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FURTHER CHARACTERIZATION OF HUMAN ERYTHROCYTE SUPEROXIDE DISMUTASE

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Summary

1. A simplified procedure for the preparation of highly purified human superoxide dismutase from erythrocytes was developed which avoided extremes of pH and ionic strength and the use of organic solvents; the properties of human and bovine proteins, prepared by the method, were compared.

2. Using the two dimensional electrophoretic procedure of O'Farrell, the human superoxide dismutase was found to consist of a single type of polypeptide.

3. The human protein was found to have a total of eight half-cystine residues per mole of protein, compared to six such residues for the bovine protein. The human protein has two sulfhydryl groups which are reactive toward mercurials when dissolved in 1 M guanidine-hydrochloride and ≈ 3 reactive sulfhydryls when the protein is dissolved in 6 M guanidine hydrochloride. The distribution of the eight sulfur atoms appears to consist of four involved in disulfide linkages, two deeply buried within the molecule and unreactive except under strongly denaturing conditions, and two which are reactive under mildly denaturing conditions. No zero-valent sulfur was found.

4. The visible optical absorption, the visible circular dichroism, and the electron paramagnetic resonance spectra are essentially identical with those of the bovine protein. No unusual absorbance was found at 330 nm. The near ultraviolet spectrum is different from that of the bovine protein, and this appears to be due to differing amino acid compositions.

5. Two fractions of superoxide dismutase activity were observed during chro-

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Abbreviations: DTNB, 5,5′-dithiobis-(2-nitrobenzoate); Gln-HCl, guanidine-HCl.

matography of partially purified solutions on diethylaminoethyl-cellulose. The minor, less mobile form, was found to revert to the less mobile species on aging; the reverse process was not observed to occur. The minor component was found to contain equimolar amounts of Zn and Cu and to have a specific dismutase activity somewhat higher than that of the purified major fraction.

Introduction

The mammalian copper protein which possesses superoxide dismutase activity [1] was first isolated from a variety of tissues in 1939 [2]. These workers developed a preparative procedure which yielded a substantially pure crystalline protein from bovine erythrocytes but which was not directly useful with red blood cells from sheep, horse or human sources. Thus, these workers observed that protein from the latter species was obtained as non-crystalline material, having a lower percentage of Cu than that obtained from beef cells.

The procedure of Mann and Keilin [2] which involves precipitation of hemoglobin by a mixture of chloroform and ethanol [3], precipitation of the copper protein and other proteins with lead acetate followed by extraction of this precipitate and subsequent specialized operations, was later modified and applied to human erythocytes [4—6]. These groups obtained the protein in reasonable yield and apparent purity. However, in each case, chloroform:ethanol precipitation was used to remove the hemoglobin. Stansell and Deutsch [7] developed an entirely new procedure for the isolation of human superoxide dismutase (then designated erythorcuprein), which did not involve use of organic solvents. These authors presented results which suggested that previous purification procedures introduced modification of the solution and electrophoretic properties of human dismutase [8]. Further, electrophoretic heterogeneity was found to increase upon aging solutions of this copper protein.

Over the past decade, the purification procedure has incurred minor modifications as work on the chemical and physical properties of the human protein has proceeded [9—12]. Hartz and Deutsch [12] suggested that the human protein, like bovine dismutase [13], consisted of two subunits which were not covalently associated via disulfide bonds. They argued that the subunits were not identical in contrast to what has now been unequivocally shown for the bovine protein [14].

There have been several conflicting reports on the sulfur components of the human protein. One group indicated the presence of eleven [4], a second group [8] observed five, one of which was reactive toward alkylating agents, and a later publication by the latter group [12] report seven half-cystine residues, with two being reactive toward iodoacetamide in 7 M guanidine-HCl.

The purposes of this communication are to described an alternative purification procedure which is useful for obtaining moderate amounts of human Zn/Cu dismutase, to delineate the distribution of the sulfur atoms between disulfide and sulfhydryl forms, and to compare the composition and spectral properties of human and bovine Zn/Cu superoxide dismutases. In the accompanying communication [15] we examine the modification of human dismutase by the classic Tsuchihashi procedure [3] for the removal of hemoglobin.

