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SULFHYDRYL REACTIVITY OF HUMAN ERYTHROCYTE SUPEROXIDE DISMUTASE

ON THE ORIGIN OF THE UNUSUAL SPECTRAL PROPERTIES OF THE PROTEIN WHEN PREPARED BY A PROCEDURE UTILIZING CHLOROFORM AND ETHANOL FOR THE PRECIPITATION OF HEMOGLOBIN

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Summary

1. During purification of human superoxide dismutase by the McCord-Fridovich procedure (McCord, J.M. and Fridovich, I. (1969) J. Biol. Chem. 244, 6049—6055) the 'extra' sulfhydryl groups react with a variety of sulfur containing compounds including zero-valent sulfur to yield several dismutase fractions containing excess sulfur atoms and having a unique absorption band in the region of 325 nm. This is shown to be artefact of the purification procedure.

2. Cysteine trisulfide and glutathione polysulfide were found to react with native human superoxide dismutase to yield derivatives having no reactive sulfhydryl groups and possessing spectral properties similar to the various fractions obtainable from the above purification procedure. A structure of the type protein-CH_2-S-$(S)_nR$ is proposed to account for the results. The value of n is variable, and the additional sulfur reactive toward thiol reagents is thought to be due to persulfides (R = H). The 325 nm band is probably due to a $n \rightarrow \sigma^*_{ss}$ transition associated with a strained S-S bound.

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Abbreviations: DTNB, 5,5′-dithiobis-(2-nitrobenzoate); GSSG, glutathione disulfide; $GS(S)_nG$, glutathione polysulfide.

Introduction

In the companion paper [1], we present a method for the chromatographic purification of human superoxide dismutase. The protein thus obtained was found to consist of a single type of polypeptide chain, and to contain four half-cysteine groups per polypeptide. It is now well known that human superoxide dismutase prepared by procedures involving more severe treatment [2—4], in particular, the use of chloroform and ethanol to precipitate hemoglobin possesses unusual spectroscopic properties [3] and contains zero-valent sulfur [4]. In this report, we will show that one type of sulfhydryl group of human superoxide dismutase is the site of this perturbation, and arises from its reaction with endogenous sulfur containing compounds under the oxidative conditions which prevail during the chloroform/ethanol treatment.

Materials and Methods

With the exceptions listed below, all methodology was as reported previously [1].

Enzyme was prepared essentially according to the method developed by McCord and Fridovich [2] for purification of bovine erythrocyte superoxide dismutase. After the acetone precipitation step, extensive dialysis was carried out against 2 mM sodium phosphate buffer at pH 6.8 (hereafter, referred to as dilute buffer). The dialyzed protein was then applied to a 2.2 × 30 cm column of DE-52 (Whatman) equilibrated with dilute buffer. After washing the column with 200 ml dilute buffer, gradient elution from 2 to 100 mM buffer, pH 6.8 (Fig. 1) resulted in 2—4 peaks having dismutase activity. After concentration by ultrafiltration in a collodion bag, the activity-containing peaks (cf. Fig. 1) were further purified by Biogel P-100 (Bio-Rad) gel permeation chromatography in 10 mM sodium phosphate buffer (pH 6.8), the column dimensions being 2.8 × 120 cm. Total dismutase activity was recovered with a yield of about 30%. Apo protein was prepared according to the procedure of Fee [5].

Zero-valent sulfur (S^0) was determined by the method of Fletcher and Robson [6]. S^0 was removed from commercial preparations of oxidized glutathione [7] by treatment with cyanide followed by passage through a column of Sephadex G-10 (Pharmacia).

Preparation of glutathione-labelled human peroxide dismutase

([^{3}H]-glycine)-L-glutathione (New England Nuclear) was added to lysate (0.25 mCi in 4.7 l) and incubated 16 h at 4°C. Assay of reduced and oxidized glutathione (GSSG) by the method of Bernt and Bergmeyer [8] revealed the presence of the latter at a glutathione/2 concentration of 1.5 mM for a specific activity of $9.7 \cdot 10^{10}$ dpm/mol. Following incubation, the superoxide dismutase was isolated by the procedure of McCord and Fridovich [2].

