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ON THE NATURE OF COPPER IN ASCORBATE OXIDASE

II. THE ROLE OF PROSTHETIC COPPER IN THE ENZYME'S FUNCTION

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SUMMARY

1. The respective roles of prosthetic Cu(I) and Cu(II) in the function of ascorbate oxidase (L-ascorbate: O_2 oxidoreductase, EC 1.10.3.3) have been examined with respect to the blue color, activity and inactivation of the enzyme.

2. It has been found that the Cu(I) fraction, representing approx. 25% of the total copper in the native enzyme, does not participate in the enzymatic activity or contribute to the blue color. That is to say, the complexing of that fraction of the protein copper by a Cu(I)-specific chelating agent does not affect the activity or the blue color.

3. The Cu(I) fraction existing in the native enzyme can not be complexed directly with the chelating agent, except when the enzyme is functioning. It is concluded, therefore, that a reversible structural modification in the conformation of the protein moiety occurs during the catalytic cycle, thereby making this non-functional Cu(I) available to the reagent. Furthermore, the configuration of the protein and the binding of its functional Cu(II) fraction, are such that the continually generated Cu(I) component of the reversible Cu(II) $\leftrightharpoons$ Cu(I) catalytic cycle is at no time available for complexing with the chelating agent.

4. It has been found that the non-functional Cu(I) fraction of the enzyme is responsible for the production of the H_2O_2 that results in the characteristic inactivation of the enzyme during aerobic function.

5. It was shown that small amounts of H_2O_2 have no inactivating effect on the resting enzyme but are strikingly effective on the functioning enzyme. The enzyme thus inactivated, loses its blue chromophore, but retains its copper. It is suggested that this H_2O_2 effect may involve directly the functional Cu(I) sites or the irreversible oxidation of some critical functional group exposed during the modification in structure of the protein moiety during catalysis.

INTRODUCTION

In the preceding paper[1], evidence was presented to support a mixed valency state for the prosthetic copper in ascorbate oxidase (L-ascorbate: O_2 oxidoreductase,

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Abbreviation: BCS, bathocuproine, disulfonated sodium salt of 2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline.

EC 1.10.3.3). The assays for Cu(I) and Cu(II) were actually made after liberation of the prosthetic copper from the enzyme, but the conditions were such as to permit an estimation of the valency state ratio in the native enzyme. The ratio of 75% Cu(II) to 25% Cu(I) found corresponds to 6 Cu(II) and 2 Cu(I) atoms per enzyme molecule. This appears to be the first quantitative experimental evidence for the existence of a mixed valency state in the prosthetic copper of ascorbate oxidase. However, MAGEE AND DAWSON[2], earlier in these laboratories, demonstrated qualitatively the presence of Cu(I) in the resting enzyme of low specific activity and suggested that it might be responsible for the low exchange with radioactive ^{64}Cu ions. Although KREUGER[3] has reported the presence of both Cu(I) and Cu(II) in the mushroom oxidase, tyrosinase, no investigation has yet been reported concerning the role of each type of copper in the catalytic activity of such copper oxidases. It is the purpose of this communication to describe an experimental approach to this problem as it concerns the heterogeneity of the prosthetic copper during the catalytic function of ascorbate oxidase.

MATERIALS AND METHODS

Materials

Ascorbate oxidase, L-ascorbic acid, BCS, the standard Cu solution, and the buffer were all prepared and used in Cu-free water as previously described[1], except that the stock concentrations of BCS and L-ascorbic acid were 28.2 mg/ml and 176 mg/ml respectively and the buffer was pH 5.6.

Enzyme assay

The complete assay of the two enzyme preparations used are given in Table I. The assay procedures have been previously described[1].

TABLE I

ASSAY OF ASCORBATE OXIDASE PREPARATIONS

Enzyme No.	*Code*	*Protein content (mg/ml)*	*Cu content (μg/ml)*	*Cu (%)*	*Specific activity* in units:*		*Absorbancy at 605 mμ*
					per mg	*per μg Cu*	
9	AAO37A	7.9	22.3	0.28	2200	790	0.430
10	AAO37B1	4.3	14.4	0.34	3020	920	0.298

* 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°.