Materials and Methods

All buffers and reagents were prepared in glass distilled water using materials of highest available purity. Enzymatic activity was determined, using a minor (half-volume) modification of the method of McCord and Fridovich [1] with a small amount of dismutase-free catalase [16] added to the cytochrome *c*. The latter prevented reoxidation of the cytochrome sometimes observed, presumably due to peroxide generated during the assay. Lysate activities were assayed in the presence of 0.1% Triton X-100, a concentration which eliminated turbidity but had no measurable effect on dismutase or xanthine oxidase activities. Dismutase activities were estimated using the formula

$$\text{Units of activity} = \left[\frac{\text{Cyt. } c \text{ redn. rate without dismutase}}{\text{Cyt. } c \text{ redn. rate with dismutase}} - 1\right]$$

Cytochrome *c* was reduced in the reference system at a rate of about 0.1 absorbance unit (550 nm) per min. Units of activity were linear with respect to dismutase concentration, and gave specific activities which were in proportion to the increased concentration of dismutase in the system.

Protein was determined by the method of Lowry et al. [17] using crystalline bovine serum albumin (Sigma) as a standard. The nitrogen content of one purified sample was determined to contain 17.95% N relative to such a Lowry protein value in reasonable agreement with other workers [6,10,11]. (We thank Dr. Irwin Goldstein, Department of Biological Chemistry, The University of Michigan, for the measurement.)

Copper, zinc, and manganese were determined in trichloroacetic acid extracts of the protein. Zinc and manganese were analyzed by using the Perkin-Elmer Model 303 atomic adsorption spectrophotometer and copper by use of bathocuproine sulfonate. Zinc chloride and copper sulfate standards were acidified to minimize hydrolysis and were found to give values identical to those obtained when standards were prepared from acid dissolution of pure metals. An extinction coefficient of 12 600 $M^{-1} \cdot cm^{-1}$ at 480 nm was determined for the reduced copper bathocuproine sulfonate complex.

Sulfhydryl groups were determined by polarographic titration with phenylmercuric acetate as previously described [18,19]. When titrations in 6 M guanidine hydrochloride (Schwartz-Mann) were carried out, EDTA (0.2—0.3 mM) was present to minimize metal catalyzed destruction of phenylmercuric acetate-reactive sulfur. The necessity of this was supported by our observation that solutions of human dismutase produced bathocuproinesulfonate reactive copper (Cu^+) in guanidine-HCl in the absence of any other reducing agent. Further, the presence of EDTA increased the amount of sulfur detected by phenylmercuric acetate, particularly the disulfide-linked form exposed in the presence of 20 mM sodium sulfite. Bis-5,5′-dithionitrobenzoate (DTNB) was also used at a concentration of 0.5—1 mM to determine sulfhydryl content. An extinction coefficient of 13 500 $M^{-1} \cdot cm^{-1}$ [20] was determined for the thionitrobenzoate anion using freshly prepared cysteine standards. Zero-valent sulfur was determined essentially as described by Fletcher and Robson [21] using Sörbo's reagent [22].

Ultraviolet and visible spectra were obtained with solutions in phosphate or

Tris buffer using the Coleman-Hitachi model 124 or the Zeiss model DMR-21 recording spectrophotometer. Circular dichroism measurements were done under similar conditions using the Durrum-Jasco instrument. $\Delta\epsilon$ was determined at 2—10 mdeg. ellipticity per cm of pen displacement using the factor of $0.0303 \cdot 10^{-3}$ cm^2 · mol^{-1} · deg^{-1}:

$$\Delta\epsilon = \frac{4\pi\theta' \times 10^{-3}}{2.303 \times 180 \text{ deg} \times c}$$

where θ' = measured ellipticity in mdeg per cm light path.

Fluorescence spectra were obtained using a Turner instrument in the laboratory of Dr. F.C. Wedler.

Electron paramagnetic resonance spectroscopy was performed using the Varian E-9 spectrometer; sample temperatures were maintained near that of liquid nitrogen.

Gel electrophoresis was carried out using the alkaline buffer system [23] except that polymerization and electrophoresis were carried out at 4°C. To effect sufficiently rapid polymerization at this temperature, the ammonium persulfate concentration was increased to 0.3% and 0.1% ammonium persulfate was layered over the gel during gelation. Gels thus prepared were preelectrophoresed in a Tris-HCl buffer system to avoid contact of protein with persulfate. Stacking gel was found to give no added resolution and was not used. Gels were stained with Coomassie blue and, for activity, by the method of Beauchamp and Fridovich [24]. Two-dimensional gel electrophoresis was carried out in Dr. F. Neidhardt's laboratory essentially as described by O'Farrell [25].

Carboxymethyl-cellulose (CM-11 or CM-23, Whatman) was precycled according to manufacturers instructions and thereafter recycled by the same method after washing with 0.2 M K_2HPO_4. Prior to use, this cellulose was equilibrated with 10 mM sodium phosphate buffer, pH 6.0. DEAE-cellulose (DE-52, Whatman) was precycled according to manufacturer's instructions and recycled by the same method after first washing with 1 M K_2HPO_4 for 1 h. This cellulose was equilibrated with 2 mM sodium phosphate (pH 7.8) prior to use. Whereas CM-cellulose appeared to be completely restored by the above treatment, DEAE-cellulose, when used for batch adsorption, acquired a greenish color which could not be removed.

