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Some Sulfhydryl Properties and Primary Structure of Human Erythrocyte Superoxide Dismutase†

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ABSTRACT: Human Cu–Zn superoxide dismutase prepared by different methods shows varying properties relevant to its sulfhydryl chemistry. A cysteine residue not found in the analogous bovine enzyme appears to be responsible for its unusual lability. Alkylation of this cysteine results in a marked increase in stability, and this form of the protein may be readily crystallized. The primary structure of the 153 amino acid residues found in the human protein has been determined, and 82% of the residues are identical with those of the bovine enzyme. A significant variation is seen in the portion of those proteins comprising residues 17–36, with eleven changes being noted.

Proteins possessing superoxide dismutase activity have been isolated from a wide variety of sources (Fridovich, 1976) since the first isolation of this protein by Mann & Keilin (1939). Properties of the bovine and human erythrocyte forms in particular have been extensively delineated (Weser, 1973). Although the bovine enzyme was originally reported to be made up of two disulfide-linked subunits (Keele et al., 1971), subsequent studies have demonstrated that these are not covalently linked either in the human (Hartz & Deutsch, 1972) or in the bovine (Evans et al., 1974; Richardson et al., 1975) forms. The former workers indicated that the subunits of the human protein, unlike the bovine enzyme, showed differences. However, more recent studies (Farb, 1977; Briggs & Fee, 1978a) have shown that the human protein also has two identical subunits.

In contrast to bovine superoxide dismutase, the human enzyme appears to undergo various transformations during isolation by methods employing the widely used ethanol–chloroform procedure (Stansell & Deutsch, 1965). This appears to be due to the presence of an additional and fairly reactive cysteine residue in the human enzyme (Hartz & Deutsch, 1972). The alkylation of this cysteine appears to markedly stabilize the protein.

Preliminary studies from our laboratory have revealed portions of the primary structures of human superoxide dismutase which included four cysteine-containing peptides separated by affinity chromatography (Farb, 1977). More recently, Barra et al. (1978) have presented data on the sequence of about two-thirds of this protein. We have now completed the sequence studies initiated by Farb (1977), and the results are presented along with the sequence for the bovine enzyme reported by Steinman et al. (1974). These two Cu–Zn forms of superoxide dismutase have sequence identities of 82%.

Experimental Section

Enzyme Preparation. Superoxide dismutase was obtained from blood provided by the Badger Regional Laboratory of the American Red Cross. Three methods were employed to isolate the enzyme from freshly washed erythrocytes. One method utilized only the chromatographic procedures employed previously by Hartz & Deutsch (1972). A second method used an initial ethanol–chloroform fractionation step to remove hemoglobin (Tsuchihashi, 1923) in a manner similar to that employed by McCord & Fridovitch (1969) in their isolation of the bovine erythrocyte enzyme. A third method used the ethanol–chloroform procedure on hemolysates obtained from 1 L of erythrocytes following a thiol–disulfide exchange reaction at 0 °C with 1.2 g of CPDS.[1] Enzymes modified in this manner could be readily crystallized. The material isolated by the method of McCord & Fridovich

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[1] Abbreviations used: CPDS, 6,6′-dithionicotinic acid; EDTA, ethylenediaminetetraacetate; BNPS-Sk, 2-(2-nitrophenylsulfenyl)-3-methyl-3-bromoindolenine; Tos-Phe-CH_2Cl, 1-tosylamido-2-phenylethyl chloromethyl ketone; PTH, phenylthiohydantoin; pMA, *p*-mercurianiline; $NaDodSO_4$, sodium dodecyl sulfate; Cm, carboxymethyl.

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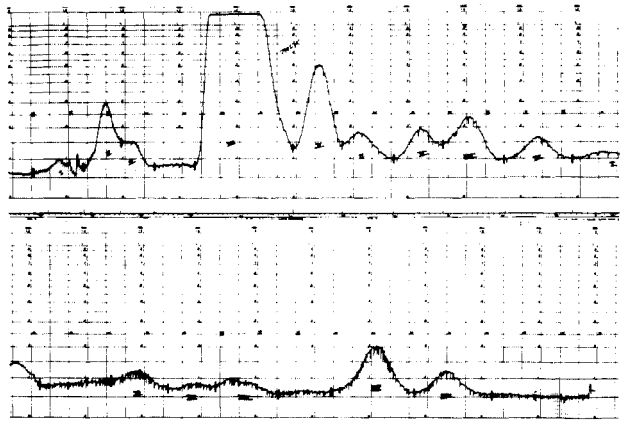

FIGURE 1: The results for the chromatographic separation of the soluble, noncysteine-containing tryptic peptides derived from 16 mg of superoxide dismutase. A 1.5 × 30 cm column of Dowex 50-X2 was utilized, and pyridine–acetate buffers were employed to establish an elution gradient from 0.2 M, pH 3.1, to 2.0 M, pH 5.5.

(1969) was usually employed in the sequence studies since it could be obtained with the least work expenditure.

