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Concerning the Quaternary Structure of Ascorbate Oxidase†

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ABSTRACT: When exposed to either sodium dodecyl sulfate or guanidinium chloride, ascorbate oxidase (molecular weight 140,000, 8–10 copper atoms) dissociates into two copper-free subunits, each of about half the molecular weight of the native enzyme. Removal of the denaturant results in aggregation of the subunits and it has not been found possible to restore the copper and activity. All evidence indicates that the two subunits of molecular weight 65,000, comprising the native enzyme, are identical. Treatment of the native enzyme with sodium dodecyl sulfate and either 2-mercaptoethanol or 2-mercaptoethylamine results in two new bands on sodium dodecyl sulfate electrophoresis. The components corresponding to these bands have molecular weights of 38,000 (A chain) and 28,000 (B chain). The same two components are obtained when disulfides of the native enzyme are cleaved with cyanide. It is proposed, therefore, that the native enzyme is a tetramer composed of two A chains and two B chains. Each AB pair is cross-linked by one or two disulfide bonds. The two resulting subunits are held together by noncovalent forces. A comparison of the spectral properties of ascorbate oxidase and *Polyporus* laccase indicates that both enzymes contain the same three types of cupric copper, but that ascorbate oxidase contains twice as much of both the type 1 and the electron spin resonance nondetectable cupric copper per mole of protein. This premise, plus the similiarity of the reactions catalyzed by the two enzymes, suggests that ascorbate oxidase has two "laccase-type" active sites per molecule of molecular weight 140,000. This hypothesis is consistent with the proposed quaternary structure of ascorbate oxidase, since each subunit can contain one active site.

Ascorbate oxidase (EC 1.10.3.3, 1-ascorbate:O_2 oxidoreductase) belongs to the group of enzymes usually referred to as the "blue" copper oxidases. The two other members of this group are laccase and ceruloplasmin. All these enzymes possess very similar spectral properties related to copper, and a recent review of the state of copper in these enzymes has appeared (Malkin and Malmström, 1970). *Polyporus* laccase remains the best understood member of the group with respect to the types of copper found in the "blue" oxidases.

Ascorbate oxidase has a molecular weight of 140,000 and a copper content of 8–10 atoms per molecule (Lee and Dawson, 1973a). The native enzyme has no detectable sulfhydryl groups, but on treatment with a suitable denaturant 10 sulfhydryl groups and four disulfide bonds are exposed (Stark and Dawson, 1962). When titrated to pH 11, ultracentrifuge experiments indicate a decrease in molecular weight to about half that of the native enzyme (Clark *et al.*, 1966), suggesting that ascorbate oxidase possesses a quaternary structure.

There appears to be a proportionality between copper content and molecular weight of the "blue" copper oxidases. The results of this investigation provide an explanation for this observation and also the many other similarities of these enzymes. Failure to appreciate the instability of disulfide bonds at high pH and the use of average molecular weights of apparently heterogeneous protein samples complicate the interpretation of earlier work on the quaternary structure of *Polyporus* laccase (Butzow, 1968) and ceruloplasmin (Poillon and Bearn, 1966).

Experimental Section

Materials. Ascorbate oxidase was prepared from zucchini squash (*Cucurbita pepo medullosa*) as previously described (Lee and Dawson, 1973a), and was homogeneous by the criteria of disc electrophoresis at pH 8.0 and 9.5 at gel concentrations of 7.5 and 5.0%.

Protein was determined by the Lowry method (Lowry *et al.*, 1951; Lee and Dawson, 1973a). Copper determinations were done according to Stark and Dawson (1958). Enzymatic activity was measured at 25° under concentration and buffer conditions previously described (Dawson and Magee, 1957), in a reaction volume of 1.5 ml using a Clark oxygen electrode on a Gilson Model KM Oxygraph.

All aqueous solutions were prepared using deionized water. Guanidinium chloride was obtained from Fisher and was recrystallized twice from absolute ethanol–benzene (2:1 v/v). Urea was Mallinckrodt reagent grade. It was recrystallized from 95% ethanol. A 10 M aqueous urea solution was prepared and deionized with Amberlite MB-3 just before use. Sodium dodecyl sulfate was obtained from Matheson Coleman and Bell. Bio-Gel A-5M 100–200 mesh was supplied by Bio-Rad Labs. All other chemical were of the highest purity available.