Mixed bed ion exchange resin was obtained from several sources including Bio-Rad (RG-501-X8), Fisher (Rexyn 300), and commercial demineralizer cartridges (e.g., ILCO research model I).

Purification procedure

All isolation steps were carried out at 4°C or with the sample in ice. Outdated whole blood or cells (3—4 units) were donated by the American Red Cross. Plasma was removed by direct decantation, where possible, or by aspiration following centrifugation. Cells were washed with 0.9% saline and lysed in 3 vols. distilled water. After centrifugation, 2 l supernatant solution were mixed with 8 l distilled water.

2.5 kg moist CM-cellulose were added and the pH adjusted with vigorous stirring to 6.0 with 1 M H_3PO_4. The suspensions was restirred with a broad paddle

at 5-min intervals. After the final resuspension for 15 min at 4°C, the mixture was passed through Miracloth ® (Calbiochem) cut to fit a large Büchner funnel. 500 g mixed bed ion-exchanger were added to the filtrate and the mixture agitated every 5 min for 30 min. During the first few minutes of agitation, it was important to monitor the pH and add appropriate amounts of NaOH or H_3PO_4 to maintain this near neutrality. Best results were obtained when conductivity was monitored and deionization continued until the conductivity reached that of 2 mM sodium phosphate at the appropriate pH (pH 6.8 and 7.8 were used in separate experiments with no significant alteration in yield or ultimate purity). At the end of deionization, exchanger was removed by filtration and the filtrate adjusted to the desired value. 250 g moist DEAE-cellulose were then added and the pH adjusted. Ion-exchanger, contained in a cloth bag or nylon stocking, was placed in the mixture and agitation performed every 5 min for 30 min. At the end of this period, the bag was rinsed with water and the DEAE-cellulose removed from the bulk solution by filtration, as described for CM-cellulose. The cellulose was suspended in 2 mM $NaPO_4$ and transferred to a 5-cm diameter column. This column was rapidly washed with up to 20 l 2 mM sodium