Gel electrophoresis of the labeled enzyme was performed as described previously [1] except that operations were at room temperature and the stacking gel was added after 3 h preelectrophoresis. Parallel gels were stained for protein, or for enzymatic activity [9], or were sliced into 5 mm segments, dissolved in H_2O_2, and counted in Aquasol (New England Nuclear) after overnight refrigeration, to avoid chemiluminescence.

Preparation of sulfhydryl-labelled derivatives of human dismutase

Enzyme prepared as previously described [1] was reacted at 25°C with various low molecular weight sulfur compounds. To maximize reactivity of the sulfhydryl groups, derivatization was performed at pH 8.0 (50 mM Tris-HCl) in 1 M guanidine-HCl. Enzyme concentration was 10 μM while the sulfur compounds were present at different levels, generally related to their solubility. After 3 h incubation, samples were dialyzed twice against 500 vols. 50 mM Tris/0.3 mM EDTA (pH 8.0).

Results and Discussion

Three independent preparations were made over a period of two years and were examined in detail. The elution pattern from DEAE-cellulose was not reproducible but generally yielded a major band, designated Fraction a and from 1—3 distinct additional bands designated b—d in order to elution. In two of the preparations the pattern was similar, only a major band, a, and a minor band, b, were observed, and the examined properties of dismutase in these two bands showed no important differences. Thus, in our summary of the properties of the various dismutase fractions (Table I) these are reported as averages.

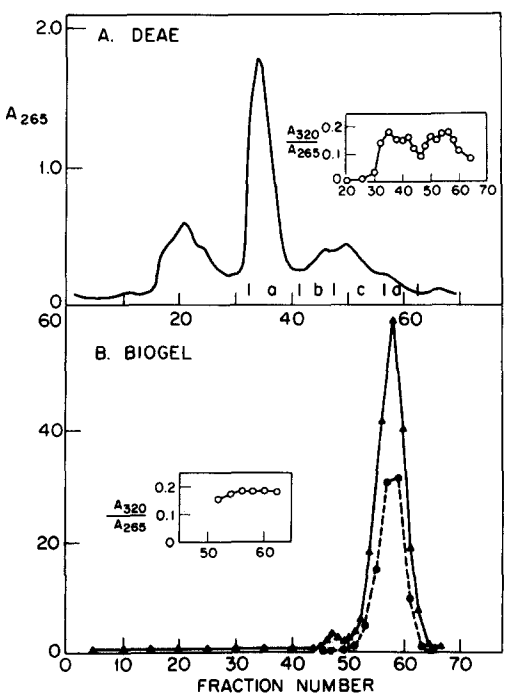


Fig. 1. Chromatographic purification of human superoxide dismutase following initial isolation by the McCord and Fridovich [2] procedure. Panel A. Elution from DE-32. Only fractions between 30 and 63 contained dismutase activity. Tubes 33—41, 42—47, 48—56, and 57—63, designated respectively, Fractions a through d, were pooled and concentrated. The inset depicts the absorbance ratio 320 nm/265 nm acrodd the elution profile. Panel N. Elution of Fraction 1 from above from Biogel P-100. Again the inset shows the 320/265 absorbance ratio over the elution profile. ———, A_{265} X 20; and - - - - - -, activity in units/μl.

TABLE I

PROPERTIES OF THE VARIOUS DISMUTASE FRACTIONS OBTAINED FROM ION EXCHANGE CHROMATOGRAPHY AFTER PARTIAL PURIFICATION BY THE PROCEDURE OF McCORD AND FRIDOVICH [2]

Fraction [a,b]	A	B	C	D	E
Copper content	1.8 ± 0.1 [c]			1.9	1.7
Zinc content	1.7 ± 0.1 [c]			1.7	1.6
Molar absorbance X 10^{-3}					
265	29.0 ± 1.4	24.3	17.0	15.2	15.9
325	8.3 ± 0.1	3.3	2.3	1.1	1.1
675	0.32 ± 0.02	0.28	0.32	0.19	0.26
Molar ellipticity X 10^3					
E_{605}	1.24 ± 0.1 [c]			1.05	0.93
Specific activity in units/ μmol [c]	120 000 ± 18 000 [c]			110 000	120 000
Sulfhydryl content					
DTNB PO_4 buffer	0.1 ± 0.01		0.34	0.8	0.7
6 M Gdn-HCl	1.7 ± 0.4	2.8	2.0	2.0	3.1
PMA [g] PO_4 buffer	0.34 [4]	1.0	0.68	1.3	3.2
6 M Gdn-HCl	3.2 [4]	4.4	3.6	3.1	2.2
Disulfide content					
SO_3/PMA [g] PO_4 buffer	0.6 ± 0	0.78	1.0	0.23	0.1
SO_3/PMA [g] 6 M Gdn-HCl	3.2 [d]	2.0	2.5	2.5	1.8
Total sulfur [e]	9.6	8.4	8.6	8.1	6.8
Total sulfur [f]	8.1	6.8	7.0	7.0	6.7

