Bioenergetics (1973) 4, 579-590

Sulfhydryl Groups of Cytochrome Oxidase*

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Received 9 January 1973

Abstract

1. The number of *p*-chloromercuribenzoate (PCMB)-reactive sulfhydryl groups per molecule was estimated to be 2 in cytochrome oxidase based on a minimum molecular weight of 69,300 daltons [12] or about 3.2 based on a minimum molecular weight of 110,000 [10].
2. The number of SH groups titrated in cytochrome oxidase with PCMB or with $AgNO_3$ was the same in the absence of sodium dodecyl sulfate (SDS), while in the presence of SDS additional SH groups became available to $AgNO_3$, but not to PCMB.
3. Removal of most of the intrinsic copper of cytochrome oxidase with $\geqslant$0.01 M NaCN (followed by passage through Sephadex G-25 to remove the copper cyanide and the excess cyanide) exposed approximately 1.5 or 2.4 additional SH groups per molecule to PCMB depending on the minimum molecular weight of the enzyme.
4. A determination of the heme of cytochrome oxidase by formation of the pyridine hemochrome revealed the same heme content as was obtained with $\Delta\epsilon$ 605 nm (reduced minus oxidized) = 7.6×10^3 cm^{-1} (g atom $Fe)^{-1}$.
5. Repeated freezing and thawing or freeze-drying was found to be detrimental to this preparation of cytochrome oxidase. However, the SH groups decreased or became unavailable to PCMB approximately in direct proportion to the decrease in the heme groups.

Our interest in the sulfhydryl groups of cytochrome oxidase as sites for the attachment of copper arose in 1961 when we studied the effect of *p*-chloromercuribenzoate on the oxidation of ferrocytochrome *c* by a

* This work was supported by a grant (AM 9762) from the United States Public Health Service and the Bureau of Biological Research.

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partially purified soluble cytochrome oxidase in air [1]. Preincubation of the cytochrome oxidase for 10 min with PCMB† caused inhibition which ranged from 15% at 1×10^{-10} M PCMB to 90% at 1×10^{-3} M. Earlier studies of the effects of PCMB and other mercurials on cytochrome oxidase were done by Slater [2], Cook and Perisutti [3], Kreke *et al.* [4], and Seibert *et al.* [5]. From these studies it became apparent that mercurials were inhibitory, and that although cysteine and other SH-containing compounds were protective, they could not reverse the inhibition. It was suggested by Barron and Singer [6] that the inhibition could probably not be explained simply by mercaptide formation.

A determination of the amino acid composition of cytochrome oxidase by Matsubara *et al.* [7] showed that seven moles of cysteic acid were obtained from one mole of cytochrome oxidase of molecular weight 93,000 after performic acid oxidation followed by acid hydrolysis. The low sulfhydryl group content prompted Orii *et al.* [8] to investigate the state of these SH groups and their relation to the activity of cytochrome oxidase. Four SH groups were found to react with $AgNO_3$ in the absence of sodium dodecyl sulfate and seven in its presence. Somewhat less than two of the SH groups reacted with PCMB (as determined with $AgNO_3$ after PCMB) when the molecule had not been opened with SDS, and the number was only increased to three in the presence of 0.5% SDS unless a large excess of PCMB was added, in which instance six of the seven SH groups appeared to react with PCMB. In these studies 1.8×10^{-3} M PCMB caused only 29% inhibition of activity, whereas $AgNO_3$ and $HgCl_2$ were much more inhibitory at about the same concentration. Tsudzuki *et al.* [9] then removed 92% of the total intrinsic copper by dialyzing against 0.01 M cyanide in phosphate buffer after treatment with 1% sodium dodecyl sulfate. The concentration of SDS (0.2 to 0.3%) which exposed the copper so that it could react with bathocuproine sulfonate was also found to be the concentration which permitted the seventh SH group to be titrated with PCMB. In their experiments it was reported that six of the seven SH groups would react with high concentrations of PCMB in the absence of SDS. The notable lack of inhibition of cytochrome oxidase activity by 1×10^{-5} M PCMB became marked in the presence of 0.18% SDS. Whereas Tsudzuki *et al.* interpreted their results as evidence for the binding of copper to SH groups, both of which were assumed to be liberated at the same concentration of SDS, the alternate explanation is that SDS simply unfolded the enzyme so that the separate copper and seventh SH group could now react with their respective agents. The increase in the SH content of Cu-free cytochrome oxidase was not determined and the purity of the preparations was not recorded.