RESULTS AND DISCUSSION

Availability of the prosthetic copper to BCS during enzyme function

Relation to blue color: In order to evaluate the changes observed in the visible absorption spectrum of ascorbate oxidase as it functioned catalytically in the aerobic oxidation of ascorbic acid while in the presence of BCS, it was necessary to make quantitative absorbancy measurements at two different wavelengths: 480 mμ (cor-

responding to the Cu(I)–BCS chelate chromophore) and 605 mμ (corresponding to the blue chromophore of the enzyme). Such measurements were always compared with those made on control systems containing all reagents, except BCS. The experiments were carried out in 5.0-ml volumetric flasks at room temperature (about 26°) and the conditions of enzyme and ascorbic acid concentration were selected so that the supply of O_2 was rapidly depleted. Consequently, the blue color of the enzyme was rapidly lost and the system remained bleached until it was re-oxygenated. This condition was necessary so as to permit a direct evaluation of the Cu(I)–BCS chromophore development, which could not be observed with the native enzyme in the absence of substrate (see preceding paper).

In each experiment four systems were set up, two as controls and two with BCS. After a suitable time had elapsed for maximal development of the absorbancy at 480 mμ in the systems containing BCS, one of these systems and one of the controls systems were oxygenated to quickly regenerate the blue color of the enzyme. The exact experimental procedure, using enzyme No. 9, was as follows:

To two of four 5.00-ml flasks containing 4.17 ml of buffer and 0.70 ml of ascorbate oxidase was added 0.10 ml of the stock BCS solution. (The blue color of the enzyme was not affected.) On addition of 0.03 ml (5.28 mg) of the stock ascorbic acid solution to each flask, the systems containing BCS rapidly became pale yellow in color whereas the controls became colorless. Each system was then measured periodically at 480 and 605 mμ over a 3-h period. Pure O_2 was then bubbled into one of the control systems and one of the systems containing BCS. The final concentration of each component (where appropriate) was as follows: prosthetic Cu, $4.9 \cdot 10^{-5}$ M; BCS, $1.0 \cdot 10^{-3}$ M; L-ascorbic acid, $6.0 \cdot 10^{-3}$ M. The results are summarized in Table II.

TABLE II

REACTIVITY OF PROSTHETIC COPPER WITH BCS DURING OXIDASE FUNCTION OF ENZYME No. 9 AND CORRELATION WITH BLUE COLOR

System	*Ascorbate oxidase Cu (μg)*	*BCS (mg)*	*Absorbancy at 480 mμ in 3 h*		*Absorbancy at 605 mμ in 3 h*		
			Before oxygenation	*After oxygenation*	*Before oxygenation*	*After oxygenation*	*Final color*
1	15.6	2.82	0.062	—*	0.008	—*	yellow
2	15.6	None	0.005	—*	0.006	—*	colorless
3	15.6	2.82	0.065	0.057	0.005	0.061**	green
4	15.6	None	0.008	0.005	0.008	0.062**	blue

* Systems 1 and 2 were not oxygenated, but left in the bleached state.

** Full absorbancy of the blue enzyme chromophore, as diluted under conditions of assay, corresponds to absorbancy 0.065.

As can be seen in the table, the absorbancy values at 480 mμ (before oxygenation) for Systems 1 and 3 developed slowly and became maximal in about 3 h. From the absorbancy at 480 mμ per μg Cu/ml determined independently on a standard copper system, these absorbancy values were calculated to correspond to 22% of the total prosthetic Cu reactive as Cu(I) with BCS in the presence of substrate*.

* This calculation was based on a Cu(I): BCS stoichiometry of 1:1, corresponding to an $\varepsilon_M = 6750$, a value one-half that used for standard Cu solutions where the complex with BCS

This figure is in complete accord with that obtained on the denatured enzyme, as previously described[1], and further substantiates the heterogeneity of the prosthetic copper in ascorbate oxidase. Subsequent oxygenation of Systems 3 and 4 fully restored the blue color of the enzyme, as shown by the absorbancy values at 605 mμ in Table II. It is of particular significance that no appreciable loss in the color intensity of the enzyme Cu(I)–BCS complex was observed for System 3 (*cf.* absorbancy value at 480 mμ before and after oxygenation). That both chromophores can coexist independently on the enzyme was revealed by the colors of the systems after oxygenation: System 4 was blue, as would be expected solely from regeneration of the blue enzyme chromophore, while System 3 was green, due to the superposition of the yellow Cu(I)–BCS complex chromophore on that of the blue enzyme chromophore. The mutual compatibility of these two enzyme chromophores after oxygenation was verified by repetition of the experiment using enzyme No. 10, with essentially identical results (see Table III). It was also found that the phenomenon was unaffected by the concentration of BCS used to complex the bound Cu(I) on the enzyme or by the concentration of ascorbic acid used to bleach the enzyme.