[a] Column A corresponds to the average values of a particular measurement on fractions a and b of the first two preparations as described in the text. Columns B and C give single values for measurements made on fractions a and c of the third preparation, respectively. Column D gives values obtained from material described in Column A but after treatment with 0.5 M 2-mercaptoethanol for several minutes followed by dialysis. Values in Column E were taken from Ref. 1 and represent those of the native protein.

[b] All values are based on a molecular weight of 32 000.

[c] All fractions, four determinations.

[d] Single measurement.

[e] Reactive toward phenylmercuric acetate in 6 M Gdn-HCl.

[f] Reactive toward DTNB as SH in 6 M Gdn-HCl and toward phenylmercuric acetate after sulfite treatment in 6 M Gdn-HCl.

[g] Phenylmercuric acetate.

In the third preparation, the DEAE-cellulose elution pattern shown in Fig. 1 was obtained, and yet another non-reproducible feature of this procedure was revealed; the examined properties of Fraction a differed from those of the two previous preparations, and these are reported separately in Table I. Only Fractions a and c of the third preparation were examined in detail. Also included in Table I are the relevant data obtained using human dismutase obtained by the chromatographic procedure [1]. While there are several features of the data in Table I which deserve comment and will be discussed later in the paper, the most important aspect is the increased amount of sulfur in the various fractions, for example, the total phenylmercuric acetate-reactive sulfur was increased by as much as 3 in one of the fractions. A second feature is the increased absorbance of these preparations at 325 and 265 nm.

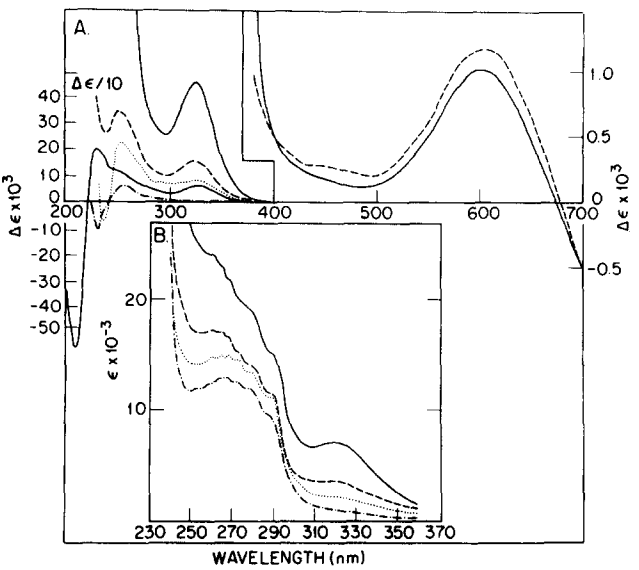


Fig. 2. Circular dichroism (A) and optical absorption (B) spectra of various fractions of human superoxide dismutase. Spectra in the visible region were obtained using approx. 1.5 mM protein; in the ultraviolet region, 15 μM; in the far ultraviolet, 1.5 μM. All solutions were pH 7.8, 2 mM sodium phosphate buffer. ———, sample described in column A, Table I; - - - - - -, sample described in column B, Table I; . . . . . ., column C, Table I; and — · —, sampleof column A, Table I treated with mercaptoethanol.

The optical and circular dichroic spectra of various dismutase fractions are presented in Fig. 2. There is a rough positive correlation of excess sulfur and 325 nm and 265 nm absorbance. The sample containing the largest amount of excess sulfur has a very prominent peak at 325 nm in the optical absorption and circular dichroism spectrum. Treatment with mercaptoethanol returned the optical properties close to those characteristic of the protein prepared by the chromatographic procedure. Again, it should be emphasized that the exact spectral properties are not quantitatively reproducible from one preparation to another.

