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ON THE NATURE OF COPPER IN ASCORBATE OXIDASE

I. THE VALENCE STATE OF COPPER IN THE DENATURED AND NATIVE ENZYME

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SUMMARY

1. The valency state of Cu in the enzyme ascorbate oxidase (L-ascorbate: O_2 oxidoreductase, EC 1.10.3.3) has been determined by liberating the copper from the enzyme in the presence of valence-specific Cu-chelating agents. The procedures were designed to prevent electronic changes in the copper valency as a result of enzyme denaturation during the assay process.

2. By means of the two Cu(I)-specific reagents, cuproine and bathocuproine, it has been found that the prosthetic copper in ascorbate oxidase exists in a mixed valency state, corresponding to 25% Cu(I) and 75% Cu(II). This ratio of 1:3 corresponds to 2 atoms of Cu(I) and 6 atoms of Cu(II) per enzyme molecule.

3. This same ratio was found for the mixed valency state in the denatured enzyme when the Cu(I) reagent, bathocuproine, and the Cu(II) reagent, cuprizone, were used simultaneously.

4. Neither bathocuproine nor cuprizone reacted directly with the native enzyme at physiological pH 7.2.

INTRODUCTION

Ascorbate oxidase (L-ascorbate: O_2 oxidoreductase, EC 1.10.3.3) is a blue, globular, conjugated protein of molecular weight 150 000 containing prosthetic copper which is non-dissociable by exhaustive dialysis and is not removed by treatment with cation-exchange resins[1]. It functions physiologically as a terminal oxidase in the catalysis of the aerobic oxidation of L-ascorbic acid, and the prosthetic copper is essential to this activity. Recent findings in these laboratories[2] have shown that after purification by DEAE-cellulose chromatography, the ultracentrifugally and electrophoretically homogenous enzyme contains 0.34% Cu, which corresponds to 8 Cu atoms per molecule. However, very little is known concerning the exact valence nature of this prosthetic copper or its mode of binding to the apoenzyme. Further-

Abbreviation: AH_2, L-ascorbic acid; BCS, bathocuproine, disulfonated sodium salt of 2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline; BCODH, cuprizone, bis-cyclohexanoneoxalyl-dihydrazone; PCMB, *p*-chloromercuribenzoate; ESR, electron spin resonance.

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more the precise role of the copper during the catalytic function of the enzyme is yet to be defined.

The reversible color changes which accompany enzyme function[3] strongly indicate a reversible Cu valency change of the type:

$$\underset{\text{blue}}{\text{Cu(II)–P}} \underset{O_2}{\overset{AH_2}{\rightleftharpoons}} \underset{\text{colorless}}{\text{Cu(I)–P}}$$

Additional evidence to support this contention is provided by radio-copper exchange experiments[4,5] where it has been found that exogenous ionic ^{64}Cu ions are incorporated significantly only while the enzyme is functioning, *i.e.* catalyzing the aerobic oxidation of its substrate. This behavior is in accord with the view that only in the reduced bleached form is the enzyme Cu exchangeable, presumably as a result of Cu(I) tetrahedral bonds being weaker than Cu(II) square coplanar bonds[6]. With this change to a weaker type of linkage during enzymatic function, the possibility of an exchange with radio-copper is enhanced[7].

It has recently been found in these laboratories[8] that all the prosthetic copper of ascorbate oxidase is not equivalent. When the pH of a dilute aqueous ascorbate oxidase solution was decreased step-wise below 4.0 and concomitantly equilibriated at 4° for 4 h before removing dissociated ionic copper by means of a cation-exchange resin, a parallel loss in both Cu content and oxidase activity occurred. Of particular significance is the fact that the titration curve thus obtained showed two distinct inflection points, one at pH 3.78 and the other at pH 3.45. It would appear, therefore, that there are two different copper-binding systems in the protein moiety of the holoenzyme.

In this, the first of two papers, we describe the resolution of the problem of the valence state of copper, both in the native and denatured enzyme, through the use of valence-specific copper reagents. The Cu(I)-specific reagents, cuproine and bathocuproine[9], and the Cu(II)-specific reagent, cuprizone[10], were used for this purpose.

In the next paper we describe the use of bathocuproine as a probe for elucidating the role of prosthetic copper during oxidase function (*i.e.* ascorbate oxidation).