Methods. Electrophoresis. Disc electrophoresis at pH 9.5 was carried out according to Davis (1964), except that a sample gel was not used and, in some cases, no stacking gel was used. Electrophoresis at pH 8.0 was performed using a buffer system described by Williams and Reisfeld (1964).

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Staining was done with either Coomassie Blue or Buffalo Black and destaining was by diffusion.

Sodium dodecyl sulfate electrophoresis was carried out using the procedure and solutions recommended by Weber and Osborn (1969), with slight modification. Gels with a final acrylamide concentration of 7.5% were used. The higher mobilities that were obtained, compared to the 10% gels of Weber and Osborn, allowed a more accurate determination of molecular weights in the range 60–100,000.

Gel Filtration. All gel filtration was carried out using Bio-Gel A-5M 100–200 mesh. Columns were 1.3 × 100 cm and a bed height of about 90 cm was used in all experiments. Fractions of 1.5 ml size were collected by drop counting or a volumetric device.

Samples were mixed with blue dextran and Dnp-alanine before application to the column. The absorbance of each fraction was measured on a Beckman DU spectrophotometer at 280, 360, and 630 nm to determine protein, Dnp-alanine, and blue dextran, respectively. From these data K_d[1] values were calculated as previously described (Fish *et al.*, 1969).

N-Terminal Determination. Dansyl chloride was used in an attempt to determine the N-terminal groups of ascorbate oxidase. Dansylation in 8 M urea was performed under the conditions previously described (Gros and Labouesse, 1969). Dansylation in 1.0% sodium dodecyl sulfate was done by dissolving about 8 mg of protein in 0.75 ml of 1.0% sodium dodecyl sulfate in 0.1 M sodium phosphate (pH 8.2). After a 3-hr incubation at 40°, the solution was cooled to room temperature and 0.2 ml of dimethylformamide was added followed by 0.2 ml of dansyl chloride (10 mg/ml in acetone). After 15 min, another 0.2 ml of dansyl chloride solution was added and the reaction was allowed to proceed for another 15 min. The protein was precipitated by adding 10 ml of acetone.

Samples of dansylated ascorbate oxidase prepared in either 1.0% sodium dodecyl sulfate or 8 M urea were hydrolyzed in constant boiling HCl under vacuum for 4, 6, and 18 hr at 110°. After removal of the HCl over NaOH pellets *in vacuo*, the residue was suspended in 0.1 M sodium citrate (pH 3.5) and extracted several times with ether. Both ether and aqueous phases were examined by thin-layer chromatography on Eastman silica gel plates (6061) in five different solvent systems.

Dansyl derivatives of ammonia and the 19 amino acids and two hexosamines comprising ascorbate oxidase were prepared and used as standards.

Spectra. Two 1-cm path length cuvettes were filled under nitrogen with a solution of ascorbate oxidase. One or two crystals of ascorbic acid were added to one of the cuvettes to reduce the enzyme (evidenced by complete bleaching of the blue color). The cuvettes were sealed and the spectrum of the oxidized minus reduced enzyme was recorded on a Unicam SP 800A recording spectrophotometer from 300 to 700 nm. Absorbance values at the maxima were measured on a Beckman DU spectrophotometer and used to calculate molar extinction coefficients.

Treatment with Sodium Dodecyl Sulfate. Standards for sodium dodecyl sulfate electrophoresis were incubated in 1.0% sodium dodecyl sulfate and 1.0% 2-mercaptoethanol in 0.01 M sodium phosphate (pH 7.0) for 3 hr at 40° prior to electrophoresis. Ascorbate oxidase was incubated in the same buffer but without 2-mercaptoethanol. In subsequent experiments, the concentration of sodium dodecyl sulfate was varied from 0.1 to 10% and the incubation time from 1 to 18 hr.