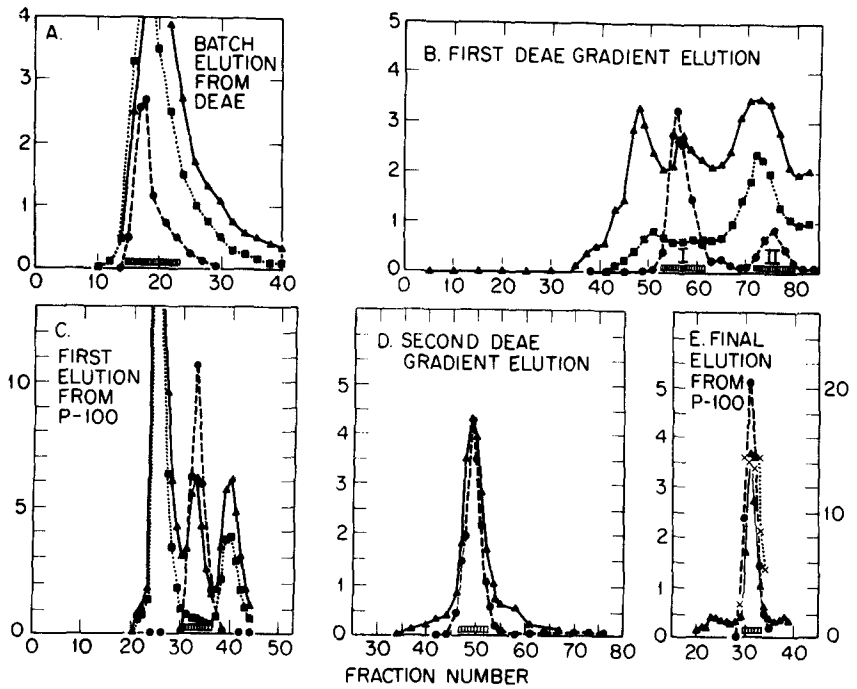

Fig. 1. Chromatographic purification of human superoxide dismutase from erythrocytes. A. Profile of elution from DE-52 after batch adsorption. Tubes 15—23 were pooled and had an average value of 10.3 ml. B. Gradient elution from DE-52. Tubes 53—61 were pooled for Fraction I while tubes 72—79 were pooled as Fraction II. The average volumes per tube were 7.4 and 7.3 ml respectively. C. Gel permeation chromatography on Biogel P-100. Tubes 31—36 were pooled and had an average volume of 6.0 ml. D. Repeat of DE-52 gradient elution. Tubes 47—52, which received 8.8 ml of eluate each, were pooled. E. Reelution from Biogel P-100. Tubes 30—33 were pooled. The average volume per tube was 6.5 ml. (△———△), in A, A_{280} X 0.4; B, A_{280} X 5; C, A_{280} X 10; D, A_{265} X 20; E, A_{265} X 10. ○———○, activity in units per microliter. - - - - - -, in A, A_{412} X 0.4; B, A_{410} X 5; C, A_{410} X 10. X. . . . . X, specific activity in units per A_{265} (Right ordinate of 1E). (□□□□□) indicates fractions pooled.

phosphate until the red color of hemoglobin was eluted. At this point the column was eluted with 500 ml 0.1 M sodium phosphate at 5—10 ml/min. 10—15 ml fractions were collected (Fig. 1a). The active fractions were combined and 50 g mixed bed ion-exchanger were added. When conductivity had reached that of 2 mM sodium phosphate at the appropriate pH, the ion exchanger was removed by filtration and rinsed with dilute buffer (care was required to avoid frothing of the filtrate). Any resulting precipitate was removed by centrifugation and the supernatant mixed with 50 g CM-cellulose previously equilibrated with 2 mM sodium phosphate. The pH was adjusted and the mixture agitated with a magnetic stirrer for 15 min. The cellulose was then removed by Büchner filtration and washed with a small amount of buffer.

Operations to this point required 8—20 h (depending on the time taken to wash hemoglobin from the DEAE-cellulose) and the resulting celar yellow sample could be stored overnight in the cold. This often resulted in some protein precipitate which was removed by centrifugation.

The solution was then applied to a 2.2 × 30 cm DEAE-cellulose column followed by washing with a 200—300 ml dilute buffer. The column was then eluted with a linear gradient of 2—100 mM sodium phosphate with 500 ml buffer in each reservoir. Flow rate was 0.5—1 ml/min and approx. 10-ml fractions were collected (Fig. 1b). Fractions I and II were separately pooled and concentrated by ultrafiltration in a collodion bag to about 2 ml. The concentrated Fraction I was applied to the top of a Biogel P-100 (Bio-Rad) column (2.8 × 120 cm) previously equilibrated with 50 mM sodium phosphate (pH 7.5). After the protein had moved 2—3 cm into the gel, the column was capped, inverted, and ascending elution carried out (Fig. 1c) with a head of about 35 cm buffer at an elution rate approx. 0.3 ml/min. Fractions were collected at 30-min intervals. Those containing dismutase activity were pooled and concentrated for rechromatography on DEAE-cellulose and Biogel P-100 with typical results being shown in Figs. 1d and 1e. The time required for the entire procedure was generally 6—8 days.

Results

Table I presents the recovery of superoxide dismutase activity in the course of purifying the human protein. These values were similar in four preparations of the human enzyme and were comparable to those obtained during isolation of bovine dismutase by the present procedure.

Since the experiment of Table I was selected for the completeness of available data, rather than for the most representative results, certain aspects differentiate the other three experiments in which recoveries were determined. The losses during CM-cellulose batch adsorption were always observed, whereas losses during batch and gradient elution from DEAE-cellulose were unique to the preparation shown.

The prevailing pH during adsorption and ion exchange chromatography, whether 6.8 or 7.8, did not affect recovery of enzyme. Once dismutase was freed of contaminating proteins, its complete recovery was obtained after DEAE-cellulose or gel-permeation chromatography. Due haste in removal of

TABLE I

RECOVERY OF DISMUTASE ACTIVITY AT VARIOUS STAGES DURING PURIFICATION OF HUMAN SUPEROXIDE DISMUTASE

Step	Total units of dismutase activity *	Equivalent mg of protein **	Percent
Lysate	340 000	97	100
1. CM-Cellulose supernatant	260 000	74	75
2. Batch adsorption supernatant	20 000	6	6
3. Pooled samples: batch elution from DEAE	170 000	46	50
4. Pooled samples: first gradient elution from DEAE	105 000	30	31
Fraction I	80 000		
Fraction II	25 000		
5. Pooled samples: first P-200 elution (Fraction I)	85 000	24	25
6. Pooled samples: Second gradient elution from DEAE	80 000	23	24
7. Pooled samples: final P-100 elution	70 000	20	21

* Units defined in Materials and Methods.

** Based on a specific activity of 3 500 units per mg.

contaminating protein and avoidance of extended refrigeration of dilute enzyme were found necessary for optimum recovery.