Sulfhydryl levels were quantitated by the method of Ellman (1958) as modified by Witwicki & Zakrzewski (1969), by the thiol–disulfide exchange procedure of Grassetti & Murray (1965) which employed CPDS, and by measurements of Cm-cysteine in hydrolysates of proteins that had been alkylated with a twofold molar excess of iodoacetate in 7 M guanidine·HCl at pH 8.4 for 1 h at 37 °C.

Peptides containing reactive sulfhydryl groups were isolated by passage over an organomercurial agarose column prepared and regenerated by the methods of Sluyterman & Wydenes (1970) with pMA as the affinity ligand. These "affinity peptides" will be designated by a nomenclature which includes the letter A; i.e., T-A-6 is a hexapeptide isolated from a tryptic digest.

Prior to proteolytic or chemical degradation, the metal-free protein in 6 M guanidine·HCl was reduced and then alkylated with iodoacetate. The metals had been removed by heating at 100 °C for 2 min at pH 3.0 in the presence of 5–10 mM EDTA. The preparation of "affinity peptides" employed reduced but nonalkylated protein.

Tryptic peptides served as source material for most of the sequence work. A 1% solution of reduced–alkylated protein in 0.15 M ammonium bicarbonate (pH 8.5) was heated at 100 °C for 2 min and then digested with 2% of its weight of Tos-Phe-CH_2Cl-trypsin (Worthington) for 24 h at 5 °C. An additional 1 or 2% (w/w) of trypsin was then added and digestion continued for 24 h at 37 °C. The digest was then lyophilized, and the peptides, soluble in pyridine–formate (pH 2.0), were recovered by lyophilization. The insoluble material was separated, washed twice with this buffer, and then subjected to gel filtration on a column of Sephacryl S-200 (Superfine, Pharmacia) in 6 M guanidine·HCl. Two fractions were resolved which were designated as T-core-I and T-core-II, respectively. Only the former fraction, which appeared to contain about 40 residues, was employed in sequence determinations. Unfractionated material was designated as T-core. It was further digested in some cases with chymotrypsin (Worthington) and thermolysin (Pierce) to produce soluble peptides. Such chymotryptic peptides are designated as T-core-C, the thermolytic ones as T-core-Th. Preparations formed by the action of both of these enzymes on core material are reported as T-core-CTh.

The soluble peptides from the endopeptidase digests were fractionated on a Dowex 50-X2 column in a manner similar

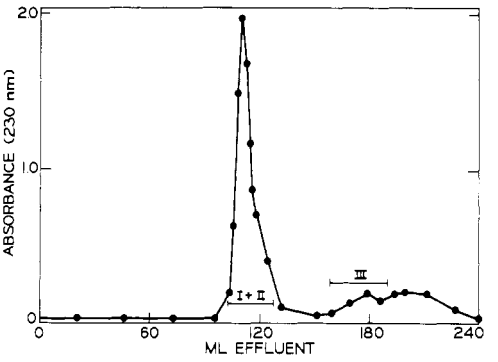

FIGURE 2: The results for the gel permeation on G-50 Sephadex (Fine) of 35 mg of superoxide dismutase that had been subjected to cleavage with BNPS-Sk. A 2.5 × 54 cm column equilibrated with 0.2 M ammonium bicarbonate (pH 8.5) was employed.

to that employed previously (Lin & Deutsch, 1973), the peptides being numbered sequentially in order of their elution, i.e., I, II, III, etc., as shown for a tryptic digest in Figure 1. These fractions were subjected to high-voltage paper electrophoresis at various pHs, usually 1.8, to provide subfractions of the Dowex 50-X2 material. These were numbered sequentially, the least cathodic peptide being number 1. A designation such as T-I-1 indicates the least cathodic component at pH 1.8 of the first tryptic peptide eluting from the Dowex 50-X2 column.

Amino acid compositions of 6 N HCl and 4 N methanesulfonic acid hydrolysates were obtained with the Durrum D-500 analyzer. It was assumed that 10% losses of serine, threonine, and tyrosine had occurred in these hydrolysates. Automated Edman degradations employed the Beckman Model 890E sequencer while a manual method was used as previously described (Lin & Deutsch, 1973).

Evidence obtained early in these studies indicated that the single tryptophan of human superoxide dismutase was located about 30 residues from the N terminus. The reduced–alkylated protein was treated with BNPS-Sk by the method of Fontana et al. (1973) to effect a 30–50% cleavage at this point. Fractionation of one of these digests over a column of Sephadex G-50 (Fine) resolved a mixture of two large peptides and a fraction containing about 30 amino acid residues as shown in Figure 2. The latter material was designated as BNPS-Sk-III and the former as BNPS-Sk-I + II. The I + II fraction appears to be made up of uncleaved protein with the material initiating at about residue 30. Since only this latter product had a free N-terminal group, the mixture could be directly employed in Edman degradations. Fraction III was digested with staphylococcal protease (Boehringer) and trypsin and also subjected to partial acid hydrolysis to provide useful peptides. The latter procedure employed 0.03 N HCl at 110 °C for 6 to 8 h.