Samples of ascorbate oxidase for gel filtration were first exposed to 5% sodium dodecyl sulfate in 0.1 M sodium phosphate (pH 7.0) and then dialyzed against a large volume of 0.02 M sodium phosphate (pH 7.0), 0.1% in sodium dodecyl sulfate. Standards were treated in the same manner except that all buffers were 1.0% in 2-mercaptoethanol. Gel filtration was carried out in 0.1% sodium dodecyl sulfate in 0.02 M sodium phosphate (pH 7.0).

Treatment with Urea. Ascorbate oxidase was dissolved in 8 M urea in 0.02 M potassium phosphate (pH 7.0). The buffer was flushed with nitrogen and the pH checked before use. After incubation under nitrogen for 3 hr at 40°, the sample was analyzed by gel filtration in 8 M urea. Samples were also analyzed by disc electrophoresis. Protein standards for gel filtration were reduced in the same buffer described above containing 8 M urea and 1.0% 2-mercaptoethanol.

Treatment with GuCl. Suitable protein standards in 5 M GuCl were reduced with 2-mercaptoethanol and then carboxymethylated as has been described (Fish *et al.*, 1969). Gel filtration of ascorbate oxidase was done in 5 M GuCl both with and without prior carboxymethylation.

Reduction of Disulfides. Ascorbate oxidase was incubated in sodium dodecyl sulfate as described above except the incubation mixture was made 1–10% in either 2-mercaptoethanol or 2-mercaptoethylamine. Incubation times were varied from 1 to 18 hr. Samples were analyzed by sodium dodecyl sulfate electrophoresis using standards prepared as described above.

Disulfide bonds in ascorbate oxidase were also cleaved with cyanide. About 1 mg of ascorbate oxidase in 1.0 ml of 0.01 M sodium phosphate (pH 7.0) was dialyzed against 200 ml of 0.1 M NaCN in 0.2 M McIlvaine's buffer (pH 7), 0.05% in sodium dodecyl sulfate, at 4° for 24 hr. The cyanide was removed by dialysis against 0.01 M sodium phosphate (pH 7.0), 0.1% in sodium dodecyl sulfate, and the resulting cyanide-free solution was examined by sodium dodecyl sulfate electrophoresis.

Hydroxylamine Treatment. A 2–3-mg sample of ascorbate oxidase in 0.01 M Tris-acetate (pH 10.0), 8 M in urea, was treated with hydroxylamine as previously described (Blumenfeld *et al.*, 1965). After dialysis at 4° against 0.01 M sodium phosphate (pH 7.6) to remove excess hydroxylamine the sample was incubated in 0.01 M sodium phosphate (pH 7.0), 1.0% in both sodium dodecyl sulfate and 2-mercaptoethanol and examined by sodium dodecyl sulfate electrophoresis.

Removal of Sodium Dodecyl Sulfate from Ascorbate Oxidase. Ascorbate oxidase was treated with 1.0% sodium dodecyl sulfate in 0.05 M Tris-acetate (pH 7.6) followed by removal of the sodium dodecyl sulfate as described by Weber and Kuter (1971). The resulting sodium dodecyl sulfate free solution was clear and colorless. A sample was subjected to disc electrophoresis at pH 9.5 using gels of 8.80, 7.82, 7.04, and 6.40% acrylamide concentration. All gels were run simultaneously and the distance of migration of each band at all four gel concentrations was measured.

To investigate the renaturation of ascorbate oxidase, the sodium dodecyl sulfate free enzyme in 6 M urea was dialyzed against 0.2 M McIlvaine's buffer (pH 5.6) and treated with a cuprous reagent (Chang, 1970) to restore the copper. After removal of excess copper by dialysis, the activity of the renatured enzyme was measured on the Oxygraph.

[1] Abbreviations used are: Dnp, 2,4-dinitrophenyl; GuCl, guanidinium chloride; Tris, tris(hydroxymethyl)aminomethane; K_d, distribution coefficient; EDTA, ethylenediaminetetraacetate; esr, electron spin resonance.