Most of the lysate hemoglobin was removed by the initial CM-cellulose batch adsorption. The small amount of remaining hemoglobin did not interfere with batch adsorption of dismutase onto DEAE-cellulose and it was completely removed by washing the DEAE-cellulose or by repetition of Step 1 after batch elution from DEAE-cellulose.

One of the interesting features of the procedure was that it revealed two distinct fractions, possessing dismutase activity, upon gradient DEAE-cellulose elution (Fig. 1b). Subsequent separate chromatography of these two fractions on Biogel P-100 revealed similar mobility on this gel for the two components. Unless noted to the contrary, all data in this section are those of the major band of dismutase activity. The minor band (Fraction II, Fig. 1b) is discussed in more detail below.

The major contaminants which eluted from DEAE-cellulose with human dismutase were a brownish protein preceeding the dismutase activity and a yellow protein which followed it. The latter appeared to correspond to a fraction termed erythroxanthin by Hartz [10]. Chromatography on Biogel P-100 (Fig. 1c) followed by repetition of DEAE-cellulose and Biogel P-100 elution (Figs. 1d and e) completed the purification.

The final Biogel P-100 peak had constant specific activity over the five fractions pooled and a constant absorbance ratio A_{265}/A_{280}, indicating homogeneity of the preparation.

Acrylamide gel electrophoresis (Figs. 2a and b), however, revealed heterogeneity in the dismutase prepared by this procedure. This did not reflect the heterogeneity observed during DEAE-cellulose chromatography, since the second peak eluted in that procedure exhibited similar electrophoretic components (Fig. 2e). Scans of Coomassie blue-stained gels (performed with cooperation of Dr. Bruce Hacker of the Departments of Biochemistry and Oncology, Albany Medical College, Albany, N.Y.) revealed no more than 5% contamina-

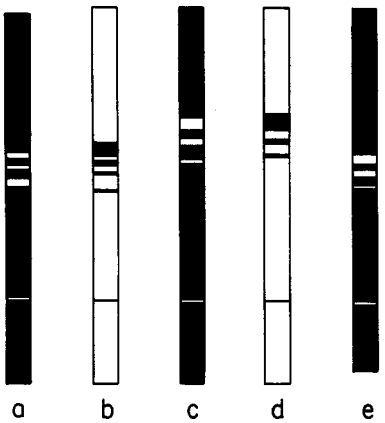

Fig. 2. Acrylamide gel electrophoresis of human and bovine superoxide dismutases. Gels A, C, and E were stained for activity [25] while B and D were stained with Coomassie blue. Human proteinwas applied (1 and 20 μg) to gels A and B, respectively, while 1 and 20 μg bovine protein was applied to gels C and D, respectively. Fraction II (Fig. 1b) was applied to gel E (5 units dismutase activity).

tion with enzymatically inactive protein assuming equal staining of all components. Bovine dismutase isolated by this procedure also exhibited hetergeneity upon gel electrophoresis (Figs. 2c and d). Its lower mobility and its heterogeneity was consistent with similar properties observed by others [1,26]. The apparent heterogeneity evident on disc electrophoresis does not reflect amino acid compositional differences. This was demonstrated by subjecting the purified protein to two dimensional electrophoresis [25]. In these experiments, only a single spot stainable by Coomassie blue was observed when the gel was loaded with approx. 1 μg protein. Less than 0.1 μg of similarly staining material could be detected. This observation strongly suggests that human protein, like the bovine protein, consists of a dimer of identical polypeptide chains.

The amino acid compositions of human and bovine dismutase are presented in Table II. (We thank Prof. C.H. Williams, Jr. for carrying out this analysis.) There are several important differences. Thus, the human protein has 4 half-cystines per dimer, the bovine protein only 3; human dismutase has no tyrosine or methionine, whereas bovine dismutase has one of each; the human protein has 2 tryptophan residues, while the bovine has none. The amino acid analysis obtained is consistent with that of Hartz and Deutsch [12] except for the total half-cystine content. Their datum suggests the presence of 3 half-cystine per subunit while our datum cannot be lower than 3.9 half-cystine per subunit. Further evidence for these additional sulfhydryl groups in human dismutase is presented below.

The composition of purified human protein is defined further in Table III. It can be seen that the metal content does not correspond to exactly one Cu and one Zn per subunit. The results which reflect four separate measurements on different preparations show that the metal binding sites are only 80—85 occupied by Cu and Zn. No detectable Mn and only trace amounts of Fe were found. It is not known whether the metal is lost during purification or not present in the cell.