Several types of treatment were found to abolish most differences between dismutase prepared by the McCord-Fridovich method and the chromatographic technique. The procedures attempted included those which might remove or reduce the copper, substances which would react with sulfhydryl or disulfide groups, those which would denature the protein, and those which would remove putative impurities. In agreement with the findings of Calabrese et al. [4], we observed that removal of the copper had no effect on the 325 nm band. Also in accord with their results, the 325 nm band was abolished by treatment with certain reducing agents or thiophiles. Sulfite, dithionite, cyanide, and 2-mercaptoethanol were effective but hydrogen peroxide and ascorbate were not, although the latter agents reduce the copper. As reported by the above group [4], concentrated guanidine-HCl (3—8 M) irreversibly denatured the protein and abolished the 325 nm band. Treatment of the protein with CM-cellulose, a step included in our chromatographic purification [1] failed to alter the 325 nm band.

The EPR spectrum of the Cu^{2+} was not greatly different in any of the frac-

TABLE II

ASSOCIATION OF ^{3}H-LABELED GLUTATHIONE AND ZERO-VALENT SULFUR WITH HUMAN SUPEROXIDE DISMUTASE FURING PURIFICATION BY THE METHOD OF McCORD AND PRIDOVICH [2]

Fractions 3—5 were retrieved from the precipitate which formed during extended dialysis of the precipitate obtained by cold acetone treatment by treating the precipitate with 0.5 M potassium phosphate buffer and chromatography on DE-32. The reason for the precipitation is not clear, but sample 4 appeared to be typical superoxide dismutase once removed from the precipitate.

Sample	Protein mg/ml	Dismutase activity	325 nm band	dpm/mg protein	GS/mol protein	S^0mol protein
1	19	+	+	560	0.2	0.03
2	3	+	+	590	0.2	—
3	20	—	+	60	0	—
4	30	+	—	910	0.3	0.4
5	116	+	(yellow)	800	0.3	0.01

tions or was it affected by the various treatments provided the excess of reagent was removed.

From Table I it can be seen that mercaptoethanol treatment substantially reduced the total sulfur in the protein bringing this to a level comparable with chromatographically prepared protein. Nevertheless approximately one additional phenylmercuric acetate-reactive sulfhydryl group remained, but not the reactivity toward 5,5'-dithiobis-(2-nitrobenzoate) which was lower and similar to that of the native protein. This apparent discrepancy is not understood.

Investigation of the reaction of dismutase with glutathione during the purification procedure

Because the above observations indicated that mixed disulfide and/or trisulfide formation may be responsible for the properties of the modified dismutase, the possibility of reaction with glutathione during isolation was examined.

Table II presents the results of an experiment in which the incorporation of labeled glutathione and zero-valent sulfur into the dismutase during isolation was measured. Small but significant amounts of glutathione are bound as well as S^0, but there is a lack of correlation between S^0 content and the other parameters. Table III depicts the ability of 2-mercaptoethanol to render the bound radioactive glutathione dialyzable. Although recoveries of radioactivity were low in the absence of mercaptoethanol the non-dialyzable component of radioactivity was at least four times greater than in the presence of mercaptoethanol. The one exception to this, in which mercaptoethanol failed to render radioactivity dialyzable, was sample 3. This component also lacked dismutase activity and exhibited far lower glutathione binding than the other samples.

The association of the glutathione with dismutase was further indicated by correlation of radioactivity with dismutase activity [9] during gel electrophoresis (Fig. 3).

Synthesis of sulfhydryl-linked derivatives of human superoxide dismutase

Table IV and Fig. 4 illustrate the results of experiments in which various sulfhydryl-linked derivatives of the protein were formed by treatment of native