MATERIALS AND METHODS

Materials

Enzyme: Ascorbate oxidase was prepared from the yellow crookneck squash *(C. pepo condensa)* according to the procedure of DAWSON AND MAGEE[11].

Substrate: AH_2 for activity determinations was used as obtained from the Merck Co. without further purification.

Copper reagents: The Cu(I)-specific reagents, cuproine (2,2′-biquinoline) and BCS, and the Cu(II)-specific reagent BCODH were obtained from the G. Frederick Smith Co., Columbus, Ohio.

Other reagents: Hydroxylamine hydrochloride, PCMB, EDTA, dibasic sodium phosphate and citric acid were used directly as obtained from various manufacturers.

Buffers: McIlvaine's buffer solutions (pH 7.2) were prepared by mixing suitable aliquots of stock solutions of 0.2 M dibasic sodium phosphate and 0.1 M citric acid[12].

Cu-free water: Stock solutions of all reagents were prepared in Cu-free water obtained by re-distilling laboratory distilled water in an all-Pyrex still[13]. The copper content of the water distilled in this manner was below 0.01.

Enzyme assay: The specific oxidase activity of the enzyme was determined by following the rate of the ascorbate oxidase–O_2–AH_2 reaction manometrically as previously described[14].

Dry weight protein content was determined according to the procedure described elsewhere[11].

Total Cu content was determined in the following way, using either cuproine or BCS: a suitable aliquot of the enzyme was denatured by acidification (below pH 3) in the presence of $NH_2OH \cdot HCl$ and the Cu reagent. In this manner the liberated copper ions were completely reduced and determined colorimetrically as the Cu(I) complex by comparison to the absorbancy per μg of Cu per ml obtained with a suitable aliquot of the standard Cu solution identically treated.

The complete assays of each enzyme preparation used in this investigation are given in Table I.

TABLE I

ASSAY OF ASCORBATE OXIDASE PREPARATIONS

Enzyme No.	*Code*	*Protein (μg/ml)*	*Cu content (μg/ml)***	*Cu (%)*	*Specific activity in units**	
					per mg ascorbate oxidase	*per μg Cu*
1	AAO36D2 (A)	4.9	17.5	0.36	2150	600
2	AAO36D2 (B)	4.6	17.6	0.38	2400	625
3	AAO36D3	5.8	21.3	0.37	1400	380
4	AAO36E1	6.0	19.5	0.33	1560	480
5	AAO36E2	3.7	12.0	0.33	2060	640
6	AAO36C1	3.0	10.4	0.34	2840	820
7	AAO36C′	6.7	19.7	0.30	2140	730
8	AAO36C2	3.2	10.2	0.32	2600	810
9	AAO37A	7.9	22.3	0.28	2200	790

* One unit is defined as that amount of ascorbate oxidase required to effect the manometric uptake of 10 μl of O_2 per min under specified conditions of pH and substrate concentration at 25° (see ref. 15).

** All preparations were exhaustively dialyzed against Cu-free phosphate–citrate buffer (pH 5.6) to remove all extraneous Cu^{2+} ions.

Preparation of reagent stock solutions

Standard copper: A carefully weighed sample of analytically pure metallic Cu was dissolved in re-distilled conc. HCl and diluted to 1 l with Cu-free water. The Cu concentration thus obtained was 24.24 μg/ml.

Copper reagents: Stock solutions of cuproine (1.5 mg/ml), BCS (5.0 mg/ml) and BCODH (5.0 mg/ml) were prepared in glacial acetic acid, Cu-free water and 50% aq. ethanol respectively.

Other reagents: Stock solutions of $NH_2OH \cdot HCl$ (5.0 mg/ml), PCMB (1.1 mg/ml) and EDTA (1.2 mg/ml) were prepared in Cu-free water. A small amount of dilute NaOH solution was required to dissolve the PCMB and EDTA.