† Abbreviations: PCMB, *p*-chloromercuribenzoate; SDS, sodium dodecyl sulfate; BCS, bathocuproine sulfonate.

Our preparation has 5 cysteine residues per heme and a minimum molecular weight of 85,000 based on an amino acid analysis [10]. Kirschbaum and Wainio [11] reported in 1966 that nitroprusside revealed 2.1 SH groups per 72,000 g of protein before treatment with EDTA and 3.8 SH groups after treatment.

Based on the assumption that the lowest minimum molecular weights reported in the literature for cytochrome oxidase, namely 69,300 [12] and 65,800 [13], are the more nearly correct ones, the number of PCMB-titrable SH groups per heme in cytochrome oxidase monomer was estimated to be about 2 in the present study. If the minimum molecular weight were 110,000 [10], the number of PCMB-titrable groups per monomer would be about 3.2. However, most of these preparations were determined to have a purity of 0.6 to 0.7 based on the heme content and to contain from 3 to 4 PCMB-titrable SH groups per heme based on a molecular weight of 69,300. Removal of most of the bathocuproine sulfonate-nonreactive copper (the intrinsic copper) with cyanide in the absence of SDS, exposed approximately 1.5 or 2.4 additional SH groups per molecule to PCMB depending on the minimum molecular weight of the enzyme.

Methods

Cytochrome oxidase was prepared from beef heart mitochondria by the method of Wainio [12]. Protein (6.25 times N) was determined by the micro-Kjeldahl method and was converted to molar concentration of oxidase with a molecular weight of 69,300 daltons [12] or 110,000 daltons [10].

Heme: protein ratios were calculated either by applying the Δ molar absorptivity for the enzyme at 605 nm or by preparing the pyridine hemochrome of the prosthetic group. The Δ absorbance at 605 nm was determined by connecting the isosbestic points at 575 nm and 622.5 nm on the curves of both the oxidized and reduced forms with a straight line and by taking the height of each curve above the straight line at 605 nm. The difference, i.e. the height of the reduced curve above its straight line minus the height of the oxidized curve above its straight line, was the absorbance from which the concentration of the heme was calculated with the aid of $\Delta\epsilon_{605\,nm} = 0.076 \times 10^5\ cm^{-1}$ (g atom Fe)$^{-1}$ as originally determined by reduction of the oxidase under anaerobic conditions with ferrocytochrome *c* [14]. The hemochrome of the prosthetic group was extracted from an alkaline solution of the enzyme with pyridine as detailed by Morrison *et al.* [15]. One ml of 0.01 N NaOH, 1.0 ml of pyridine (spectrographic grade), and 0.5 ml of distilled water were added to 0.5 ml of enzyme or to an equivalent amount of freeze-dried enzyme (0.5 ml equivalent of solids) after it had been

redissolved in 0.5 ml of distilled water. The molar absorptivity at 587 nm = 26×10^3 $cm^{-1} mole^{-1}$ was used to calculate the concentration of the pyridine hemochrome in the clear solution.

The sources of the reagents were: *p*-chloromercuribenzoate, J. T. Baker Chem. Co., Phillipsburg, N.J.; NaCN and $AgNO_3$, Merck and Co., Rahway, N.J.; pyridine Fisher Scientific Co., Fair Lawn, N.J.; sodium deoxycholate, Difco Laboratories, Detroit, Mich.; sodium bathocuproine sulfonate, G. F. Smith Chem. Co., Columbus Ohio.

Total copper in cytochrome oxidase was determined by atomic absorption spectroscopy employing the Perkin-Elmer model 303 spectrometer. The copper reference was obtained from Fisher Scientific Co., Fair Lawn, N.J. Bathocuproine sulfonate-reactive copper was determined by the method of Zak [16] following reduction of the copper with dithionite. BCS-nonreactive copper was determined by difference, i.e. total copper minus BCS-reactive copper.

The activity of cytochrome oxidase was assayed spectrophotometrically at pH 6.0 in 0.1 M phosphate buffer (0.112 M cation) by following the rate of oxidation of ferrocytochrome *c* at 550 nm in air as described by Wainio *et al.* [17]. Horse heart cytochrome *c* of 97% purity (Type VI, Sigma Chem. Co., St. Louis, Mo.) was used.

Deoxycholate in the final preparation of cytochrome oxidase was determined by the method of Szalkowski and Mader [18] as adapted by Kremzner [19]. The sodium deoxycholate standard was purified by precipitation from hot ethanol.