The sequences of peptides were determined by a combination of methods including manual and automated Edman degradations and studies of the sequential release of amino acids with various exopeptidases. The PTH derivatives from the Edman degradations were usually converted to their corresponding amino acids by the method of Smithies et al. (1971). High-pressure chromatography using the Du Pont 830 liquid chromatograph was also employed in some instances to identify PTH derivatives.

The exopeptidases employed included leucine aminopeptidase and carboxypeptidases A and B (Worthington and Boehringer products), aminopeptidase M (Pierce), and carboxypeptidase C (Nutritional Biochemicals). From 5 to 20 nmol of a given peptide were treated with an approximately

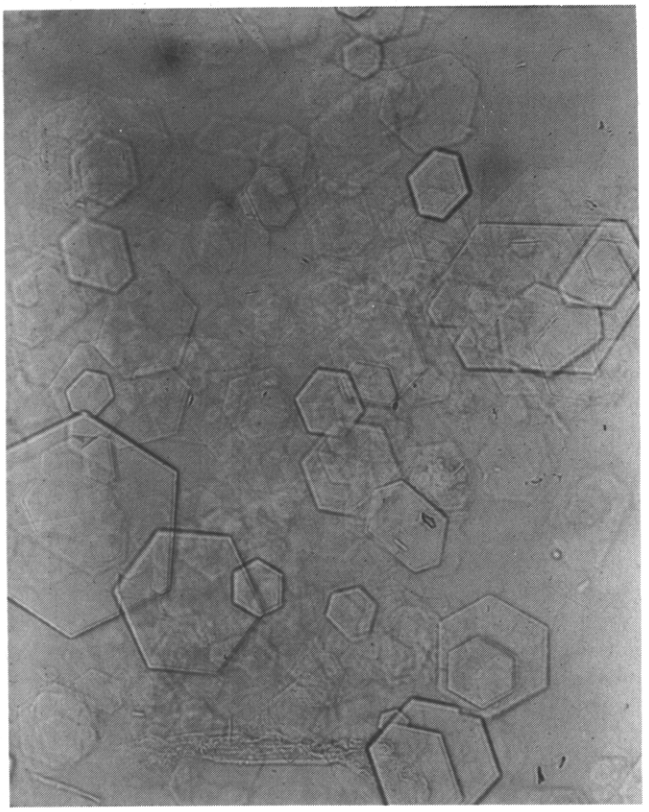

FIGURE 3: Photomicrograph of CPDS-superoxide dismutase crystallized from 2.7 M ammonium sulfate at pH 4.6 (magnification ~400×).

equal weight of the enzyme at 37 °C in 0.2 M ammonium bicarbonate (pH 8.0). Samples were taken at intervals, usually after 30, 60, and 120 min of incubation, lyophilized, and assayed for their contents of free amino acids with the Durrum D-500 analyzer.

Results

Enzyme prepared by the chromatographic method was found to contain from 1.5 to 1.9 mol of sulfhydryl groups/32 000 g as measured by its reactivity with iodoacetate or by disulfide exchange with CPDS. This indicates the presence of one freely reactive sulfhydryl in each of its two subunits. In contrast, material isolated by the procedure employing ethanol–chloroform showed no such reactive cysteine. A second sulfhydryl group per subunit could be alkylated under denaturing conditions (6 M guanidine·HCl, pH 8) in the chromatographically prepared enzyme but not in the ethanol–chloroform fractionated protein. Both types of enzyme preparations showed the presence of two sulfhydryl groups per subunit when subjected to disulfide exchange at 25 °C with CPDS at pH 4.5 in the presence of $NaDodSO_4$ (0.4%) and EDTA (10 mM).

The ability to specifically alkylate the readily reactive cysteine in the chromatographically prepared enzyme, the cysteine reacting only under denaturing conditions and the two cysteines linked by a disulfide bond following reduction, permitted selective radiolabeling of these cysteines (Farb, 1977). In some cases radioautographic or paper electrophoresis methods were employed in locating these peptides on unstained peptide maps.

Superoxide dismutase isolated from hemolysates by the ethanol–chloroform procedure after a thiol–disulfide exchange reaction with CPDS was found to contain 1.9 mol of carboxypyridine/32000 g of protein. Such derivatized enzyme, in contrast to underivatized protein, readily crystallized upon dialysis against water at 0 °C or from 2.7 M ammonium sulfate, pH 4.6, at room temperature. Crystals of the type formed under the latter conditions are shown in Figure 3.

