 165. Boxed residues are identical.

Sequencer analysis of CB3 established the sequence from residues 154 to 200 and additionally residue 202. The remaining residues were established by peptide C6, the carboxyl-terminal chymotryptic peptide.

The sequence at the carboxyl terminus was confirmed by analysis of intact protein and CB3 with carboxypeptidase Y. Superoxide dismutase (50 nmol) was digested at a substrate/enzyme ratio of 100:1. Samples corresponding to 5 nmol of protein were taken at 5-min intervals up to 20 min and thereafter every 20 min up to 3 h. At times 5, 10, 40, 80, and 160 min, the following amounts of amino acids were detected: lysine, 0.7, 1.0, 2.5, 30, and 4.1 nmol; alanine, 0.2, 0.6, 1.7, 2.5, and 3.6 nmol; glutamic acid, 0.1, 0.4, 1.0, 1.4, and 1.8 nmol. These results, taken with the independently

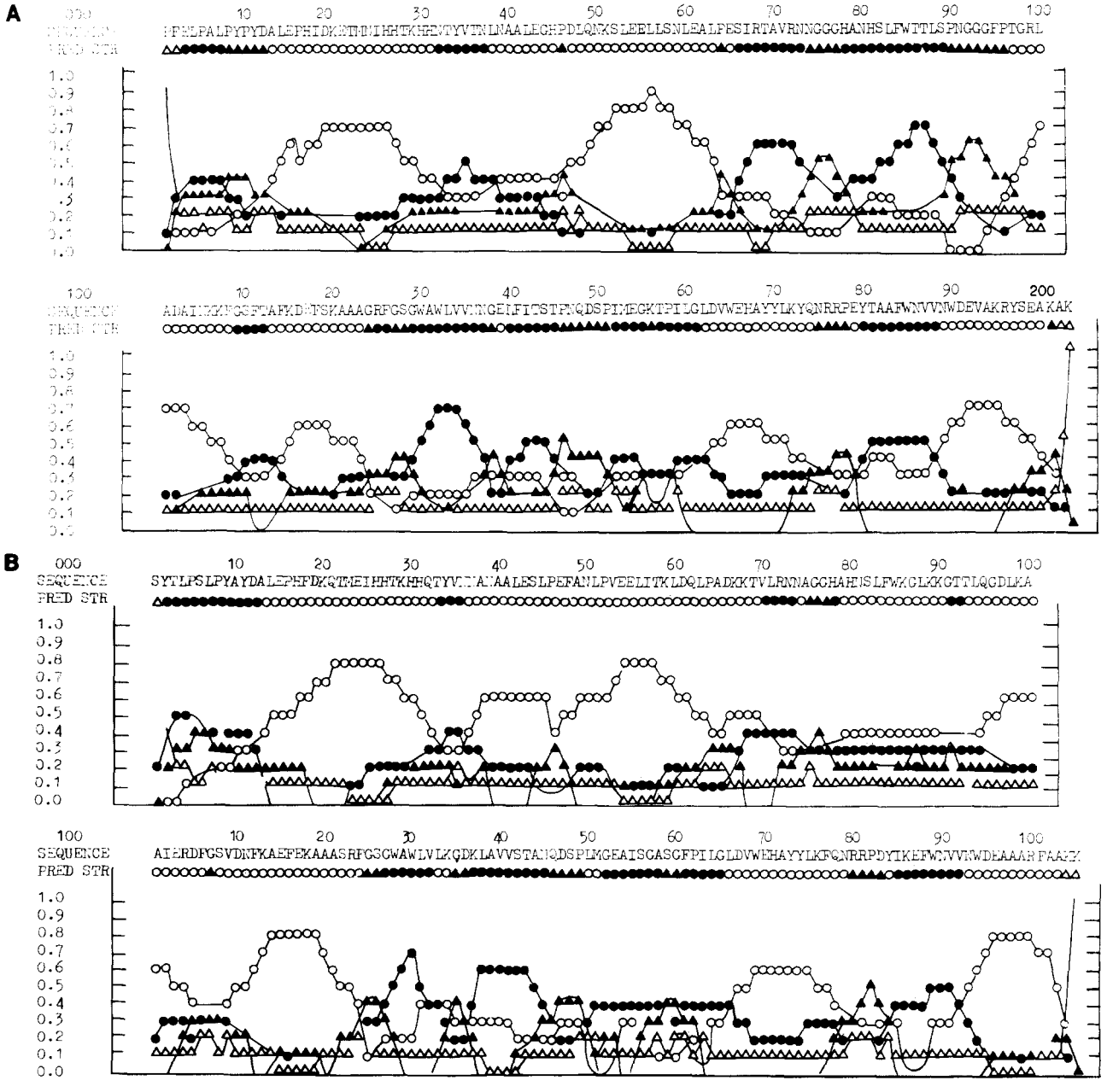

FIGURE 5: Secondary structures of the manganese superoxide dismutases from *B. stearothermophilus* (A) and *E. coli* B (B) predicted by McLachlan's method. The vertical axis is a probability scale from 0 to 1: (○) α helix; (●) β sheet; (△) β bend; (▲) irregular.

established sequences of CB3 and C6, are consistent with the C-terminal sequence -E-A-K-A-K.