RESULTS AND DISCUSSION

Cuprous copper in the denatured enzyme by cuproine assay

The cuproine reagent gives a deep purple color with Cu(I) in glacial acetic acid[9] ($\lambda_{max} = 540$ mμ; $\varepsilon_M = 6000$). Since the conditions of its application lead to the dissociation of prosthetic copper, precautions must be taken to insure the maintenance of each Cu atom in the valence state it possesses at the moment of release from the apoenzyme. It has been shown that the presence of reducing functional groups such as –SH in Cu proteins promotes non-specific reduction of liberated Cu(II) to Cu(I), thereby giving erroneous cuprous assay values[16]. STARK AND DAWSON[17] has demonstrated that native ascorbate oxidase contains no free –SH groups but that 10–12 –SH groups are exposed as the result of denaturation. Hence it was necessary to prevent the possible interference of liberated –SH groups during the cuproine assay of denatured ascorbate oxidase. For this purpose, two modifications of the usual analytical conditions were used: (a) PCMB was introduced as a specific reagent for blocking any exposed –SH groups, thereby destroying their reducing capacity; (b) EDTA was introduced as a specific reagent for instantaneous chelation of the liberated Cu(II) ions, thereby rendering them unavailable for subsequent reduction *in situ*.

The exact experimental conditions adopted for each type of modified cuprous assay were as follows, using enzyme No. 4 as representative:

(1) Cuproine modified by PCMB: To 0.5 ml of stock PCMB solution was added 0.75 ml of ascorbate oxidase, followed by 1.0 ml of the acidic cuproine stock solution; an instantaneous pink color resulted. After diluting to 5.0 ml with 50% acetic acid, duplicate samples were read at 540 mμ in a Beckman spectrophotometer, model DU, within 1 h. The final concentration of each component was: ascorbate oxidase protein, $6.0 \cdot 10^{-6}$ M; –SH (estimated), $6.0 \cdot 10^{-5}$ M; PCMB, $30 \cdot 10^{-5}$ M; cuproine, $1.2 \cdot 10^{-3}$ M.

(2) Cuproine modified by EDTA: The conditions were the same as those described above except for the substitution of 0.5 ml of stock EDTA solution for the PCMB. The final EDTA concentration was $40 \cdot 10^{-5}$ M for a total prosthetic Cu concentration, $4.0 \cdot 10^{-5}$ M.

With each cuprous assay, by either of the modified procedures described above, a total copper assay was performed simultaneously (in duplicate) on a suitable aliquot of the enzyme, as previously described (see enzyme assay).

The results obtained using four different ascorbate oxidase samples are summarized in Table II. They indicate that when the cuproine reagent is applied directly to the denatured enzyme without modification (Column 3), the initial Cu(I) assay is significantly higher (by 5–10%) than when the assay procedure is modified by the presence of either PCMB or EDTA (Columns 4 and 5). Furthermore, the absorbancy at 540 mμ in the unmodified systems was found to increase steadily over 24 h (*e.g.*, with enzyme No. 3, an increase from 0.102 to 0.156 was observed). This effect can be ascribed to the intrinsic reducing capacity of the –SH groups exposed on the apoenzyme by denaturation.

The inclusion of PCMB to block these –SH groups does reduce this effect, but does not eliminate it entirely. For instance, with enzyme No. 4 the absorbancy at 540 mμ (Column 4) was found to increase slowly from 0.089 to 0.118 over 24 h.

TABLE II

SHOWING THE EFFECT OF PCMB AND EDTA ON THE CUPROINE ASSAY OF Cu(I) IN DENATURED ASCORBATE OXIDASE

Enzyme No.	*Total prosthetic Cu used (μg)*	*Per cent of total copper reacting as Cu(I) with**		
		cuproine alone	*cuproine + PCMB***	*cuproine + EDTA****
1	14.0	30	25	—
2	14.0	31	—	22
3	16.0	33	—	23
4	14.6	—	30	24

* Based on absorbancy readings at 540 mμ taken within 1 h of the addition of the enzyme to the reagent.

** A 5 M excess relative to the estimated –SH exposed on denaturation.

*** A 10 M excess relative to the prosthetic Cu liberated on denaturation.

Hence, reducing functional groups other than –SH (possibly tyrosyl) are most likely responsible for this additional reduction *in situ* of liberated Cu(II) ions, even in the presence of PCMB. However, this effect can be eliminated completely by the inclusion of EDTA in the system to selectively chelate all Cu(II) ions released from the apoenzyme on denaturation. For instance, with enzyme No. 4 the absorbancy at 540 mμ (Column 5) remained constant over 8 h, then decreased gradually from 0.068 to 0.052 after 24 h, due to the slow breakdown of the Cu(I)–cuproine complex in the presence of EDTA. Hence, the EDTA modification of cuproine gives a more reliable assay of the Cu(I) initially released from denatured ascorbate oxidase than does the PCMB modification. The results with enzymes Nos. 2, 3 and 4 (Column 5) show excellent agreement and indicate that approx. 25% of the total enzyme copper reacted as Cu(I) immediately upon release from the native protein by exposure to acidic pH.