TABLE III

SPECTROPHOTOMETRIC ASSAY OF ENZYME No. 10, BOTH FUNCTIONING (IN ABSENCE AND PRESENCE OF BCS) AND RESTING (IN PRESENCE OF BCS)

System No.	*Ascorbate oxidase Cu (μg)*	*BCS (mg)*	*Enzyme state*	*Final treatment*	*Absorbancy at 480 mμ*	*Absorbancy at 605 mμ*	*Final color*
1	14.4	2.82	functioning	unoxygenated	0.051	0.010	yellow
2	14.4	None	functioning	unoxygenated	0.002	0.004	colorless
3	14.4	2.82	functioning	oxygenated	0.048	0.058*	green
4	14.4	None	functioning	oxygenated	0.003	0.058*	blue
5	14.4	2.82	resting	unoxygenated	0	0.060*	blue

* Full absorbancy of the blue enzyme chromophore, as diluted under conditions of assay corresponds to absorbancy 0.060.

The fact that the original intensity of blue color of the enzyme was restored by oxygenation, even when approx. 25% of its total copper was still tied up in a stable complex with BCS, clearly shows that a significant fraction of the copper of the ascorbate oxidase is not involved in its characteristic blue chromophore. Of particular interest is the fact that this fraction of the prosthetic copper which becomes available for complexing by BCS in the presence of substrate (functioning enzyme) is completely unavailable to the BCS in the native (resting) enzyme. On the basis of evidence presented in the preceding paper[1], it is likely that this same faction of prosthetic copper is present in the native enzyme as Cu(I). Hence, during enzymatic function some structural modification occurs which results in this Cu(I) becoming available for BCS complex formation. It would appear therefore that only about 75% of the

involves uncoordinated Cu(I) ion in a 1:2 complex[4]. Dialysis data to be presented subsequently will show that the enzyme Cu(I)–BCS complex formed, as indicated in Table II, involves Cu(I) still bound to the apoprotein. Since the usual coordination number of copper is four, and it may be presumed that at least two of these coordination positions are occupied by protein ligands, a 1:2 complex involving four ligands from BCS would not be expected.

total copper, presumably present as Cu(II), is involved in the blue color of the native enzyme, with the other 25% being present in a non-chromophoric (colorless) chelate system.

Relation to activity: In order to determine whether the same type of relationship existed between the non-chromophoric prosthetic copper and the enzyme's activity, the foregoing experiment was repeated and activity determinations were made, as well as absorbancy measurements, following the oxygenation stage, both before and after exhaustive dialysis. The experimental procedure, using enzyme No. 10, was identical to that previously described, except that a fifth system containing only the BCS and resting enzyme (no substrate) was included as an additional control. The final concentration of each component (where appropriate) was as follows: prosthetic Cu, $4.7 \cdot 10^{-5}$ M; BCS, $7.0 \cdot 10^{-4}$ M; L-ascorbic acid, $6.0 \cdot 10^{-3}$ M. After allowing adequate time for full color development in the functioning systems containing BCS, two of the systems were oxygenated as previously described. The absorbancy at 480 and 605 mμ for each system at this stage was then measured (see Table III). The values at 480 mμ for Systems 1 and 3 corresponded to 18% of the total copper reactive as Cu(I) by BCS complex formation. This figure for the fraction of total copper available to BCS during enzymatic function is in satisfactory agreement with the 22% value previously determined using enzyme No. 9 (see Table II). Aliquots of each system were then taken for the determination of enzyme activity in the usual manner[5]. As can be seen from the results in Column 2 of Table IV, there was no loss of activity during the BCS incubation period. It is also noteworthy that the presence of a small amount of BCS in the highly diluted enzyme aliquot used for activity assay in Systems 1 and 3 did not affect the observed activity. These results therefore provide strong evidence that all the prosthetic copper in ascorbate oxidase