A residue by residue justification of the sequence presented in Figure 1 is given in the supplementary material.

Discussion

Independent Mass Spectrometric Study. A mass spectrometric study of *B. stearothermophilus* superoxide dismutase has been carried out independently of this work (A. D. Auffret, T. J. A. Blake, and D. H. Williams, unpublished results). Sequences have been obtained which cover 131 of the 203 residues of the protein, although the longest overlapped sequence was 18 residues long. These data in almost all instances serve to confirm the sequence presented here. In addtion, microheterogeneous sequences were noted at two residues and four small peptides were noted which do not correspond with our sequence. It seems likely that these may have arisen from a contaminant in the enzyme preparation. The enzyme used in the mass spectrometric study was prepared prior to the enzyme used in the work presented here. The homogeneity of our material was checked by polyacrylamide gel electrophoresis in two independent systems and by amino-terminal analysis of the protein. We have not isolated any peptides inconsistent with the sequence presented here. The high degree of homology between the sequences of the *E. coli* and *B. stearothermophilus* enzymes (Figure 4) which were determined entirely independently lends further support to the sequence presented here. There is no correspondence between the sequences of four peptides detected by mass spectrometry and sequences in the *E. coli* enzyme.

Sequence Comparisons. Sequence comparisons between the various classes of superoxide dismutase help us to determine how it has evolved. Thus, the amino-terminal sequences of the Fe and Mn superoxide dismutases show great similarities between the two groups, indicating a common ancestry (Harris & Steinman, 1977; Harris et al., 1980a; Walker et al., 1980a). Moreover, it is not possible to distinguish between Fe and Mn enzymes on the basis of these sequences (Walker et al., 1980a).

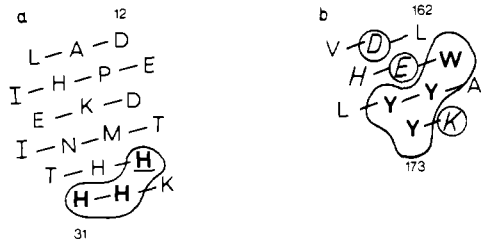

FIGURE 6: Sequence of two of the predicted helixes of superoxide dismutase plotted on a helix surface with 3.5 residues/turn. Sequence reads downward right to left as indicated by the lines as if projected onto a sheet of paper wrapped round the helix axis and then unrolled. (a) Helix 1 of the *B. stearothermophilus* enzyme (residues 12–31). The encircled area contains three histidine residues in close proximity in the helix. These residues are conserved in all species of Mn and Fe superoxide dismutases sequenced hitherto. (b) Penultimate helix of *B. stearothermophilus* enzyme (residues 162–173) showing a cluster of aromatic residues in heavy type and charged residues in italic and encircled. A histidine residue in italic is conserved in both the *B. stearothermophilus* and *E. coli* enzymes.

These studies also indicated a lack of sequence homology between the Mn/Fe class and the Cu/Zn enzyme from bovine erythrocytes. This conclusion, based on partial sequences of Fe/Mn enzymes, has been confirmed by the determination of the complete sequences of the Mn enzymes from *E. coli* B (Steinman, 1978) and *B. stearothermophilus*. Hence, the Cu/Zn enzyme probably has evolved independently of the Fe and Mn superoxide dismutases.

The two Mn enzymes are highly homologous in sequence; 121 residues (60%) are identical (Figure 4). Conserved amino acids are to be found throughout the polypeptide and especially in the regions encompassing residues 4–43 and 158–196 in the *B. stearothermophilus* sequence. Regions including insertions and deletions cannot be important for catalysis and may well be external regions in the three-dimensional structures of the respective enzymes.

We have also attempted to predict the secondary structures of the Mn enzymes from their primary structures by the method of McLachlan (1977). This method applies the laws of statistical inference to amino acid frequency data from proteins of known structure. It calculates the probability of the α helix, β sheet, bends, and irregular structure along the length of a given sequence. The method was applied to proteins of known structure such as adenylate kinase, phage T4 lysozyme, and pancreatic trypsin inhibitor and correctly predicted 55–69% of their secondary structures. In addition, a much clearer structural pattern emerged with serum albumin (McLachlan & Walker, 1977).

We first applied the method to the sequence of the Cu/Zn enzyme from bovine erythrocytes. Crystallographic analysis of this protein has demonstrated that it contains a β-barrel structure composed of eight antiparallel β strands (Richardson et al., 1975). Seven of these strands and intervening β bends were correctly predicted by McLachlan's method (Walker et al., 1980a).