Analysis of the various cysteine components is presented in Table III. Under

TABLE II

AMINO ACID COMPOSITIONS OF HUMAN AND BOVINE SUPEROXIDE DISMUTASES [a]

Amino acid	Human	Bovine [b]
Half-cystine	4 (3.3) [c]	3
Aspartic acid	18	17
Threonine	8	12
Serine	10	8
Glutamic acid	13	11
Proline	5	6
Glycine	26	25
Alanine	10	9
Valine	14	15
Methionine	0	1
Isoleucine	8	9
Leucine	9	8
Tyrosine	0	1
Phenylalanine	4	4
Lysine	11	10
Histidine	8	8
Argenine	4	4
Tryptophan	2 [d]	
Molecular weight	15 900	15 600

[a] Residues per subunit.
[b] Taken from Abernethy, Steinman, and Hill [13].
[c] Taken from Hartz and Deutsch [12] all other values are in agreement with their earlier results.
[d] Determined by the method of Edelhoch [28].

TABLE III

SOME PROPERTIES OF HUMAN SUPEROXIDE DISMUTASE

	Expressed as mol per 32 000 daltons	Number of determinations
Copper content	1.7 ± 0.1	4
Zinc content	1.6 ± 0.2	2
Molar extinction		
ϵ_{265}	15 900 ± 800	4
ϵ_{325}	1 100 ± 500	4
ϵ_{675}	260 ± 20	4
Molar ellipticity		
$\Delta\epsilon_{605} \times 10^3$	0.93 ± 0.07	4
Specific enzymatic activity		
Units/μmol	120 000 ± 4000	4
Sulfhydryl content		
DTNB PO_4 buffer	0.7 ± 0.5	3
1 M Gdn-HCl	1.8 ± 0	2
6 M Gdn-HCl	3.1 ± 0.3	4
PMA PO_4 buffer	2.2 ± 0.1	2
1 M Gdn-HCl	1.5 ± 0	2
6 M Gdn-HCl	3.2 ± 0.2	4
Disulfide content		
(SO_3^{2-}, PMA) PO_4 buffer	0.1 ± 0.1	2
1 M Gdn-HCl	0.3 ± 0.1	2
6 M Gdn-HCl	1.8 ± 0.6	4
Total reactive sulfur		
(PMA in 6 M Gdn-HCl)	6.8 ± 0.6	4

* Phenylmercuric acetate.

non-denaturing conditions, both 5,5′-dithiobis-(2-nitrobenzoate) and phenylmercuric acetate react with up to 2 SH per mole but this is quite variable as indicated. Under mild denaturing conditions, however, both 5,5′-dithiobis-(2-nitrobenzoate) and phenylmercuric acetate react with approx. 2 SH and the values presented are quite reproducible. Under strongly denaturing conditions, 6 M guanidine-HCl, slightly greater than 3 SH are found per mol protein, or 1.5 per subunit. Given the difficulties inherent in these experiments (cf. Ref. 19), this number can reasonably be thought of as representing 4 SH per molecule. The data concerned with disulfide linkages suggests that there are two per molecule which are exposed only under strongly denaturing conditions. The total half-cystine content determined by these methods was 6.8 ± 0.6, a number which is entirely consistent with the 8 half-cystines found by amino acid analysis. The above results suggest that each subunit of human superoxide dismutase possesses one more SH group than bovine dismutase and that this group is quite reactive toward mercurials. By contrast, the two SH groups of the bovine protein are only exposed in the presence of a strong denaturant [19].

In the accompanying communication [15], we report that under the extreme conditions of removing hemoglobin by chloroform/ethanol precipitation, glutathione can become covalently associated with the human dismutase. It was important to determine whether any glutathione was associated with protein prepared by the chromatographic procedure. Thus, one preparation was carried out following incubation of 25 μCi [*Gly*-^{3}H]glutathione (New England Nuclear) with 2 l lysate for 16 h at 4°C. Assays of oxidized and reduced glutathione performed on perchloric acid extracts of lysate [27], before and after incubation, indicated reduced and oxidized glutathione to be present at 102 μM and 39 μM, respectively, at the start of incubation and at 71 μM and 50 μM, respectively, at the end of this period. Although 200 000 dpm appeared in the eluant from DEAE-cellulose-batch adsorbed protein, only 55 000 dpm survived subsequent retreatment with CM-cellulose. Furthermore, no radioactivity was detected in the final solutions of dismutase, either in Fraction I or Fraction II.

Several samples were tested for the presence of zero-valent sulfur with consistently negative results.