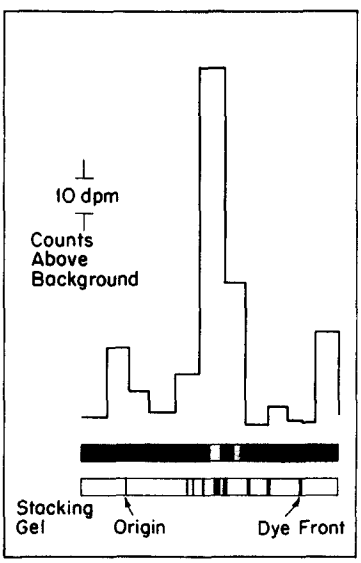
TABLE III

RELEASE OF BOUND GLUTATHIONE FROM HUMAN SUPEROXIDE DISMUTASE

The experiment was conducted as follows: a small volume of radioactivity labeled protein was placed inside 1 cm diameter dialysis tubing. This was tied off and placed in a tube containing 5 mM potassium phosphate buffer with or without 0.1 mM 2-mercaptoethanol. The total volume was 1.5 ml. The tube was sealed with Parafilm® and followed to stand at room temperature with occasional shaking for 1.5 h. After this time precise volumes were withdrawn from the dialysis bags, transferred to the counting vials and the volume of water in the vials brought to 1.0 ml with glass distilled water. Similarly, 1.0 ml of the external solutions was transferred to counting vials. ^3H was then determined by standard scintillation counting procedures.

Sample *	2-mercapto-ethanol	dpm inside dialysis bag	dpm outside dialysis bag	dpm added	% inside	% recovered
1	—	365	8	993	37	38
	+	94	851		10	95
3	—	868	616	2124	41	70
	+	776	1326		37	99
4	—	1216	41	1926	63	65
	+	132	1577		7	89
5	—	1313	30	1975	66	68
		378	1573		19	99

* Same as in Table II.



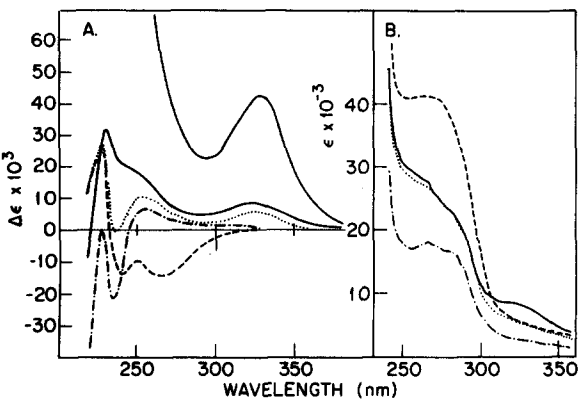


Fig. 3. Comparison of Coomassie blue stain, activity and radioactivity of acrylamide gels following electrophoresis of modified human superoxide dismutase labeled with ([^{3}H]glutathione. 100 μg peak 2 of the soluble fraction (see Table II) was run on duplicate gels for Coomassie blue staining. These were then sliced and counted. 10 μg protein run in parallel was stained for dismutase activity [9].

Fig. 4. Circular dichroic (A) and optical absorption (B) spectra of native human superoxide dismutase treated with various sulfur containing compounds. The concentration of protein was 10 μM and samples were prepared as described in Table IV. - - - - - -, GSSG; . . . . . , GS(S)$_n$SG; — · —, cystine; ———, cystine trisulfide. (The lower solid line was recorded at 1/5 gain.)

TABLE IV

EFFECT OF VARIOUS SULFUR COMPOUNDS ON THE SPECIFIC ACTIVITY AND REACTIVE SULFHYDRYL CONTENT OF NATIVE HUMAN SUPEROXIDE DISMUTASE

The sample volume was 1.2 ml and was 9 μM dismutase, 0.3 mM EDTA, 50 mM Tris-HCl pH 8.0, 1 M Gdn-HCl, and ± reagent indicated. After incubation for 3 h at 25°C the samples were dialyzed (2X) vs. 100 ml buffer of EDTA solution before SH and activity determinations. SH analyses were done in the presence of 1 M Gdn-HCl.

System	Specific activity * $X10^{-3}$	SH/mol protein
Buffer alone	110	1.3
Buffer and 1 M Gdn-HCl	94	1.0
1 mM dithiothreitol	82	1.1
0.46 mM cysteine	82	0
10 mM GSSG	76	0
0.46 mM cysteine trisulfide	82	0
Approx. 0.1 mM glutathione polysulfide	70	0

* Per μmol protein.

dismutase with sulfur containing compounds. Dithiothreitol had little effect on the optical and chiroptical properties of native dismutase. In the case of reaction with cystine, 5,5′-dithiobis-(2-nitrobenzoate)-reactive sulfhydryls disappeared but no alteration of the ultraviolet optical or circular dichroism spectrum was observed.