The amperometric titration of SH groups with $AgNO_3$ was accomplished with the aid of a rotating Pt electrode. The reference electrode was Hg-HgO-saturated $Ba(OH)_2$ which has a potential of –0.10 V when measured against the saturated calomel electrode [20]. Cysteine was the standard in the presence of 0.001 M EDTA and under N_2. For 0.2, 0.3 and 0.4 μmole of cysteine, the number of μmole of 0.001 M $AgNO_3$ were 0.24, 0.35 and 0.475, respectively. Thus, an average factor of 0.84 was applied to the titration of the protein with $AgNO_3$ in the presence of EDTA and N_2 when the number of μmole of SH groups taken for analysis was between 0.2 and 0.4.

Sulfhydryl groups were also determined with PCMB according to the method of Boyer [21]. The concentration of the PCMB solution was 1×10^{-4} M and its concentration was determined by employing the molar absorptivity at 232 nm of 1.69×10^4. The titrant was added in 0.1 ml increments to a known amount of enzyme in a volume of 3 ml. The increments of absorbance at 255 nm due to mercaptide formation were determined with a Beckman DU spectrophotometer. Absorbance changes at 255 nm due to equal increments of the PCMB solution added to a 3 ml volume of water alone were recorded in another experiment. The latter were subtracted from the values determined in the presence of the enzyme. A plot of Δ absorbance at 255 nm *vs.* equivalents of PCMB

added yielded a curve which plateaued when the SH groups had been titrated [22].

The effect on the number of SH groups of attempting to remove the copper as a cyanide complex was studied by three different methods:

1. *The column method.* Two ml of cytochrome oxidase (2.3×10^{-5} M) were incubated for 1 h with 0.1 ml of NaCN of the appropriate concentration to give a final concentration of from 1×10^{-3} to 1×10^{-1} M. A column of Sephadex G-25 (1×70 cm) was then prepared. It was washed with approximately 500 ml of 1×10^{-3} M EDTA and was equilibrated with 400 ml of 0.1 M phosphate buffer, pH 8.0, containing 5 mg sodium deoxycholate per ml. The absorbance of the effluent at 255 nm was zero at the end of the equilibration period. The sample was placed on the column which was then developed with approximately 80 to 90 ml of 0.1 M phosphate buffer, pH 8.0, containing 5 mg deoxycholate per ml. The absorbance by the oxidized cytochrome oxidase as it was eluted was determined at 418 nm. Six ml of the concentrated effluent were collected when the absorbance became intense. Analyses of SH groups, heme, copper and activity were made on this 6 ml sample.

2. *The dialysis cell method.* Plastic dialysis cells of 5 ml capacity on each side were obtained from The Chemical Rubber Co. Cleveland, Ohio. The dialysis membrane was seamless regenerated cellulose dialysis tubing purchased from Oxford Laboratories, San Mateo, Calif. It was boiled five times with ion-free water, and soaked in five changes of 1×10^{-3} M EDTA for a total of 48 h. One ml of enzyme (4.6×10^{-5} M) + 1.2 ml of 0.1 M phosphate buffer, pH 8.0, + 2.2 ml of 2×10^{-3} or 2×10^{-1} M NaCN in 0.1 M phosphate buffer, pH 8.0, were placed on one side of the assembled cell. The other side received 4.4 ml of 0.1 M phosphate buffer, pH 8.0, which was either 1×10^{-3} or 1×10^{-1} M with respect to NaCN and contained 9.2 mg of sodium deoxycholate per ml (the same amount of deoxycholate as in the enzyme). Dialysis was continued for 8 h with the cell held on a slowly turning sheel (6 rpm) with two changes of the 4.4 ml of the buffer-NaCN-deoxycholate solution. The enzyme solution was then placed on a Sephadex G-25 column as described above and it was eluted in the same way.

3. *The dialysis sac method.* Seamless regenerated cellulose dialysis tubing (28 mm width-flat) was prepared as outlined above. One ml of enzyme + 1.2 ml of 0.1 M phosphate buffer, pH 8.0, + 2.2 ml of 2×10^{-2} M NaCN in 0.1 M phosphate buffer, pH 8.0, were placed in the dialysis sac. The filled sac was placed in a 1-liter Erlenmeyer flask containing 1 liter of 0.1 M phosphate buffer, pH 8.0, which was 1×10^{-2} M with respect to NaCN and contained 9.2 mg of sodium deoxycholate per ml. The Erlenmeyer flask was shaken on a shaking

machine for 12 h with one change of the buffer-NaCN-deoxycholate solution at 6 h. At the end of 12 h the buffer-NaCN-deoxycholate was replaced with 0.1 M phosphate buffer, pH 8.0, containing 9.2 mg of sodium deoxycholate per ml. Shaking was continued for an additional 36 h with two changes of the buffer-deoxycholate solution. The enzyme was not filtered through a Sephadex G-25 column.