TABLE IV

SHOWING THE NON-INVOLVEMENT OF THE 25% Cu(I) FRACTION IN ENZYME No. 10 (AS COMPLEXED BY BCS) IN OXIDASE FUNCTION

System No.	*Per cent activity found before dialysis**	*Enzyme state during dialysis***	*Per cent recovery after dialysis*		*Final color*
			Activity	*Prosthetic Cu*	
1	102	functioning***	24	95	yellow§§§
2	102	functioning***	20	99	colorless§§§
3	95	resting§	92	109	green
4	100	resting§	91	109	blue
5	102	resting§,§§	86	108	blue

* For control purposes, the original activity of a suitable diluted aliquot of enzyme No. 10 was assayed at the same time the activities of systems 1–5 were determined.

** All systems were dialyzed, with stirring, against a total of 4.5 l of 0.1 M phosphate–0.05 M citrate buffer (pH 5.8) over a period of one week.

*** The enzyme was maintained in a functioning and reduced (bleached) state by the inclusion of $6.0 \cdot 10^{-3}$ M ascorbic acid in the dialyzing buffer and an adequate supply of air.

§ The lack of ascorbic acid in the dialyzing buffer and the presence of air maintained the enzyme in the usual blue (oxidized) state.

§§ This enzyme system had never functioned at any time.

§§§ Both Systems 1 and 2 were oxygenated before evaluation of color visually; no blue color could be regenerated in either case.

is not catalytically equivalent because in Systems 1 and 3 a significant fraction of the total Cu was tied up as Cu(I) in the BCS complex and yet the enzyme still demonstrated its full activity. Hence it may be concluded that a fraction close to 25% of the enzyme's prosthetic copper is not directly involved in either its oxidase activity or characteristic blue color.

It was next of interest to ascertain whether or not the complexing of this Cu(I) fraction by BCS had weakened the copper–protein bond sufficiently to render it dissociable by dialysis. Consequently, Systems 3, 4 and 5 were dialyzed with stirring against 8 600-ml changes of phosphate–citrate buffer (pH 5.8) over a one-week period at refrigerator temperature. Systems 1 and 2 were similarly dialyzed but the dialyzing buffer contained enough ascorbic acid to keep the enzyme continually bleached (*i.e.* in the reduced form) during the entire dialysis period. The activity and copper content values for each system were then determined in the usual manner. The results are shown in Columns 4 and 5 of Table IV*. The copper recovery values in Table IV show that during the dialysis there was no loss of copper from either the oxidized or reduced enzyme, even when nearly one-quarter of the copper was complexed with BCS (*cf.* Systems 1 and 3 with Systems 2 and 4). Hence it is clear that the Cu(I)–BCS complex is tightly bound to the protein and the resulting enzyme complex is stable to prolonged dialysis, as revealed by the copper assay and qualitatively by the persistence of the yellow color in both Systems 1 and 3 after dialysis (see Column 5 of Table IV). On the other hand, when a Cu(I)–BCS complex, produced with a standard Cu solution, was subjected to the same dialysis procedure, a rapid loss in color was observed, *i.e.* the free (non-protein-bound) BCS complex was readily diffusible. It is to be noted that the activity recovery values (see Column 4, Table IV) showed a marked dependence on the state of the enzyme during dialysis. In all cases (Systems 3–5) where the enzyme did not function during dialysis, there was a satisfactory (86–92%) recovery of original activity. However, in the systems (1 and 2) where the enzyme continually catalyzed the aerobic oxidation of ascorbic acid during dialysis, there was a pronounced loss of activity. This was not unexpected since it has been recently shown[7] that micro-amounts of H_2O_2 generated by a secondary catalytic reaction are responsible for the inactivation of ascorbate oxidase that is commonly observed as the enzyme functions in an aerobic system.