The secondary structures of the Mn enzymes shown in Figure 5 predicted by the same procedure not surprisingly are very similar to each other but differ substantially from that of the bovine erythrocyte enzyme. It appears unlikely that they contain the eight-stranded β-barrel structure characteristic of the Cu/Zn enzyme. Thus, this supports the notion that the Cu/Zn enzyme has evolved independently of Fe and Mn enzymes.

In general, concordant secondary structures are obtained for the two Mn proteins. The only significant difference is in the region of residues 80–90: an α helix is weakly predicted (40% probability) in the *E. coli* enzyme and a β sheet (70% probability) in the *B. stearothermophilus* protein, although the sequences in this region are similar. The insertions in the *E. coli* sequence are to be found in β structures and those in the *B. stearothermophilus* enzyme in a β bend preceding an α helix.

On closer inspection of the α-helical regions a number of interesting features can be discerned. Thus, the first helix (residues 12–31) is almost identical in both proteins. It contains four histidine residues and a lysine residue which are in close proximity to each other in the α helix (Figure 6a). Three of the four histidine residues are conserved in all Mn/Fe enzymes sequenced hitherto (Harris et al., 1980a). Moreover, nuclear magnetic resonance experiments (A. Cass and H. A. O. Hill, unpublished work) implicate histidine in manganese binding. Thus, it may be that this cluster of histidine residues provides some of the ligands required to bind the single tervalent manganese present in the dimeric *B. stearothermophilus* enzyme in an octahedral complex.

Another histidine residue conserved in both enzymes is in the penultimate helix (residues 162–173, *B. stearothermophilus*; residues 168–179, *E. coli*). This helix is also highly conserved. In addition to the histidine residue, which is next to two acidic residues, the helix contains a patch of four aromatic amino acids (Figure 6b).

X-ray crystallographic analysis of the protein in progress will ultimately decide upon the validity of these features predicted from the amino acid sequence.

Structural analysis may also provide an insight into the means by which this particular protein is thermostabilized. It is certainly not possible to do so on the basis of the sequence alone as the structural changes needed to thermostabilize a protein are subtle and may only be discernible by comparison of independently determined structures of mesophile and thermophile enzymes. In the case of the tetrameric enzyme D-glyceraldehyde-3-phosphate dehydrogenase from *B. stearothermophilus*, significant contributions to thermal stability are gained from extra ionic bonds between subunits, although other relatively minor structural changes are also important (Harris & Walker, 1977; Walker, 1979; Walker et al., 1980c).

Acknowledgments

The late J. I. Harris initiated studies on *B. stearothermophilus* superoxide dismutase in this laboratory. We acknowledge his help and interest. We thank K. A. Edwards for amino acid analyses.

Supplementary Material Available

Details and figures of purification of peptides obtained from proteolytic digests and tables giving residue by residue basis for sequence assignment (17 pages). Ordering information is given on any current masthead page.

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Purification and Properties of the Inducible Enzyme Cyanase†

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ABSTRACT: Cyanase (cyanate hydrolase EC 3.5.5.3) has been purified 270-fold to a high state of purity from *Escherichia coli* B. The native enzyme has a molecular weight of ~150000 as estimated by sucrose density gradient centrifugation and gel-filtration chromatography on Bio-Gel P-300. The enzyme is an oligomer composed of apparently identical subunits which have a molecular weight of ~15000 as estimated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Amino acid analyses showed that the enzyme contains no tryptophan and a single histidine residue, based on a subunit molecular weight of 14661. Catalytic hydrolysis of cyanate was found to be dependent on the presence of bicarbonate and to be affected by ionic strength. The concentration of bicarbonate required to give half-maximal activity in the presence of 2 mM potassium cyanate was 0.1 mM. The apparent K_m for cyanate in the presence of 3 mM bicarbonate is 0.6 mM. The initial product of the reaction is carbamate (or a related, unstable compound and/or carbamate precursor) which subsequently decomposes to ammonia and bicarbonate.

Studies in our laboratory and elsewhere have indicated that cyanate is a site-specific inhibitor of certain enzymes (Anderson & Carlson, 1975; Chollet & Anderson, 1978; Fan & Plaut, 1974; Shen & Colman, 1975; Sluyterman, 1967; Schroeder et al., 1969). As a result of these and current related studies and also because of recent interest in the potential use of cyanate as an antisickling agent (Brewer, 1976), we initiated an investigation of the properties of the enzyme cyanase (EC 3.5.5.3) reported to be present in rat and guinea pig tissues (Holtham & Schutz, 1948), bacteria (Taussig, 1960, 1965; Guilloton & Hargreaves, 1972), and plants (Lisanti, 1963; Lotti, 1963). This enzyme catalyzes hydrolysis of cyanate to ammonia and bicarbonate.

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