Results

The use of absolute molar absorptivity values to calculate the concentration of cytochrome oxidase in solution has been unsatisfactory in our experience. The presence of varying amounts of contaminating substances, presumably lipids, often contributed to the nonspecific absorbance. It is for this reason that we originally calculated equivalents of cytochrome oxidase from the Δ absorbance at 605 nm [14]. The value that we determined for $\Delta\epsilon_{605\ nm}$ by reduction with ferrocytochrome *c* under anaerobic conditions was $7.6 \times 10^3\ cm^{-1}$ (g atom $Fe)^{-1}$. In order to satisfy ourselves that this was a true measure of the heme content, we compared this method with that of preparing the pyridine hemochrome of the prosthetic group and employing $\Delta\epsilon$ at 587 nm. The results of the two methods when applied to several preparations are presented in Table I. The averages for the two methods

TABLE I. A comparison of the concentration of the heme of cytochrome oxidase as calculated from the Δ molar absorptivity of the enzyme at 605 nm and from the molar absorptivity of the pyridine hemochrome of the prosthetic group at 587 nm.

Preparation no.	State of the preparation	Concentration of the heme	
		Per the enzyme at 605 nm[a]	Per the pyridine hemochrome at 587 nm[b]
		$\times 10^{-5}$ M	$\times 10^{-5}$ M
21-2	Freeze-dried	1.09	1.29
VII-1	Freeze-dried	2.71	2.52
	Freeze-dried	1.43	1.52
30-2	Freeze-dried	3.00	3.38
31-1	Unfrozen	2.21	2.24
32-1	Unfrozen	2.52	2.45
32-1	1-time frozen	2.44[c]	2.31[c]
32-1	Freeze-dried	2.41[c]	2.35[c]
32-1	4-times frozen	1.66[c]	1.86[c]
Average		2.16	2.23

[a] $\Delta\epsilon_{605\ nm} = 7.6 \times 10^3\ cm^2$ (g atom $Fe)^{-1}$.
[b] $\Delta\epsilon_{587\ nm} = 26 \times 10^3\ cm^2\ mole^{-1}$.
[c] Excluded from the average.

are in the ratio 1 : 1.03. We conclude that the use of $\Delta\epsilon$ at 605 nm is an accurate method for determining the heme content of cytochrome oxidase.

Amperometric titration with Ag^+ revealed that one preparation of cytochrome oxidase (freeze-dried, heme: protein = 0.55) after preincubation under N_2 for 15 min contained 5.6 moles SH per mole of heme before treatment with SDS and 8.5 moles SH per mole of heme after treatment with 0.5% SDS based on a molecular weight of 69,300.

Sulfhydryl groups were then determined in the same preparation with PCMB. The average of 10 determinations yielded a value of 5.8 moles SH per mole of heme based on a molecular weight of 69,300. Thus, the number of sulfhydryl groups accessible to Ag^+ and to PCMB in the absence of SDS was the same. However, the addition of 0.5% SDS did not expose additional SH groups to PCMB, while the addition of 4 M urea increased the number of PCMB-titrable SH groups by 100 to 200%.

It was decided to continue the determination of SH groups with PCMB rather than with $AgNO_3$ because the intended use of cyanide to remove the copper of cytochrome oxidase precluded titration with Ag^+. When the reaction of HCN with Ag^+ was followed by amperometric titration, it was apparent why this method is a quantitative one for determining μmolar amounts of cyanide. The stoichiometry of the reaction appeared to be 1:1.

A study was made of the effects of repeated freezing and thawing and of freeze-drying on the heme concentration and the SH group : heme ratio of several preparations of cytochrome oxidase. The results obtained with two preparations based on a molecular weight of 69,300 are presented in Table II. Freeze-drying decreased the heme content by different amounts. With some preparations the decrease was 20% or more, while with other preparations the decrease was only 4%. Repeated freezing and thawing was much more detrimental. However, the surprising finding was that the PCMB-titrable SH groups disappeared or became unavailable in the same proportion as did the heme groups. The SH : heme ratios remained essentially unchanged. This convinced us that we could combine all values for SH: heme ratios into a single figure and relate them to their respective heme : protein ratios.