Relation to inactivation: It has been suggested[7] that the generation of H_2O_2 during the enzymatic oxidation of ascorbic acid is the result of a slow, secondary reaction involving non-enzymatic copper catalysis. That is to say, earlier studies of the reaction inactivation of ascorbate oxidase have focused attention on the possibility that all of the prosthetic copper of ascorbate oxidase is not functionally equivalent. The experimental findings described above have confirmed this view. Consequently, it was of interest to seek experimental evidence as to whether the Cu(I) fraction of the enzyme's prosthetic copper played a unique role in the reaction inactivation mechanism. With this view in mind, the following experiments were performed to investigate the reaction inactivation in two different enzyme systems: one containing the 25% non-enzymic Cu(I) fully complexed by BCS and the other without this

* During the prolonged dialysis some dilution of the enzyme systems was unavoidable. A suitable correction factor was obtained by determining the change in total protein concentration resulting from dialysis. For this purpose, a modification of the Folin–Lowry colorimetric procedure was employed[6].

BCS modification. The exact procedure, using enzyme No. 10, was as follows: the two ascorbate oxidase systems were set up, allowed to function and then regenerated by oxygenation as previously described, using 0.5 ml enzyme aliquots. They are hereafter designated as System A (with BCS-green) and System B (without BCS-blue). Suitable diluted aliquots of each system were then used in all subsequent parallel activity determinations. Four pairs of measurements were made, each of which differed only in the concentration of the enzyme employed to catalyze the substrate oxidation. The conditions in each of the four cases were such that reaction inactivation would be maximal, while the contribution of substrate inhibition to it would be negligible[7]. In general, this was accomplished by following manometrically the rate of oxygen uptake as a given quantity of the enzyme catalyzed the aerobic oxidation of several successive increments of ascorbic acid[8].

The results obtained using the smallest amount of enzyme are indicated graphically in Fig. 1 and the conditions of the experiment are described in the legend. With larger amounts of enzyme the results were similar, but the elapsed time for the inactivation effect to become apparent increased. In other words, the protective effect of the BCS modification against enzyme inactivation, which can be seen by comparing the oxygen uptake totals of Curves A and B, was most apparent with the lower concentrations of enzyme, *i.e.* when the rate of enzymatic oxidation was

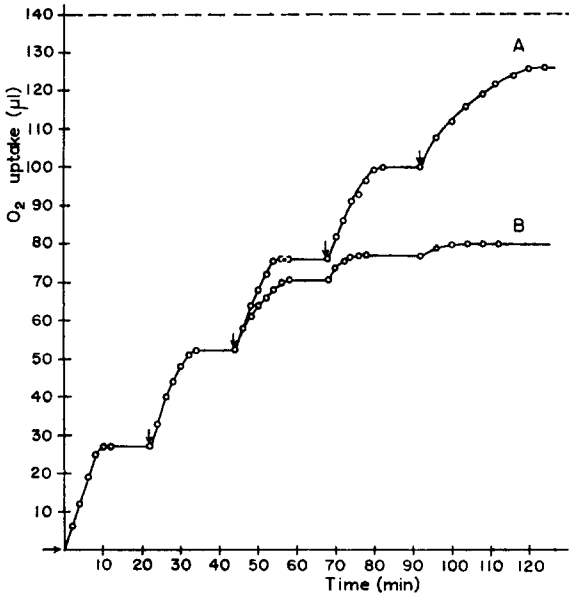

Fig. 1. Illustrating the activity of the BCS modified (Curve A) and the native enzyme (Curve B) resulting from the step-wise addition of substrate. Oxygen uptake was measured manometrically at 25° in $4 \cdot 10^{-2}$ M phosphate–citrate buffer (pH 5.8). Both reaction vessels contained initially the same amount of enzyme: that equivalent to $7.3 \cdot 10^{-4}$ µg of enzyme Cu. However, in the case corresponding to Curve A, approx. 25% of the enzyme's prosthetic copper had been complexed by BCS, as previously described. Each system also contained initially an 0.02-ml increment of ascorbic acid (0.44 mg = $1.0 \cdot 10^{-3}$ M) equivalent to an O_2 uptake of 28 µl for complete oxidation. The systems were made up to a final volume of 2.40 ml with copper-free water. Successive increments of ascorbic acid, each equivalent to the original, were added as indicated. The dotted line corresponds to the theoretical O_2 uptake for the five increments of ascorbic acid used.