Table I: Amino Acid Composition of Human Superoxide Dismutase[a]

amino acid	residues[c]	residues (sequence)
half-cystine[b]	3.8 (4)	4
aspartic acid	18.7 (19)	18
threonine	7.8 (8)	8
serine	9.2 (9)	10
glutamic acid	14.7 (15)	13
proline	5.4 (5)	5
glycine	24.9 (25)	25
alanine	10.2 (10)	10
valine	13.5 (14)	15
isoleucine	7.6 (8)	8
leucine	8.7 (9)	9
phenylalanine	3.6 (4)	4
histidine	7.3 (7)	8
lysine	10.6 (11)	11
tryptophan[d]	0.6 (1)	1
arginine	3.7 (4)	4
residues	153	153

[a] The average of results obtained in hydrolysates of both chromatographic and ethanol–chloroform preparations prepared with 6 N HCl and 4 N methanesulfonic acid. [b] Average of determinations for *S*-carboxymethylcysteine and -cysteic acid. [c] Residues per 16 000 g of protein. [d] From 4 N methanesulfonic acid hydrolysates.

The above results indicate that the loss of the readily reactive sulfhydryl groups in human superoxide dismutase prepared by the ethanol–chloroform procedure and the failure of the enzyme to crystallize if not alkylated prior to fractionation relate to a structural lability dependent on the two readily reactive cysteine residues. More detailed information on these variations has been presented by Farb (1977).

The amino acid composition of human superoxide dismutase, which served as the basis of the sequence determination, is presented in Table I and is the average of results obtained in 6 N HCl and 4 N methanesulfonic acid hydrolysates of the protein. These data are essentially the same as those previously presented by Hartz & Deutsch (1969) and Carrico & Deutsch (1969). The composition of the various peptides employed in the sequence determinations, their charges at pH 6.5 in some cases, and their positions in the molecule are shown in Table II. The amino acid composition for BNPS-Sk-III is given without residue numbers. This stems in part from the incomplete hydrolysis experienced in the 20-h hydrolysate times employed and some apparent destruction of hydroxyamino acids by the BNPS-Sk. It was useful in the generation of other smaller peptides whose compositions could be better defined, and the sequence analyses of these substantiated that peptide BNPS-Sk-III comprised residues 1–32.

The results for the experiments establishing the sequence of human superoxide dismutase are presented in Figure 4. Since this protein is strongly homologous to the analogous bovine enzyme (Steinman et al., 1974), many peptides could be initially aligned from their compositions without the necessity of preparing overlapping ones. For this reason and also to show the extent of the indicated homology, the sequence of the bovine protein is presented as part of Figure 4.

A tryptic (T) peptide, T-II-3, that contained no free N-terminal grouping sequentially released lysine and threonine upon digestion with a mixture of carboxypeptidases B and A. The report of the presence of two acetate groups in the human