A limited study of Fraction II (Fig. 1b) was also carried out with purification carried only as far as two elutions from DEAE-cellulose and one from Biogel P-100. Partial reversion of this protein to Fraction I-mobility was consistently observed during rechromatography on DEAE-cellulose. In contrast, rechromatography of Fraction I on DEAE-cellulose never resulted in appearance of the Fraction II-like component, though this was specifically sought in three separate preparations. It contained approximately equal amounts of Cu and Zn, no detectable Mn, and had a specific activity (per gatom Cu) which was 20—30 percent greater than the major (Fraction I) peak and the determinations of two different preparations were in agreement. Acrylamide gel electrophoresis (Fig. 2e) revealed heterogeneity and appeared to indicate concentration of activity in the more mobile bands. As the possibility existed that Fraction II existed as a mixed disulfide with low molecular weight sulfhydryl component (cf. Ref. 15) an experiment was performed in which this Fraction was treated with 10 mM 2-mercaptoethanol followed by chromatography on DEAE-cellu-

lose. This treatment failed to produce more than the normal amount of reversion to Fraction I type protein.

Spectral properties

The circular dichroism spectra of human dismutase in the visible and ultraviolet regions are shown in Fig. 3a, and optical adsorption spectra are depicted in Fig. 3b. The absorption spectra do not differ substantially from those of human hepatocuprein reported by Carrico and Deutsch [9] except for complete absence of an absorbance band near 335 nm. The $E^{1\%}_{1cm}$ at 675 nm is 0.089 ± 0.006 and 4.8 ± 0.2 at 265 nm, both values being very similar to the earlier report [9].

The uncorrected fluorescence spectrum of human dismutase (not shown) exhibited excitation and emission maxima at 282 and 360 nm, respectively. This fluorescence was due to the tryptophan for which we determined a value of 2.9 per mol, using the method of Edelhoch [28]. In contrast, no tryosine was detectable by this method, and this is consistent with the amino acid analysis.

EPR spectra of human and bovine proteins are compared in Fig. 4. It is evident that they are identical, the human protein exhibiting $g_{//} = 2.26$, $g_m = 2.08$, and $A_{//} = 123$ gauss. It should be noted that the latter is much smaller than given in an early report [29] as has also been noted by Bannister et al. [34]. The lineshapes of these spectra differ significantly from those reported by Car-

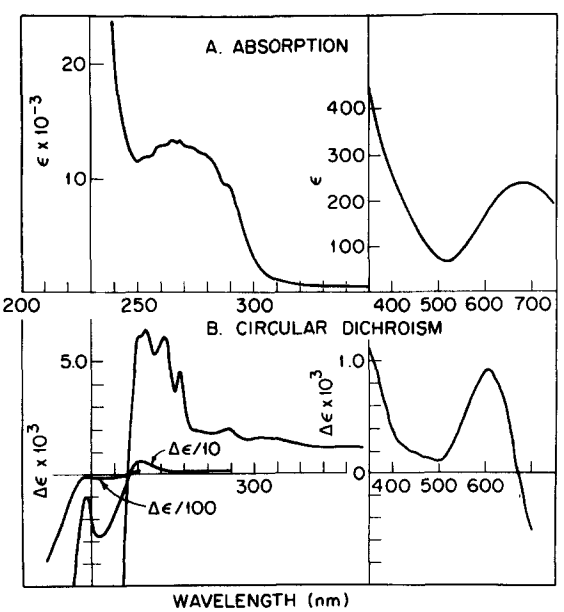

Fig. 3. Optical spectra of human superoxide dismutase. A. Absorption spectrum. The spectra were obtained at 25°C with 0.2 mM enzyme in 0.05 M sodium phosphate buffer (pH 7.5). The ultraviolet region was scanned with the sample in a 2-mm light path cuvette. B. Circular dichroism spectrum. The sample for the X 1 spectrum was identical to that used for the absorption spectrum. The spectra indicated as $\Delta\epsilon/10$ or $\Delta\epsilon/100$ were obtained using a sample diluted to 5.5 μM in 0.05 M sodium phosphate buffer (pH 7.5).

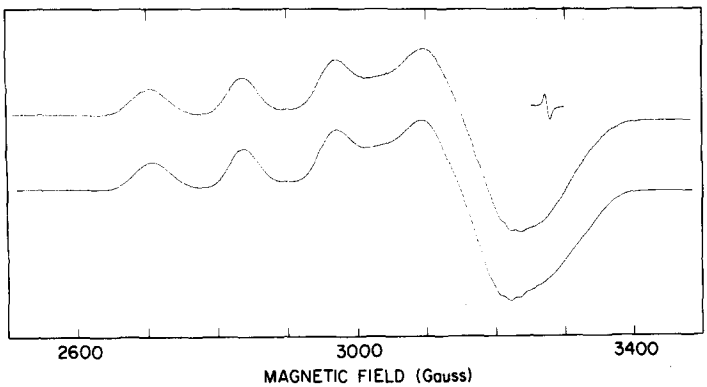

Fig. 4. Electron paramagnetic resonance spectra of human and bovine dismutases. Spectra were obtained at 140°K at a microwave frequency, 9.181 GHz; microwave power, 50 mW; modulation amplitude 10 G; scan rate, 500 G/min; time constant, 30 ms; gains of 200 and 20 for the enzyme from human end bovine source, respectively. Sample copper concentrations were 0.3 mM for human and 3 mM for bovine proteins.

rico and Deutsch [9] and by Hartz [10] obtained under similar conditions.