relatively slow. In fact, when an amount of enzyme (equivalent to $23.0 \cdot 10^{-4}$ μg of copper) large enough to bring about a very rapid oxidation of ascorbic acid was employed, there was essentially no inactivation of the enzyme observed in either the native or the BCS-modified case. The data in Table V are presented to show the results obtained in terms of the initial reaction velocity, the inactivation O_2 total, and the inactivation time for an intermediate case of enzyme concentration (equivalent to $11.5 \cdot 10^{-4}$ μg of enzyme copper). It is apparent from the curves of Fig. 1 that the native enzyme (Curve B) was extensively inactivated, which resulted in only about 60% of the oxygen uptake corresponding to the complete oxidation of the total ascorbic acid added. Furthermore, it is apparent that the inactivation did not manifest itself significantly until about an hour of oxidation had elapsed, *i.e.*, during the oxidation of the third increment of ascorbic acid. This behavior is in accord with the observation made earlier in this laboratory[7] that the inactivation is due to the slow accumulation of microamounts of H_2O_2. Consequently, no inactivation was apparent or anticipated after the addition of the first or second increment, where there was more than adequate enzyme to rapidly and completely oxidize the ascorbic acid enzymatically. Instead, a period of time was required for the nonenzymatically involved prosthetic copper to slowly produce sufficient H_2O_2 to exert an inactivating influence. It is strikingly apparent from Curve A that each of the increments of ascorbic acid was completely oxidized, with essentially no enzyme inactivation. The total oxygen uptake of Curve A corresponded to more than 90% of that anticipated for complete substrate oxidation. In the case of Curve A the enzyme

TABLE V

SHOWING THE INITIAL REACTION VELOCITY, THE INACTIVATION TOTAL* AND THE TIME REQUIRED FOR INACTIVATION DURING THE INCREMENTAL ADDITION OF SUBSTRATE TO A FIXED AMOUNT OF THE BCS-MODIFIED (A) AND THE NATIVE ENZYME (B) UNDER AEROBIC CONDITIONS**

Ascorbic acid increment	*Initial reaction velocity (μl O_2/min)*		*Inactivation total (μl O_2)****		*Time required for inactivation (min)*	
	A	*B*	*A*	*B*	*A*	*B*
1	7.3	7.0	25.9	25.8	4	6
2	7.3	7.0	27.2	27.8	6	6
3	7.3	6.6	26.6	27.7	6	6
4	6.7	6.0	27.3	27.1	8	8
5	5.3	5.7	27.3	24.5	8	10
6	5.0	1.2	24.6	11.2	8	10
7	4.5	1.0	26.7	6.6	8	8
8	3.5	0	24.3	0	10	0
9§	3.0	—	26.0	—	12	—

* "Inactivation total" defined as an oxygen uptake lower than the theoretical value for complete oxidation of added substrate.

** The experimental conditions were the same as described in the legend of Fig. 1, except that the amount of enzyme was greater and corresponded to $11.5 \cdot 10^{-4}$ μg of prosthetic copper.

*** Each $1.0 \cdot 10^{-3}$ M increment of ascorbic acid corresponded to a theoretical O_2 uptake of 28 μl.

§ System A was allowed to incubate 3 h at 25° after the eighth increment. The native enzyme was still active with the ninth increment, although considerably reduced in catalytic efficiency.

had been previously treated with BCS and approx. 25% of its prosthetic copper existed (as previously shown) in a stable Cu(I)–BCS complex. Hence, it is evident that this same Cu(I) fraction of the enzyme's prosthetic copper must be involved in the slow, secondary production of the inactivating agent, H_2O_2. This same conclusion is justified in an even more striking and detailed way by the data presented in Table V, where the experiments involved larger amounts of enzyme and more increments of ascorbic acid.

In order to elucidate further the manner in which H_2O_2 exerts its inactivating effect on ascorbate oxidase, the following experiment, involving three systems was performed. In one case, the resting enzyme (no substrate present) was incubated with a small amount of H_2O_2 in a refrigerator for a period of 20 h prior to blue color and activity assay. The system was then exhaustively dialyzed and re-assayed for blue color, activity and Cu content. In a second case, the functioning enzyme (containing substrate, L-ascorbic acid) was identically incubated with H_2O_2 and likewise assayed before and after dialysis. The third case, which was used as a control, was a functioning enzyme system to which no H_2O_2 had been added. The experimental details are given in the legend to Table VI.