Cuprous copper in the denatured enzyme by bathocuproine assay

The bathocuproine reagent (BCS) gives an intense orange color with Cu(I) (see ref. 9) (λ_{max} = 480 mμ; ε_M = 13 500). Since the Cu(I) complex forms best at neutral pH, it was necessary first to release the prosthetic copper from the apoenzyme by reducing the pH below 3 with dilute HCl, in the presence of all reagents, and then to back titrate with dilute NaOH to neutral pH. For reasons previously stated, the effect of EDTA on the cuprous assay was investigated.

The exact conditions were as follows, using enzyme No. 5 as representative: To a system containing 0.5 ml of 0.2 M HCl, 0.1 ml of the stock BCS, and 0.25 ml of the stock EDTA (where appropriate) was added a 0.5-ml aliquot of the blue enzyme. To the resulting colorless solution was then added 0.2 M NaOH drop-wise to a phenolphthalein pink end point (pH 8–10); 1.0 ml of phosphate–citrate buffer (pH 7.2) was then added to dissipate the indicator color. The resulting orange solution was diluted to 5.0 ml with Cu-free water and duplicate samples were read at 480 mμ within 1 h. The final concentration of each component was as follows: ascorbate oxidase protein, $2.5 \cdot 10^{-6}$ M; prosthetic Cu, $2.0 \cdot 10^{-5}$ M; BCS, $18 \cdot 10^{-5}$ M;

EDTA, $20 \cdot 10^{-5}$ M. Total Cu content was determined simultaneously as previously described (see enzyme assay).

The results obtained, using four different ascorbate oxidase samples, are summarized in Table III. They substantiate and are in excellent agreement with the results obtained by cuproine assay (*cf.* Table II). That is to say, when modified by EDTA, the BCS reagent accounts for approx. 25% of the total enzyme copper as Cu(I) under denaturing conditions.

TABLE III

SHOWING THE EFFECT OF EDTA ON THE BATHOCUPROINE ASSAY OF Cu(I) IN DENATURED ASCORBATE OXIDASE

Enzyme No.	*Total prosthetic Cu used (μg)*	*Per cent of total Cu reacting as Cu(I) with**	
		BCS alone	*BCS + EDTA***
5	7.2	43	23
6	6.2	—	20
7	7.9	—	27
8	5.6	61	21

* Based on absorbancy readings taken at 480 mμ within 1 h of the addition of the enzyme to the reagent.

** A 1 M excess relative to the prosthetic Cu liberated on denaturation.

Simultaneous determination of Cu(I) and Cu(II) in the denatured enzyme by combined bathocuproine–cuprizone assay

Since the Cu(II)–EDTA complex is colorless, its presence does not interfere with either the cuproine or BCS colorimetric assay of Cu(I) liberated from denatured ascorbate oxidase. However, its lack of visible absorption makes it unsuitable for a colorimetric determination of Cu(II). The cuprizone reagent (BCODH), on the other hand, forms an intense, blue complex ($\varepsilon_M = 16\,000$) with Cu(II) ion that absorbs maximally at a wavelength ($\lambda = 600$ mμ) that is sufficiently far removed from the absorption of the orange Cu(I)–BCS complex ($\lambda = 480$ mμ) so that their absorption spectra do not overlap significantly. Consequently, BCODH (Cu(II)-specific) and BCS (Cu(I)-specific) may be used simultaneously for the assay of both Cu-valence states liberated from the denatured enzyme. Since the maximum intensity of the Cu(II)–BCODH complex is attained only in the limited pH range 7–9 (see ref. 10), a neutralization step following the acid denaturation of the enzyme was required.