TABLE I: Electrophoretic Mobility of Reduced and Unreduced Proteins in Sodium Dodecyl Sulfate.[a]

Protein	Disulfides per 100 Residues	Electrophoretic Mobility		Ratio of Mobilities Disulfides Intact/ Disulfides Reduced	Ratio of Stokes' Radii[c] Disulfides Reduced/ Disulfides Intact
		Disulfides Intact	Disulfides Reduced[b]		
Lysozyme	3.1	0.956	0.936	1.02	
Chymotrypsinogen	2.0	0.759	0.745	1.02	1.36
Pepsin		0.595	0.580	1.03	
Ovalbumin	0.3	0.548	0.530	1.04	1.07
Bovine serum albumin	2.8	0.408	0.359	1.14	1.19

[a] All mobilities were measured relative to the marker dye, Bromphenol Blue. [b] Disulfides were reduced by incubation in 1.0% 2-mercaptoethanol at 40° for 2 hr. [c] Values for the Stokes' radii of reduced and unreduced proteins in sodium dodecyl sulfate were taken from Fish *et al.* (1970).

Results

Sodium Dodecyl Sulfate Treatment. When ascorbate oxidase is treated with sodium dodecyl sulfate in the absence of a reducing agent, a single band is observed on sodium dodecyl sulfate electrophoresis. The molecular weight of the protein corresponding to this band is 68,000 ± 2000. Variations in the concentration of sodium dodecyl sulfate (0.1–10%), and the time of incubation (1–18 hr), have no effect on the electrophoretic results. These results suggest that the enzyme is composed of two half molecular weight subunits.

However, this molecular weight value was obtained by comparisons with standard proteins that had been reduced (all disulfide bonds cleaved). Since ascorbate oxidase has been shown to contain disulfide bonds (Stark and Dawson, 1962) (four per enzyme molecule or two per subunit, assuming two identical subunits) and these disulfide bonds were not cleaved in this molecular weight determination, the apparent molecular weight of 68,000 required further experimental examination.

To investigate the effect of disulfide bonds on sodium dodecyl sulfate electrophoretic mobility, the mobilities of several proteins were measured both with and without reduction of their disulfide bonds. All data were obtained in a single electrophoresis experiment to avoid possible variations in gel composition between different sets of gels (Table I). Of the five proteins listed in Table I, four have virtually identical mobilities whether or not disulfides are reduced. The ratio of mobilities does not parallel either the disulfide content or the inverse ratio of the Stokes' radii. For the first four proteins, the same molecular weight would have been calculated for either reduced or unreduced samples.

Failure to reduce disulfides apparently decreases the Stokes' radius of the protein–sodium dodecyl sulfate complex (Fish *et al.*, 1970), and results in a decreased binding ratio of sodium dodecyl sulfate to protein (Pitt-Rivers and Impiombato, 1968). Since the net charge on the complex results almost entirely from the binding of dodecyl sulfate anion, a decreased net charge resulting from a decreased binding ratio may act to counter the smaller size of unreduced protein–sodium dodecyl sulfate complexes, thus rendering electrophoretic mobility insensitive to the presence or absence of disulfides. It may be concluded therefore that, in selected cases, electrophoretic mobility of unreduced protein–sodium dodecyl sulfate complexes can be used to obtain reliable molecular weight values.

The molecular weight of ascorbate oxidase calculated from gel filtration data in 0.1% sodium dodecyl sulfate was found to be 63,000. Phosphorylase A, bovine serum albumin, ovalbumin, pepsin, and lysozyme were used as standards.

Urea Treatment. Gel filtration of ascorbate oxidase in 8 M urea gave a single, sharp, symmetrical peak corresponding in molecular weight to 110,000, as judged by comparison with known protein standards (details are available in Strothkamp, 1973). No other protein peaks were detected. The single peak would indicate that dissociation of the enzyme does not occur in 8 M urea. Disc electrophoresis also gave a single band. The addition of EDTA to the electrophoresis incubation mixture had no effect on the results.

GuCl Treatment. In analyzing the 5 M GuCl gel filtration data, two relationships between molecular weight and K_d were used. Plots of both log molecular weight *vs.* K_d and $K_d^{1/3}$ *vs.* (molecular weight)$^{0.555}$ (Fish *et al.*, 1969) yielded straight lines for the standards. Both calibration curves yield a molecular weight of 58,000 for ascorbate oxidase.