Glutathione freed of S^0 [7], by contrast, reacts with human dismutase to produce profound changes in both optical and chiroptical properties of the near ultraviolet region. A hyperchromicity reflected by a doubling of the intensity of the full range of ultraviolet absorbance (Fig. 5b) accompanies disappearance of 5,5′-dithiobis-(2-nitrobenzoate)-reactive sulfhydryl groups (Table IV). The circular dichroism spectrum (Fig. 5a) is essentially the summation of the spectra of glutathione-disulfide (10) and dismutase. The difference in ellipticity at 260 nm between control (protein) and glutathione-protein complex ($66 \cdot 10^2$ deg · mol^{-1} protein · cm^{-2}) is consistent with incorporation of 2 glutathione per molecule of protein. Cysteine trisulfide, when reacted with human dismutase, reproduced (Fig. 5) the optical and chiroptical properties of certain of the protein preparations (Fig. 2). This derivative contained 1.9 S^0 per mol protein, and while cysteine content was not measured, the formation of two trisulfide linkages offers a reasonable explanation of the data. The E_{325} for this derivative per S^0 was about 4000, a value much higher than that reported by Calabrese et al. [4].

Glutathione polysulfide (prepared by reacting oxidized glutathione with S_nBr_n [11]) reacted with human dismutase to produce ultraviolet absorption and circular dichroism spectra similar to those of modified dismutase forms (Fig. 2). None of these derivatives resulted in significant alteration of dismutase activity from the control level (Table IV).

While it is clear that native human protein reacts with polysulfides to give products having optical properties similar to those of certain fractions of dismutase, the protein product thus obtained still differs in its sulfur content and disposition from those isolated by the McCord-Fridovich procedure. In particular,

a mixed polysulfide structure cannot explain the large amount of 5,5′-dithiobis-(2-nitrobenzoate)- and phenylmercuric acetate-reactive sulfur unless some true persulfide, S-SH, is also present.

The reaction between native dismutase and the polysulfide compounds appears to be very favorable. Thus, disulfide and trisulfide compounds could serve to attach reporter groups such as spin labels and fluorophores to the protein.

Conclusions

The 325 nm absorption band found in certain preparations of human superoxide dismutase is an artefact resulting from the reaction of a variety of sulfur containing substances with the partially exposed sulfhydryl group of this protein. A structure of the type $P\text{-}CH_2\text{-}S\text{-}(S)_n\text{-}R$ is proposed where $n \geqslant 0$ and R can be a hydrogen atom but otherwise varies according to the concentration and reactivity of substances present in the medium. It is further suggested that the quantitative variation of the absorbance from one preparation and fraction to another reflects the distribution of possible values of n and the nature of R. Similar non-specific reactions have been observed by others [12].

The absorption at 325 is proposed to arise from a type $n \rightarrow \sigma^*$ type transition in a dihedrally strained polysulfide or disulfide as has been extensively documented in the chemical literature [13—20].

There are a number of reasons to suspect that this sulfhydryl group and its reactivity toward sulfur containing compounds is not related to any physiological function of this protein: (a) bovine [21] and equine [22] superoxide dismutases possess only one SH per subunit and it is deeply buried within the three-dimenisonal structure [23]. By contrast, the Zn/Cu protein from turkey liver [24] has 14 half-cysteines per mole. At least two of these are sufficiently exposed that protein polymerization occurs in the presence of oxygen. The isozyme II from wheat germ [25] has only four half-cysteine per molecule, and we conjecture that these exist as two disulfides leaving no free SH groups. (b) When care is taken to avoid oxidizing conditions such as those which prevail during the chloroform/ethanol prepcipitation of hemoglobin, the human protein can be isolated without any mixed disulfide formation at the 'extra' sulfhydryl [1]. Hartz [26] and Carrico and Deutsch [27], who were the first to report the presence of the 325 nm band in this protein, found that the development of the band did not require the extreme conditions of chloroform/ethanol treatment for its development. Thus, human cerebrocuprein was found to have this band when isolated by a chromatographic procedure, whereas hepatocuprein similarly isolated did not have the band. Both proteins are, of course, one and the same [27]. (c) Formation of a mixed disulfide group has no effect on the metal binding site of the protein (ref. 4 and this work). It is thus proposed that this SH is rather far from the metal binding region of the molecule.

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