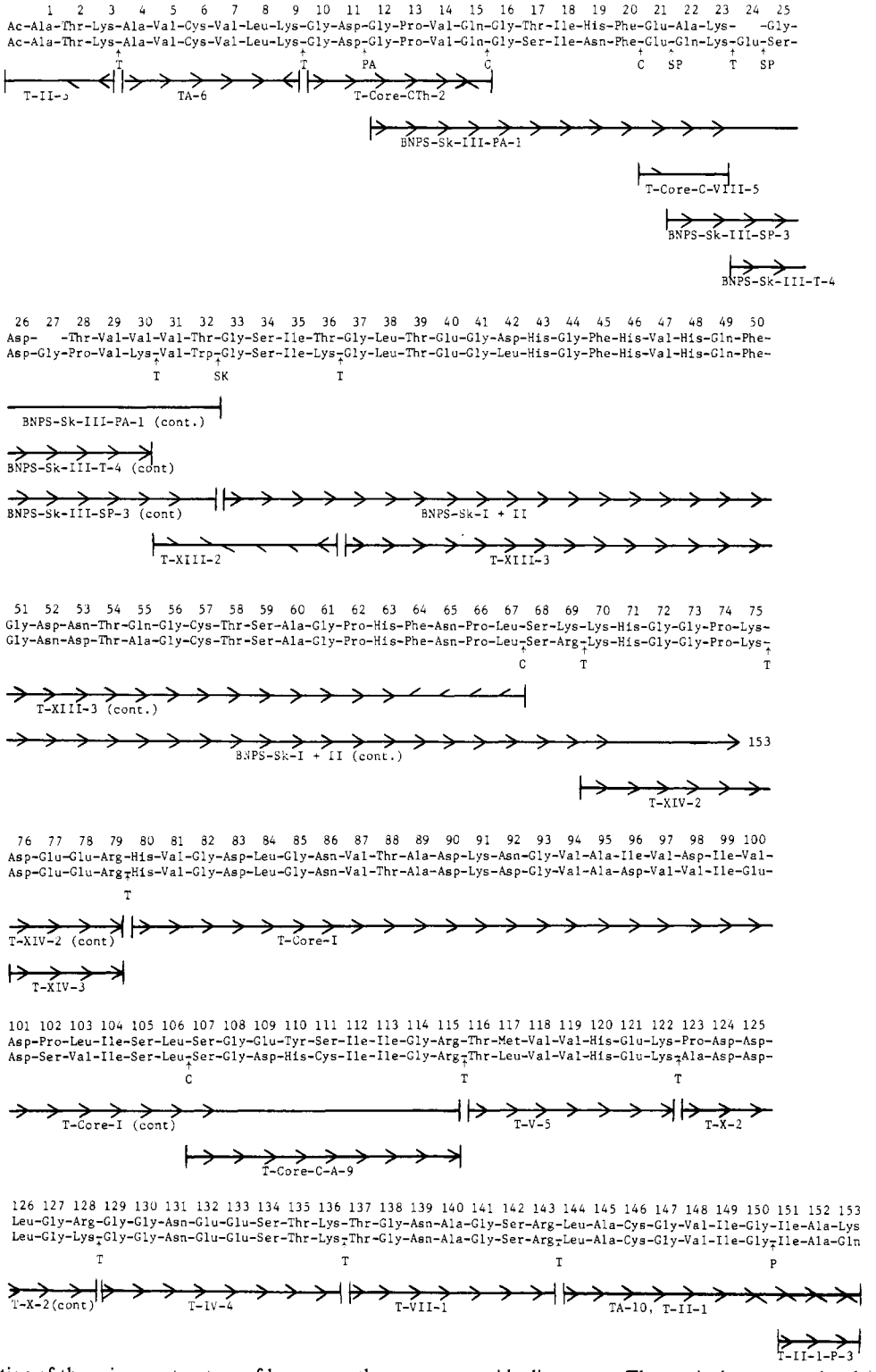

FIGURE 4: Delineation of the primary structure of human erythrocyte superoxide dismutase. The vertical arrows under the structure indicate points of cleavage by trypsin (T), chymotrypsin (C), pepsin (P), staphylococcal protease (SP), BNPS-Skatole (SK), and partial acid hydrolysis (PA). The methods used to degrade a given peptide in establishing its sequence are indicated by the arrows and half-arrows on the underlined sequences of each peptide: Edman degradation (⇒); leucine aminopeptidase (⇝); carboxypeptidases B (⇐), A (↼), and C (↽). The upper sequence is for bovine superoxide dismutase, the lower for the human enzyme.

enzyme by Hartz & Deutsch (1972) along with these data indicates that this peptide probably had an acetylalanyl amino terminus and comprised the first three residues of the protein.

Edman degradation of the soluble tryptic TA-6 isolated by affinity chromatography (Farb, 1977) gave a sequential release of alanine, valine, Cm-cysteine, valine, and leucine. Treatment of it with carboxypeptidase B released lysine. This hexapeptide was assigned to positions 4–9.

Much of the material covering residues 10–115 was found in tryptic core material. The BNPS-Sk-III material, which comprised the first 32 residues of the protein, also proved useful in determining the structure of this portion of the protein.

A hexapeptide, T-core-CTh-2, was isolated after digestion of tryptic core with a mixture of chymotrypsin (C) and thermolysin (Th). Six cycles of Edman degradation gave the results shown in Figure 4 and established the sequence for