The exact procedure, employing enzyme No. 7, was as follows: To a system containing 0.1 ml of stock BCS, 0.5 ml of stock BCODH and 0.5 ml of 0.2 M HCl was added 0.5 ml of ascorbate oxidase. After incubating 15 min, 0.2 M NaOH was added drop-wise to the phenolphthalein end point; 1.0 ml of phosphate–citrate buffer (pH 7.2) was then added to dissipate the indicator color. A purplish-green solution resulted, as a superposition of the colors of the individual Cu complexes, *i.e.* Cu(II)–BCODH (blue) and Cu(I)–BCS (orange). After diluting to 5.0 ml with Cu-free water, the absorbancy at both 480 and 600 mμ was read periodically over 24 h. The final concentration of each component was as follows: ascorbate oxidase

protein, $4.5 \cdot 10^{-6}$ M; prosthetic Cu, $3.1 \cdot 10^{-5}$ M; BCODH, 18×10^{-4} M; BCS, $1.8 \cdot 10^{-4}$ M. Suitable standards were similarly prepared for each type of Cu complex, using an aliquot of standard Cu solution equivalent to the amount of liberated prosthetic copper of each valence state expected on denaturation of the enzyme. (This is necessary so as to determine the absorbancy correction to be made at the respective λ_{max} of each type of Cu complex due to the slight mutual absorbancy of the other.)

The results are summarized in Table IV. They can best be interpreted by the following considerations.

TABLE IV

SIMULTANEOUS DETERMINATION OF Cu(I) AND Cu(II) IN DENATURED ENZYME No. 7 BY COMBINED BATHOCUPROINE–CUPRIZONE ASSAY

Time (h)	*Valence of copper determined (μg/ml)*		*Total Cu (μg/ml)*	*Per cent of total Cu reacting as*		*Per cent deviation from theoretical copper*
	Cu(I)	*Cu(II)*	*Cu(I)+Cu(II)*	*Cu(I)*	*Cu(II)*	
0.5	6.0	13.5	19.5	30	70	0
2	7.4	12.3	19.7	38	62	0
8	8.2	8.6	16.8	42	44	14
24	10.2	4.7	14.9	53	24	23

(a) Up to 2 h the total Cu content (as the sum of Cu(I) + Cu(II)) agreed remarkably well with that determined independently by BCS assay alone (see Table I), thus assuring that all prosthetic copper was fully complexed by one reagent or the other. However, the percentage of the total copper reacting as either Cu(I) or Cu(II) did not stay constant during this time, but shifted by 8% in favor of the Cu(I)–BCS complex. This phenomenon can be attributed to the known stability difference between the two Cu complexes *i.e.* since the Cu(I)–BCS complex is more stable, the Cu(II)–BCODH complex slowly dissociates, thereby allowing the released copper to be reduced and recomplexed by BCS. Since the total copper still corresponded to 100% of the theoretical, regardless of this gradual shift in favor of Cu(I) ion, one can obtain that fraction of the prosthetic copper reacting as either Cu(I) or Cu(II) ion instantaneously on release from the apoenzyme by extrapolating back to zero time the plot of percentage total copper reacting as either ion *vs.* time (up to to 2 h). When this was done, values of 27% Cu(I) and 73% Cu(II) were obtained. These values are in good agreement with those obtained by either cuproine or BCS assay of Cu(I), as previously described.

(b) After 2 h the total copper showed progressively increasing deviation from 100% theoretical. As can be seen from the table, the Cu(II) value decreased faster after this time than the Cu(I) value increased. The explanation lies in the known instability of the blue color of the Cu(II)–BCODH complex—it has been reported[10] to be stable only up to 2 h, fading thereafter at the rate of about 1% per h. Hence after 2 h not only was the Cu(II)–BCODH complex continuing to lose its copper *via* dissociation and reduction to the more stable BCS chelate system, but the intensity of color of the intact Cu(II)–BCODH complex was slowly deteriorating, leading to low values of Cu(II) assay. Consequently, only the Cu(I) assay values can be considered reliable after 2 h elapsed time from the initial denaturation of the enzyme.

The excellent corroboration of these results with those obtained independently with either the cuproine or BCS reagent (as modified by EDTA) conclusively establishes the existence of both Cu(I) and Cu(II) in ascorbate oxidase, in the ratio 1:3. In terms of total prosthetic copper, this corresponds to 2 Cu(I) and 6 Cu(II) atoms per molecule.

Direct copper assay on the native enzyme

In order to confirm, if possible, the 3:1 ratio of Cu(II) to Cu(I) found, as previously described, by liberating the copper from the enzyme in the presence of valence-specific chelating agents, it seemed advisable to attempt the assay directly on the native (intact) copper-protein chelate complex.