When the oxidase was S-carboxymethylated in GuCl, the gel filtration pattern revealed a symmetrical peak corresponding to a single component of molecular weight 58,000. The uncarboxymethylated sample also yielded a gel filtration pattern corresponding to 58,000 but the peak was not symmetrical. The lack of symmetry indicated the presence of a small amount of higher molecular weight material, probably arising *via* oxidation of sulfhydryl groups.

Disulfide Reduction. Sodium dodecyl sulfate electrophoresis of ascorbate oxidase after treatment with 2-mercaptoethanol to effect disulfide reduction showed three bands corresponding to components having molecular weights of 68,000, 38,000, and 28,000. Variations in the concentration of 2-mercaptoethanol and sodium dodecyl sulfate as well as the time of incubation caused only minor variations in the relative intensities of the three bands. The two lowest molecular weight components always exhibited bands of about equal intensity. When 2-mercaptoethylamine was used as the reducing agent, the same three bands were obtained. However, the highest molecular weight band was greatly reduced in intensity with a corresponding increase in intensity of the other two bands.

Previous work had shown that the conditions of cyanide treatment used to prepare apoascorbate oxidase result in quantitative reduction of disulfide bonds (Chang, 1970). In the present investigation, ascorbate oxidase was exposed to these identical conditions except for the addition of 0.05% sodium dodecyl sulfate. When examined by sodium dodecyl sulfate electrophoresis, only the two bands corresponding to

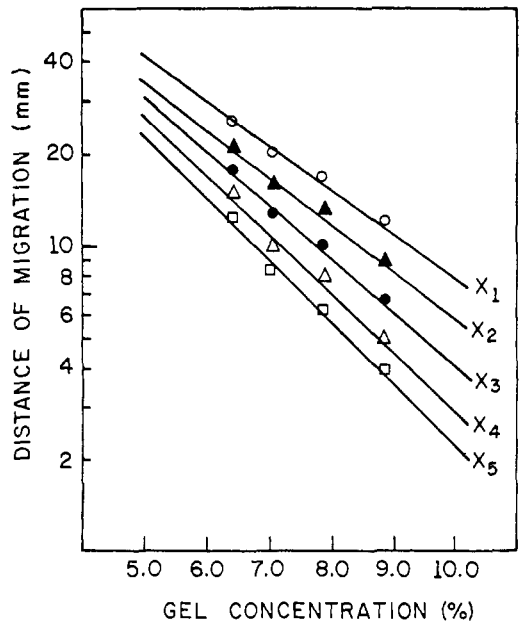

FIGURE 1: A plot of the distance of migration *vs.* the acrylamide gel concentration for each of the five bands of the aggregation pattern obtained on disc electrophoresis. The best straight line through each set of points was determined by the method of least squares. The symbols X_1, X_2... are defined in the text.

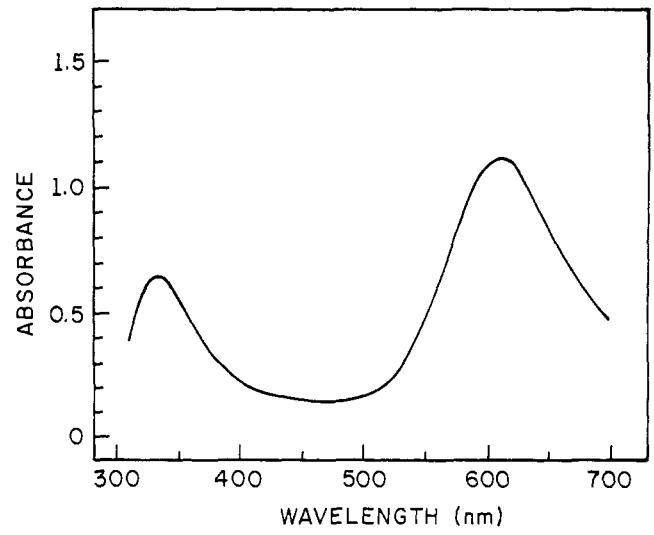

FIGURE 2: The oxidized minus reduced spectrum of ascorbate oxidase in 0.01 M potassium phosphate (pH 7.6). The enzyme samples used to obtain this spectrum as well as the data in Table IV had a copper content of 0.42%. This corresponds to an average of 9 ± 1 copper atoms per molecule. There was no change in the spectrum over the pH range 5.7–8.5.

the lowest molecular weights were seen. The 68,000 band was completely absent and the molecular weights of the other two components were the same as determined after the above thiol treatment.