Table II: Compositions of Peptides of Human Superoxide Dismutase

amino acid	T-II-3	T-A-6	T-Core-CTh-2	BNPS-Sk-III-PA-1	BNPS-Sk-III	T-Core-C-VIII-5	BNPS-Sk-III-SP-3	BNPS-SK-III-T-4	T-XIII-2	T-XIII-3	T-XIV-2	T-XIV-3	T-Core-1	T-Core-C-A-9	T-V-5	T-X-2	T-IV-4	T-VII-1	T-A-10	T-II-1	T-II-1-P-3
CmCys		(0.97) 1			(+)					(0.8) 1			(0.7) 1	(0.8) 1					(1.1) 1	(2.5) 1	
Asp			(2.0) 1	(2.5) 2	(1.81)		(0.8) 1	(2.6) 1		(3.1) 3	(1.1) 1	(1.8) 1	(+6.2) 7	(1.0) 1		(4.5) 2	(2.9) 1	(2.8) 1			
Thr	(6.4) 1				(0.76)					(3.1) 3			(1.0) 1		(6.0) 1		(2.2) 1	(2.4) 1			
Ser				(1.2) 2	(0.84)		(2.1) 1	(2.4) 1	(3.0) 1	(1.4) 1			(3.3) 3	(0.8) 1			(2.4) 1	(2.7) 1			
Glu			(1.7) 1	(5.6) 4	(2.3)	(6.0) 2	(2.9) 2	(2.3) 1		(2.1) 2	(2.1) 2	(4.1) 2	(1.1) 1		(7.6) 1		(4.2) 2		(2.0) 1	(3.0) 1	(7.4) 1
Pro			(2.4) 1	(3.4) 2	(1.6)		(1.4) 1	(2.5) 1		(2.0) 2	(0.9) 1										
Gly			(4.1) 2	(3.7) 3	(3.6)		(2.3) 1	(2.7) 1	(3.3) 1	(5.8) 6	(2.2) 2		(4.2) 5	(1.9) 2		(2.4) 1	(4.4) 2	(5.3) 2	(3.9) 2	(5.9) 2	
Ala	(7.6) 1	(1.1) 1			(1.2)					(2.0) 2			(1.7) 2				(1.6) 1	(2.4) 1	(3.3) 2	(5.8) 2	(7.3) 1
Val		(2.0) 2	(1.8) 1	(3.2) 3	(2.6)		(2.8) 2	(2.2) 1	(2.3) 1	(1.0) 1			(3.9) 6		(10.8)2				(1.5) 1	(1.8) 1	
Ile				(1.7) 1	(0.69)				(2.6) 1				(2.6) 4	(1.3) 2					(2.7) 2	(4.8) 2	(6.5) 1
Leu		(1.2) 1			(0.70)					(2.9) 3			(1.8) 2		(1.9) 1				(1.7) 1	(2.6) 1	
Tyr																					
Phe																					
His				(1.5) 1	(0.57)					(3.0) 3											
Lys	(6.7) 1	(0.9) 1		(2.0) 2	(1.9)	(2.7) 1	(2.9) 2	(1.9) 1	(2.6) 1	(3.7) 4	(1.0) 1		(2.5) 2	(0.8) 1	(5.3) 1						
Arg											(1.8) 2		(0.9) 1		(6.3) 1	(1.9) 1	(2.1) 1				
Trp											(1.0) 1	(1.9) 1	(1.0) 1	(1.0) 1				(2.0) 1			
charge, pH 6.5		0			(+)	0	0	−1		−1		−2		−0.5	+0.5	−1	−1	+1	−2	−2	
no. of residues	3	6	6	(21)	(32)	3	11	7	6	31	10	4	36	9	7	6	8	7	10	10	3
sequence position	1–3	4–9	10–15	12–32	1–32	21–23	22–32	24–30	31–36	37–67	70–79	76–79	80–115	107–115	116–122	123–128	129–136	137–143	144–153	144–153	151–153

positions 10–13. Only glutamine was released upon digestion with carboxypeptidase A. Residue 11 appeared to be aspartic acid since partial acid hydrolysis of the peptide BNPS-Sk-III effected a cleavage at this position. The composition of one of these partial acid hydrolysis products designated as BNPS-Sk-III-PA-1 suggested that it comprised residues 12–32. The results of 12 cycles of Edman degradation which established this are shown in Figure 4. A neutral tripeptide, T-core-C-VIII-5, was found to release glutamic acid upon treatment with leucine aminopeptidase and thus possessed a Glu-Gln-Lys sequence. This further confirmed the sequence of residues 21–23. This result indicated that BNPS-Sk-III should be cleaved by staphylococcal protease (Ryden et al., 1974) at residue 21. Peptide BNPS-Sk-III-SP-3, isolated from such a digest by high-voltage paper electrophoresis at pH 6.5, appeared to comprise residues 22–32. The results for Edman degradation of BNPS-Sk-III-SP-3 gave the sequence shown in Figure 4 for residues 22–31. This was confirmed by the results obtained for other peptides derived from BNPS-Sk-III. These included a tryptic heptapeptide designated as BNPS-Sk-III-T-4. It had a charge of −1 at pH 6.5 and should thus contain two acidic residues. Seven cycles of Edman degradation were performed to give the sequence shown for residues 24–30 in Figure 4. These studies revealed that there are two additional residues not noted in the bovine enzyme in this part of the protein and that there are also three substitutions.

A soluble tryptic hexapeptide, T-XII-2, that contained tryptophan was isolated from an unstained peptide map. Treatment of it with leucine aminopeptidase sequentially released valine and tryptophan while the results of treatment with a mixture of carboxypeptidases B and A established its C-terminal sequence as being -Gly-Ser-Ile-Lys. This sequence for residues 31–36 is the same as that of the analogous portion of the bovine enzyme except for a substitution of tryptophan for threonine at residue 32.

Edman degradation of the BNPS-Sk-I + II fraction derived from 10 mg of protein, which as was earlier indicated is a mixture of unsplit enzyme and material initiating at residue 33, was successful in establishing the sequence of 38 residues. No amino acids were found in the alkaline hydrolysates of the residues corresponding to positions 57 and 59. These were assigned to cysteine and serine since they both are labile to the hydrolysis conditions used to convert the thiazolinone derivatives to their corresponding amino acids. An increase in the level of ammonia in the hydrolysate of the thiazolinone for cycle 17 was the basis of its assignment as an amide. The positions of 18 of the residues found in BNPS-Sk-I + II had been earlier determined in a tryptic affinity peptide designated as TA-33 and found to initiate at residue 37 (Farb, 1977).