TABLE VI

A COMPARISON OF THE INACTIVATING EFFECT OF H_2O_2 ON FUNCTIONING *versus* RESTING ENZYME*

System	*Initial enzyme state*	*Incubation conditions*	*Before dialysis*		*After dialysis*§	
			*Absorbancy at 605 mμ***	*Activity recovered (%)*	*Absorbancy at 605 mμ***	*Activity recovered (%)*
1	Resting	With H_2O_2	0.066	97	0.057	82
2	Functioning	With H_2O_2	0.015***	21	0.023***	0
3	Functioning	No H_2O_2	0.062***	98	0.063***	86

* All systems contained a 0.5-ml aliquot of enzyme No. 10. Systems 2 and 3 were made $2.0 \cdot 10^{-2}$ M in ascorbic acid and Systems 1 and 2 were made $1.0 \cdot 10^{-2}$ M in H_2O_2, using suitable stock solutions, before all systems were made up to 2.5 ml with phosphate–citrate buffer (pH 5.6). The final prosthetic copper concentration corresponded to $4.6 \cdot 10^{-5}$ M.

** Theoretical absorbancy at 605 mμ for full blue color, 0.060.

*** Pure O_2 was bubbled into both systems for 5 min, followed by centrifugation to remove haze and debris.

§ All systems were exhaustively dialyzed with stirring at refrigeration temperature against 4 1-l changes of phosphate–citrate buffer (pH 5.8) over a 4-day period.

As can be seen from the table, H_2O_2 has no significant inactivating effect on the resting enzyme (System 1) under the conditions described in the legend. After the 20-h incubation period, the enzyme had lost none of its blue color and possessed 97% of the original activity. There was no significant loss in copper following dialysis and the slight losses in color and activity were undoubtedly due to environmental denaturation incurred during dialysis. On the other hand, when the enzyme was allowed to function by catalyzing the oxidation of ascorbic acid in the presence of H_2O_2 during the 20-h incubation period (System 2), there was a 79% loss in activity and a very appreciable loss in blue color. Furthermore, this H_2O_2-induced inactivation did not affect the Cu–protein bond because after dialysis essentially all the

prosthetic copper was found inside the dialysis sac. These results for System 2 should also be compared with the control, in which no H_2O_2 was present (System 3).

The fact that functioning ascorbate oxidase (System 2) is considerable more sensitive to the inactivating effect of H_2O_2 than is resting ascorbate oxidase (System 1) is the most salient feature of Table VI. It provides further evidence for the enhanced vulnerability of the enzyme while in the reduced form (wherein the functional prosthetic copper is presumably present as Cu(I)). It should be recalled that earlier studies in these laboratories[2,9] have revealed that the copper of ascorbate oxidase is rapidly exchangeable with radioactive ^{64}Cu ions to a significant extent only when the enzyme is functioning (*i.e.* when the functional copper is presumably cycling reversibly between its two oxidation states). The above results involving H_2O_2 show again that the susceptibility of the enzyme to attack by other species is greater when the enzyme is performing its catalytic function than when it is resting. As pointed out previously, it seems likely that in addition to a reversible change in the oxidation state of copper, a reversible structural modification in the protein moiety of the enzyme also occurs during its catalytic function. Thus it will be recalled that the Cu(I)-chelating agent, BCS, was found to be totally unreactive with the resting enzyme[1], while the 25% Cu(I) fraction of the prosthetic copper (presumably non-functional) became available to BCS when the enzyme functioned. This behavior, therefore, would support the premise that a critical alteration in the enzyme's conformation occurs during oxidase function, of such a nature as to expose the previously inaccessible non-functional Cu(I) sites to the BCS reagent. Such a modification in the protein's conformation might expose functional groups sensitive to peroxide oxidation and thereby account for the observed inactivation. On the other hand, it may be that the H_2O_2inactivating effect involves a direct attack on the enzymatically functional Cu(I) in the reduced enzyme to produce a stable complex that is no longer reversible to the active blue Cu(II) form by molecular oxygen.

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