An extensive study of the sulfhydryl group content of old and new preparations of cytochrome oxidase with PCMB (Figure 1) revealed that the relationship between the heme : protein molar ratio on the ordinate and the SH : heme molar ratio on the abscissa as determined at the same time is not linear. The number of PCMB-titrable SH groups per heme based on a molecular weight of 69,300 decreases to a value of 2 as the purity of the enzyme, i.e. the heme : protein molar ratio, increases to the limiting value of 1.0. If the minimum molecular weight were 110,000 [10], the number of PCMB-titrable SH groups per molecule would be about 3.2. Orii *et al.* [8] found for several of their preparations that the

TABLE II. The effect of repeated freezing and of freeze-drying on the heme concentration and the sulfhydryl group to heme ratio of cytochrome oxidase.

Preparation no.	State of the preparation	Heme concentration[a]	SH: heme ratio[b]	Activity
		$\times 10^{-8}$ M		sec^{-1} (mole heme)$^{-1}$ (3 ml)$^{-1}$
28-1	Unfrozen	4.33		
	1-time frozen	3.44	3.9	
	2-times frozen	2.96	3.8	
	3-times frozen	2.29	4.0	
	4-times frozen	1.93	3.5	
	Freeze-dried	3.43	4.1	
32-1	Unfrozen	2.52	4.1	3.73
	1-time frozen	2.44	3.7	2.81
	4-times frozen	1.66	4.0	2.67
	Freeze-dried	2.41	3.5	3.14

[a] Calculated from $\Delta\epsilon_{605\ nm} = 7.6 \times 10^3$ cm^2 (g atom Fe)$^{-1}$.
[b] Based on a molecular weight of 69,300

number of SH groups per molecule of molecular weight 93,000 decreased from 6.7 to 4.1 as the ratio, absorbance at 280 nm : absorbance at 421 nm for the oxidized enzyme as an index of increasing purity, decreased from 3.06 to 2.61. In the absence of direct data, it must be assumed that their purest preparations had heme : protein molar ratios which were no higher than 0.72 [23, 24] based on a molecular weight of 69,300. From our Figure 1 it would be estimated that a preparation with 4.1 SH groups per heme would have a heme : protein molar ratio of 0.6.

The effects of the attempted removal of the copper of cytochrome oxidase treated with NaCN was studied by the three procedures detailed under Methods: the column, the dialysis cell and the dialysis sac. In the column method the NaCN was removed by filtration through Sephadex G-25 following simple incubation of the enzyme with 1×10^{-3}, 5×10^{-3}, 1×10^{-2} or 1×10^{-1} M NaCN for 1 h. The results are presented in Table III. Only at 1×10^{-2} and 1×10^{-1} M NaCN were one or more additional PCMB-titrable SH groups exposed per heme. At the same two concentrations of NaCN all of the bathocuproine sulfonate (BCS)-reactive copper and most of the BCS-unreactive copper (the enzyme copper) was removed. It is surprising that the activity was not lowered in direct proportion to the amount of copper removed. For example 1×10^{-1} M NaCN removed 86% of the BCS-nonreactive copper but lowered the activity by only 18%. The total copper, i.e. the BCS-nonreactive copper that remained, was in a 0.21 : 1 ratio to the heme. 1×10^{-1} M NaCN decreased the BCS-nonreactive copper by 83%

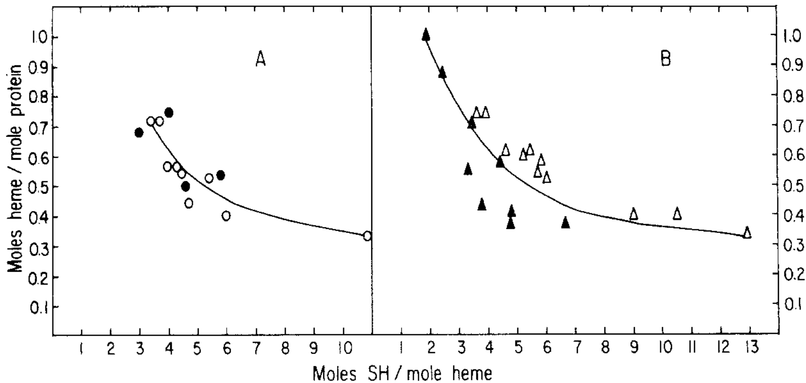

Figure 1. The number of *p*-chloromercuribenzoate-titrable SH groups in preparations of cytochrome oxidase of varying purity. The heme : protein ratio of 1.0 which may represent the pure enzyme is based on a molecular weight of 69,300 daltons. In A are presented the results obtained with fresh (●) and one-time frozen (○) preparations, and in B are presented the results obtained with new freeze-dried preparations (▲) and with old, pre-1968 freeze-dried preparations (△) all stored at -30°.