A soluble tryptic peptide, T-XIII-3, appeared to comprise residues 37–67 and to have been released at its C-terminal end by a chymotryptic cleavage at residue 67. The sequential release of amino acids by carboxypeptidase C established that its C-terminal sequence was -Phe-Asn-Pro-Leu. This permitted assignment of residue 65 as asparagine. The hydrolysis products of the thiazolinone for the Edman cycle of BNPS-Sk-I + II corresponding to residue 52 showed a marked increase in ammonia, indicating that the residue in question was an amide, i.e., asparagine. A decrease in ammonia for the PTH product of cycle 17 indicated that the residue was aspartic acid.

The tryptic decapeptide, T-XIV-2, found in a short-time (5 h) digest was subjected to 10 cycles of Edman degradation and gave the sequence shown for residues 70–79 in Figure 4. Another peptide of this digest, T-XIV-3, was revealed to have

the sequence shown for residues 76–79. Since it possessed a charge of −2 at pH 6.5, it appeared that no amides were present.

Peptide T-core-I appeared to constitute residues 80–115. Twenty-eight cycles of Edman degradation gave the results shown in Figure 4. Cycles in which no amino acid was detected in the alkaline hydrolysates of the thiazolinone were presumed to be serine. The PTH derivatives corresponding to residues 83, 86, 90, 92, 96, 100, and 101 were determined to be those of aspartic acid or asparagine and glutamic acid (residue 100) by high-pressure liquid chromatography.

A nonapeptide, T-core-C-A-9, was used to obtain the sequence of residues 107–115. These results show that the cysteine residue of this peptide was a substitution of a serine in the bovine enzyme. This cysteine is the readily reactive one discussed earlier. This peptide has a charge of −0.5 at pH 6.5. The presence of a carboxymethylcysteine and an aspartic acid residue accounts for this charge.

Peptide T-V-5 gave the sequence shown for residues 116–122 in Figure 4. This peptide has a charge of +0.5 at pH 6.5, which indicates that the glutamyl residue is present as the acid. Edman degradation of the tryptic hexapeptide T-X-2 gave the sequence of residues 123–128. The latter peptide possessed a net charge of −1 at pH 6.5, which indicates that both of the aspartyl residues were present as the free acids.

Octapeptide T-IV-4 provided the sequence for residues 129–136. It had a charge of −1 at pH 6.5, and thus one of its three potential acidic residues should exist in the form of an amide. An increase in ammonia in the hydrolysate of the thiazolinone of the third Edman cycle indicated that the residue was asparagine. A soluble tryptic heptapeptide, T-VII-1, appeared to be identical with the homologous region of the bovine enzyme and furnished the structure for residues 137–143. It possessed a charge of +1 at pH 6.5 and thus contained an amide residue. The fourth affinity peptide, TA-10, previously designated as TA-17 by Farb (1977), lacked both lysine and arginine and was presumed to comprise the carboxyl-terminal portion of the protein. Ten cycles of Edman degradation on it established the sequence noted for the analogous region of the bovine protein. Farb (1977) had presented data indicating that this material was a carboxyl-terminal heptadecapeptide that entailed an extension of seven amino acid residues beyond that noted for the bovine enzyme. Various studies were instituted to determine the validity of this suggestion. A peptide, T-II-1, prepared from reduced–alkylated protein, with an amino acid composition similar to that of the affinity peptide from this region, was also isolated from various tryptic digests. Twelve cycles of Edman degradation on it gave the sequence noted in Figure 4 for residues 144–153. The 11th and 12th cycles were devoid of any thiazolinones, and no significant peptide residue was noted in the reaction cup of the sequencer. Digestion of T-II-1 with carboxypeptidase A sequentially released glutamine, alanine, isoleucine, and glycine. Treatment of heat-denatured holoenzyme with carboxypeptidase A sequentially released glutamine, alanine, and isoleucine. Digestion of peptide T-II-1 with chymotrypsin, pepsin, and thermolysin released only peptides that were expected from the 144–153 sequence shown in Figure 4. One of these, tripeptide T-II-1-P-3, had the composition shown in Table II and gave the Edman degradation result for residues 151–153 shown in Figure 4. This portion of the molecule of human superoxide dismutase is identical with that of the bovine enzyme except for glutamine being the carboxyl-terminal residue instead of lysine. A similar finding has been reported by Barra et al. (1978).

Table III: Variations in the Primary Structures of Human and Bovine Erythrocyte Superoxide Dismutases

residue no.	amino acid		residue no.	amino acid	
	bovine	human		bovine	human
17	Thr	Ser	69	Lys(67)	Arg
19	His	Asn	92	Asn(90)	Asp
21	Gly	Glu	96	Ile(94)	Asp
22	Ala	Gln	98	Asp(96)	Val
24[a]		Glu	100	Val(98)	Glu
25	Gly(24)[a]	Ser	102	Pro(100)	Ser
27		Gly	103	Leu(101)	Val
28	Thr(26)	Pro	109	Glu(107)	Asp
30	Val(28)	Lys	110	Tyr(108)	His
32	Thr(30)	Trp	111	Ser(109)	Cys
36	Thr(34)	Lys	117	Met(115)	Leu
42	Asp(40)	Leu	123	Pro(121)	Ala
52	Asp(50)	Asn	128	Arg(126)	Lys
55	Gln(53)	Ala			

[a] The numbering for the human and bovine enzymes changes at this point due to two subsequent deletions in the latter protein whose sequence has been reported by Steinman et al. (1974). The numbers in parentheses are for the bovine enzyme residues as given by these authors.