As mentioned above, this isolation technique was applied to bovine erythrocytes. The bovine protein had less affinity for DEAE-cellulose, eluting at a phosphate concentration of 30—50 mM, compared to the human protein which eluted near 50 mM phosphate. Due to this lower affinity, bovine dismutase was more readily freed of the erythroxanthin [10] which we observed to be present in bovine as well as in human erythrocytes. The final preparation was identical in every respect examined to enzyme isolated by the method of McCord and Fridovich [1].

Discussion

The major differences between the present purification procedure and that of Hartz and Deutsch [12] are the use of carboxymethyl cellulose to remove hemoglobin in the present study and avoidance of low pH as would be encountered in isoelectric focusing [12]. Deionization by mixed bed ion-exchanger prior to batch adsorption has been used [20] to facilitate binding of catalase to DEAE-cellulose. The nearly complete removal of hemoglobin by carboxymethyl cellulose and the ease with which this resin can be regenerated recommends the use of this procedure. Provided care is taken to maintain constant pH, the presence of mixed bed ion exchanger during adsorption of proteins having limited affinity for ion exchange resins may be of general use in increasing the efficiency of such a process.

The inherent limitation of our procedure is that relatively large quantities of CM-cellulose are required restricting the amount of the starting material which can be used. Loss of approx. 25% of the total dismutase activity occurs during the first treatment with CM-cellulose. By contrast, the second treatment of enzyme with CM-cellulose results in complete recovery of enzymatic activity. The loss of activity in the first CM-treatment may be due entirely to the removal of the hemoglobin which was shown to possess a very weak dismutase

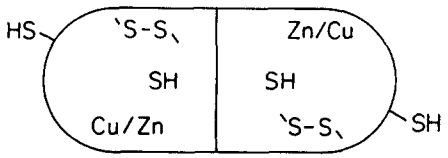

Fig. 5. Schematic representation of the cysteine residues in Zn/Cu superoxide dismutase. The one disulfide appears to be preserved, the internal SH group is present in bovine and other dismutases which have 6 half-cystines per molecule, the external SH groups are present in human dismutase which has 8 half-cystines, (cf. Ref. 15).

activity [31] although certainly a portion of this loss results from entrapment within the bulk of the resin.

Most of the properties reported here are similar but not identical to those previously reported [7—12]. The ultraviolet adsorption spectrum is notable for the absence of absorption bands at 325 nm and 250 nm associated with impurities or variant forms of the enzyme [9,15,32].

The low metal content of all preparations reported here, in the companion paper [15] and elsewhere for the bovine protein [26], are a troublesome feature. We cannot ascribe this result to impurities since such gross contamination with non-dismutase, copper-free protein would be evident in the O'Farrell gel. In addition, the final Biogel P-100 peak exhibited constant specific activity and spectral properties across its 5-fraction width indicating no heterogeneity with respect to molecular weight. The low metal content and the heterogeneity observed on polyacrylamide gel electrophoresis may be related. Given the many combinations of filled and unfilled metal sites and occupied subunits electrophoretic heterogeneity would certainly be observed if the bound metals contribute to the total charge of the protein molecule. Exactly such a situation has been studied for the bovine protein [33] using polyacrylamide gel electrophoresis to estimate the protein bound metal ion distribution.

The visible and EPR spectral properties are essentially identical to that of the bovine protein. However, it is evident that the Cu^{2+} site of the human protein is much more sensitive toward denaturation under mild conditions.

In summary, the previous results and those of the present report suggest that human superoxide dismutase will have a three-dimensional structure very similar to that of the bovine protein [14]. The disposition of the two sulfhydryl groups in each subunit deserves comment. The single sulfhydryl group in the bovine protein (Cys-6) is part of the β-sheet structure and is relatively unreactive [14]. The results of this paper suggest that one of the SH groups of the human protein is more reactive than the other. A schematic model of the cysteine groups is shown in Fig. 5. In the accompanying paper, we show that the 'extra' SH group of the human protein is quite reactive even toward relatively large molecules and is the part of the structure responsible for the 320 nm absorption band observed in many preparations of the human protein.

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