and lowered the activity by 56%. Neither the dialysis cell method nor the dialysis sac method exposed additional SH groups, whereas the former method did not remove much of the BCS-reactive or of the BCS-nonreactive copper even though the sample was finally filtered through Sephadex G-25.

Discussion

In this study the value of 2 for PCMB-titrable SH groups per heme as the purity of the enzyme, i.e. the heme : protein ratio based on a minimum molecular weight of 69,300 daltons, increased to a limiting value of 1.0, would appear to support Orii *et al.* [8], who found that PCMB titrated less than two SH-groups in the absence of sodium dodecyl sulfate and $AgNO_3$ titrated more. However, in our experiments, PCMB and $AgNO_3$ titrated the same number of SH groups in the absence of SDS, at least when the number was 5.6-5.8 based on a molecular weight of 69,300. This raises the question whether some of the inaccessibility of the SH groups to PCMB in the experiments of Orii *et al.* might not have been due to the polymeric nature of their enzyme, which has a molecular weight of 530,000 [23] in Emasol 1120. Our enzyme has a molecular weight of 228,000 which is unchanged in added SDS, while the enzyme becomes even more polymeric when transferred into Tween 80 [10].

TABLE III. Effect of treatment with cyanide on sulfhydryl groups, copper content and activity of cytochrome oxidase

Method	Concentration of NaCN	Number of determinations	SH groups per heme[b]		Δ SH groups per heme	Number of determinations	BCS[a]—non-reactive copper per heme[b]		Number of determinations	Activity	
			Before NaCN	After NaCN			Before NaCN	After NaCN		Before NaCN	After NaCN
	M									sec^{-1} (mg Protein/3 ml)$^{-1}$	
Column	1×10^{-3}	2	3.59	3.61	+0.02	1	1.30	1.25			
	5×10^{-3}	2	3.73	4.35	+0.62	2	1.44	1.23	1	1.00	0.85
	1×10^{-2}	6	3.28	4.78	+1.50	5	1.48	0.21	3	1.42	1.16
	1×10^{-1}	1	3.26	4.40	+1.14	1	1.42	0.24	1	1.54	0.67
Dialysis cell	1×10^{-3}	1	3.74	3.62	−0.12	1	1.40	1.30			
	1×10^{-1}	1	3.74	3.98	+0.24						
Dialysis sac	1×10^{-2}	2	4.05	3.76	−0.29						

[a] Bathocuproine sulfonate.
[b] Based on a molecular weight of 69,300

Tzagoloff *et al.* [25] reported a molecular weight of 290,000 for their enzyme and no apparent effect of SDS on the molecular weight.

When in these experiments the bathocuproine sulfonate-nonreactive copper was removed with NaCN, the average number of additional PCMB-titrable SH groups exposed per heme was approximately 1.5. The concentration of cyanide needed was 1×10^{-2} M or greater. In the experiments of Tsudzuki *et al.* [9], the relation between copper and SH groups was presumptive. They found it possible to remove all of the intrinsic copper by dialysis against cyanide, but it was with SDS rather than with cyanide that they exposed the apparently critical SH group. The concentration of SDS (0.2 to 0.3%) which permitted a seventh SH group to be titrated with PCMB was also the concentration of SDS which exposed the copper so that it could react with bathocuproine sulfonate. Since the intrinsic copper was considered to be bathocuproine sulfonate-nonreactive, it was assumed by Tsudzuki *et al.* that the SDS had liberated the intrinsic copper from the protein to chelate with the BCS. There is no evidence from other studies that SDS can free intrinsic copper, although it must be considered that SDS might expose the copper so that one or more of its natural ligands could be replaced by BCS [26]. It is unlikely, however, that BCS could replace SH as a ligand for copper. In other studies, we have estimated the log of the apparent binding constant for the copper of the enzyme to be 31 [27] which indicates a very strong binding.

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