Discussion

The sequence of human Zn–Cu superoxide dismutase shows a strong homology to the analogous bovine enzyme (Steinman et al., 1974); 126 of the 153 residues of the human enzyme or 82% are identical with the 151 residues found for the bovine protein. The differences noted for these two sources of superoxide dismutase are presented in Table III. None of the substitutions appear to be close to the sites in the bovine enzyme (Richardson et al., 1975) responsible for binding of the zinc. All of the substitutions, except those at positions 22, 30, 32, 42, 55, and 96, can be accounted for by single base changes in their respective codons. Most of these residues appear to be located on the surface of the protein if it is assumed that similar secondary–tertiary structures are obtained for the human and bovine enzymes (Richardson et al., 1975). Although 82% of the residues of the bovine and human proteins are homologous, two regions show marked differences. The first 16 residues are identical, but this is followed by a segment of 20 amino acids having 11 changes. The reason for such a sudden loss of identity is difficult to explain but does not appear to involve a frame shift. Residues 17–36 form a part of the enzyme that has been shown to be opposite to the region of hydrophobic interaction of subunits in the bovine enzyme (Richardson et al., 1975). The human enzyme has a proline at position 28 not present in the bovine protein (Steinman et al., 1974). This region appears to be the turning point of an antiparallel, β-pleated sheet structure in the latter enzyme, and the presence of a proline in the human protein at this point would not be expected to markedly influence such a structural change.

The three variant residues at positions 109–111 include a substitution of cysteine for the serine of the bovine enzyme. These three residues would, however, be expected to effect major electrostatic changes at another of the expected subunit–subunit interfaces of the protein. These may be the major factors in conferring marked lability on the human enzyme as compared with the bovine form. The derivatization of the cysteine at position 111 converts the human protein into a more stable form and one that can be readily crystallized even after the derivatized protein has been separated by the ethanol–chloroform procedure. The accessibility of this cysteine residue of human superoxide dismutase has also been noted by Briggs & Fee (1978b). As shown by Farb (1977) and also by Briggs

& Fee (1978b), this cysteine does not exist as a persulfide as reported by Calabrese et al. (1975). Briggs & Fee (1978b) have indicated that the results reported by the latter workers are artifacts arising from oxidative conditions employed during isolation by procedures including removal of the hemoglobin by denaturation with solutions of ethanol–chloroform.

The sequence for human superoxide dismutase reported in the present study is similar to those portions of the partial structure given by Barra et al. (1978) except for residue 47, at which position these workers report a glycine. We have found a valine at this position, which is in agreement with what is noted for the analogous position in the bovine enzyme.

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Proton Nuclear Magnetic Resonance Studies of the Effects of Ligand Binding on Tryptophan Residues of Selectively Deuterated Dihydrofolate Reductase from *Lactobacillus casei*†

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ABSTRACT: We have prepared a selectively deuterated dihydrofolate reductase in which all the aromatic protons except the C(2) protons of tryptophan have been replaced by deuterium and have examined the ^{1}H NMR spectra of its complexes with folate, trimethoprim, methotrexate, $NADP^+$, and NADPH. One of the four Trp C(2)-proton resonance signals (signal P at 3.66 ppm from dioxane) has been assigned to Trp-21 by examining the NMR spectrum of a selectively deuterated *N*-bromosuccinimide-modified dihydrofolate reductase. This signal is not perturbed by NADPH, indicating that the coenzyme is not binding close to the 2 position of Trp-21. This contrasts markedly with the ^{19}F shift (2.7 ppm) observed for the ^{19}F signal of Trp-21 in the NADPH complex with the 6-fluorotryptophan-labeled enzyme. In fact the crystal structure of the enzyme·methotrexate·NADPH shows that the carboxamide group of the reduced nicotinamide ring is near to the 6 position of Trp-21 but remote from its 2 position. The nonadditivity of the ^{1}H chemical-shift contributions for signals tentatively assigned to Trp-5 and -133 indicates that these residues are influenced by ligand-induced conformational changes.

Dihydrofolate reductase (EC 1.5.1.3) catalyzes the reduction of dihydrofolate to tetrahydrofolate by using NADPH as a coenzyme. The enzyme is of considerable pharmacological interest, being the target for "antifolate" drugs such as methotrexate and trimethoprim. As part of a wider program aimed at understanding the factors influencing the binding of ligands to dihydrofolate reductase, we have been examining the NMR spectra of complexes of the enzyme with its coenzyme, substrates, and substrate analogues. The success of such studies depends on the detection of signals from nuclei

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