Application of the BCS reagent: To 0.75 ml of enzyme No. 6 was added either 40 or 80 mg of BCS dissolved in 1.0 ml of Cu-free water, followed by dilution to 5.0 ml with phosphate–citrate buffer (pH 7.2). Duplicates of the resulting solution were measured periodically at 480 mμ against suitable blanks containing all reagents except enzyme. The final concentration of each component was: prosthetic Cu, $2.46 \cdot 10^{-5}$ M; BCS, 14.2 or $28.4 \cdot 10^{-3}$ M.

The results are summarized in Table V. It is evident that at neither level of BCS concentration did any significant absorbancy at 480 mμ develop over a 20-h period due to Cu(I)–BCS complex formation.

TABLE V

SHOWING THE UNREACTIVITY OF Cu(I) IN NATIVE ENZYME No. 6 TO THE BCS REAGENT AT pH 7.2

System	*Total enzyme Cu (μg)*	*BCS reagent (mg)*	*Absorbancy at 480 mμ***		
			1 h	*4 h*	*20 h*
Blank	0	40	(0.083)***	(0.086)	(0.107)
Ascorbate oxidase	7.8	40*	0.002	0.002	0.005
Ascorbate oxidase	7.8	40	0.002	0.008	0.006
Blank	0	80	(0.175)***	(0.171)	(0.193)
Ascorbate oxidase	7.8	80*	0.012	0.010	0.008
Ascorbate oxidase	7.8	80	0.009	0.007	0.005

* 40 and 80 mg of the BCS reagent correspond to a 580 and 1160 molar excess of the reagent relative to the enzyme copper.

** Complete formation of the Cu(I)–BCS complex corresponding to the copper present would give an absorbancy of 0.330 (based on $\varepsilon_M = 13\,500$).

*** The blank, containing 40 or 80 mg of yellow BCS, was measured against distilled water; the increase in absorbancy of about 0.02 absorbancy units between 4 and 20 h in both cases can be attributed to a slow autoxidation of the reagent[9].

Application of the BCODH reagent: There were certain complicating factors which had to be considered in applying the BCODH reagent to the native enzyme: (a) The reagent itself is water-insoluble and is only limitedly soluble in 50% aq. ethanol. Thus an upper limit was imposed on the concentration of the reagent that could be used without exceeding the toleration of the enzyme to the denaturing

action of ethanol. (b) The wavelength of maximum absorption of the Cu(II)–BCODH complex (λ = 600 mμ) coincides almost exactly with that of the blue chromophore of the enzyme itself (λ = 605 mμ). Thus, although a diluted form of the enzyme was used, a correction for the residual absorbancy of the blue enzyme chromophore at 600 mμ had to be applied to each Cu(II)–BCODH assay. With these considerations in mind, the following procedure was adopted, using enzymes Nos. 7 and 9.

To the appropriate aliquot of enzyme (0.2 ml for No. 7 or 0.5 ml for No. 9) was added 3.0 ml of phosphate–citrate buffer (pH 7.2). Either 0.5 ml (No. 7) or 1.0 ml (No. 9) of a stock BCODH solution (10 mg/ml in 50% ethanol) was then added, followed by dilution to 5.0 ml with buffer. In this manner, the final ethanol concentration was either 5% (No. 7) or 10% (No. 9). The final concentration of each of the other components was as follows. Enzyme No. 7: prosthetic Cu, $1.26 \cdot 10^{-5}$ M; BCODH, $3.6 \cdot 10^{-3}$ M; enzyme No. 9: prosthetic Cu, $1.58 \cdot 10^{-5}$ M; BCODH, $7.2 \cdot 10^{-3}$ M. The resulting pale blue solutions were measured periodically at 600 mμ against suitable blanks containing all reagents except enzyme. The results are summarized in Table VI. It is evident that no significant absorbancy due to Cu(II)–BCODH complex formation developed over 10 h at either level of BCODH concentration.

TABLE VI

SHOWING THE UNREACTIVITY OF Cu(II) IN NATIVE ASCORBATE OXIDASE TO THE BCODH REAGENT AT pH 7.2

System	*Total enzyme Cu (μg)*	*BCODH reagent (mg)*	*Absorbancy at 600 mμ***		
			0.5 h	*4 h*	*10 h*
Blank	0	5	(0.005)***	(0.003)	(0.002)
Ascorbate oxidase (No. 7)	4.0	5*	0.011§	0.009	0.008
Blank	0	10	(0.008)	(0.007)	(0.008)
Ascorbate oxidase (No. 9)	5.0	10*	0.003	0.003	0.002

* 5 and 10 mg of the BCODH reagent correspond to a 290 and 460 M excess of the reagent relative to the enzyme copper.

** Complete formation of the Cu(II)–BCODH complex corresponding to the prosthetic Cu present would give an absorbancy of 0.200 (No. 7) or 0.250 (No. 9) (based on ε_M = 16 000).

*** The blank, containing 5 or 10 mg of BCODH, was measured against distilled water and shows essentially complete transparency in both cases.

§ Absorbancy has been corrected in all cases by 0.025 unit, corresponding to the residual absorbancy of the blue enzyme chromophore at this wavelength.

On the basis of the data shown in Tables V and VI, it is evident that the direct application of valence-specific Cu reagents to the native enzyme did not result in chelate formation with either valence state of the prosthetic copper. Hence, it must be concluded that in the native enzyme the prosthetic copper was not accessible to either of these chelating agents, even though they were applied in high molar excess. Presumably, the complete unreactivity of both Cu(I) and Cu(II) in the native enzyme can be attributed to either: (a) a steric hindrance of the protein configuration such as to mask the sites of bound copper and provide an effective barrier to the penetration of either reagent, or (b) complete saturation of the coordination positions of bound copper, thus preventing interaction with either reagent. (a and

b are not mutually exclusive and both may contribute to the overall unreactivity of the enzyme's prosthetic copper.)

In any case, the evidence obtained by application of the valence-specific Cu reagents to the denatured enzyme systems is in no way invalidated by this finding with the native enzyme. Its weight is too convincing to be attributable to an artifact. In order to obtain an unequivocal resolution of the valence state of copper in native ascorbate oxidase, one must use a physical method which does not require dissociation of bound copper from the protein moiety. An ESR study would be fruitful in this regard. Until such a study is made, the argument for the existence of a mixed valency state of prosthetic copper in native ascorbate oxidase, in the ratio 75% Cu(II): 25% Cu(I), must rest on the chemical evidence presented herein.

REFERENCES

[1] F. DUNN AND C. R. DAWSON, *J. Biol. Chem.*, 189 (1951) 485.
[2] K. TOKUYAMA AND C. R. DAWSON, unpublished results.
[3] C. R. DAWSON, *Ann. N.Y. Acad. Sci.*, 88 (1960) 353.
[4] M. JOSELOW AND C. R. DAWSON, *J. Biol. Chem.*, 191 (1951) 11.
[5] R. J. MAGEE AND C. R. DAWSON, *Arch. Biochem. Biophys.*, 99 (1962) 338.
[6] L. PAULING, *Nature of the Chemical Bond*, 2nd. Ed., Cornell University Press, 1948, p. 104.
[7] C. R. DAWSON, in W. MCELROY AND B. GLASS, *A Symposium on Copper Metabolism*, The Johns Hopkins Press, Baltimore, 1950, pp. 18–47.
[8] B. GREENWALD AND C. R. DAWSON, unpublished results.
[9] H. DIEHL AND G. F. SMITH, *The Copper Reagents: Cuproine, Neocuproine and Bathocuproine*, G. F. Smith Co., Columbus, Ohio, 1958.
[10] R. PETERSON AND M. BOLLIER, *Anal. Chem.*, 27 (1955) 1195.
[11] C. R. DAWSON AND R. MAGEE, *Methods of Enzymology*, Vol. 2, Academic Press, New York, 1955, p. 831.
[12] *Handbook of Chemistry and Physics*, 34th. Ed., 1952–53, p. 1541.
[13] S. AMES AND C. R. DAWSON, *Ind. Eng. Chem.*, 17 (1945) 249.
[14] K. TOKUYAMA AND C. R. DAWSON, *Biochim. Biophys. Acta*, 56 (1962) 427.
[15] W. POWERS, S. LEWIS AND C. R. DAWSON, *J. Gen. Physiol.*, 27 (1944) 167.
[16] G. FELSENFELD, *Arch. Biochem. Biophys.*, 87 (1960) 247.
[17] G. STARK AND C. R. DAWSON, *J. Biol. Chem.*, 237 (1962) 712.