N-Terminal Determination. Analysis of both the aqueous and ether extracts of the ascorbate oxidase hydrolysate gave no α-dansyl derivative. Dansylation in both 8 M urea and 1.0% sodium dodecyl sulfate gave the same results. The only fluorescent products were ε-dansyllysine, *O*-dansyltyrosine, dansylamide, and dansylsulfonic acid.

Hydroxylamine Treatment. Sodium dodecyl sulfate electrophoresis of hydroxylamine-treated ascorbate oxidase and a control resulted in identical electrophoresis patterns.

Removal of Sodium Dodecyl Sulfate from Ascorbate Oxidase. The electrophoretic pattern of ascorbate oxidase in 6 M urea after removal of sodium dodecyl sulfate indicated aggregation of the subunit had occurred. Removal of urea by dialysis resulted in a slight amount of precipitation. Attempts to restore the copper and activity of the sodium dodecyl sulfate free protein using the same procedure employed for apoascorbate oxidase failed to give any measurable activity.

Each of the five bands in the electrophoretic pattern obtained after sodium dodecyl sulfate removal was given a symbol for identification purposes. In order of decreasing mobility they are, X_1, X_2, X_3, X_4, and X_5. A plot of log distance of migration *vs.* gel concentration for each of these five bands gave a straight line (Figure 1). The lines intersect at a gel concentration of about 1.7%.

The slope of each line is referred to as the retardation coefficient, K_R (Rodbard and Chramback, 1970). Values of K_R for each of the five bands and also for "native" ascorbate oxidase in 6 M urea were calculated.

This same pattern of aggregation results when ascorbate oxidase in 5 M GuCl is dialyzed against buffer to remove the GuCl and then examined by disc electrophoresis. When this aggregated material is dissolved in 1.0% sodium dodecyl sulfate and subjected to sodium dodecyl sulfate electrophoresis, a single band is found corresponding to the half molecular weight subunit.

Visible Absorption Spectrum of Ascorbate Oxidase. The oxidized minus reduced spectrum of ascorbate oxidase from 300 to 700 nm is shown in Figure 2. The band at 335 nm, previously seen only as a shoulder on the 280-nm aromatic absorption, is well resolved. Table II lists extinction coeffi-

TABLE II: Spectral Properties of *Polyporus* Laccase and Ascorbate Oxidase.

			Type 1 Copper			Diamagnetic Copper		
	Mol Wt	Cu atoms/ Molecule	λ_{max} (nm)	ϵ^a	Ratio of ϵ^d	λ_{max} (nm)	ϵ	Ratio of ϵ^d
Laccase[b] (*Polyporus* versicolor)	62,000	4	610	4600	1.0	330	3000	1.0
Ascorbate oxidase (squash)	140,000	8–10	610	9600	2.1	335	5500	1.8
Ascorbate[c] oxidase (cucumber)	132,000	8	607	9700	2.1			

[a] Extinction coefficient per mole of protein. [b] Taken from Malkin and Malmström (1970). [c] Taken from Nakamura *et al.* (1968). [d] The ratio of each extinction coefficient to that of *Polyporus* laccase.

TABLE III: The Molecular Weight of Ascorbate Oxidase under Various Conditions.

Type of Treatment	Method of Molecular Weight Determination	Molecular Weight(s) Obtained
0.1–10% SDS[d]	SDS electrophoresis	68,000
0.1% SDS	Gel filtration	63,000
5 M GuCl	Gel filtration	58,000
SDS plus	SDS electrophoresis	28,000
2-mercaptoethanol or		38,000
2-mercaptoethylamine		69,000
SDS plus cyanide	SDS electrophoresis	28,000
		38,000
8 M urea	Gel filtration	110,000
pH 11[a]	Sedimentation equilibrium	65,000
Native ascorbate oxidase[b]	Sedimentation and diffusion	140,000
Native ascorbate oxidase[c]	Gel filtration	132,000

[a] Taken from Clark *et al.* (1966). [b] Taken from Lee and Dawson (1973a). [c] Taken from Wilson (1966). [d] SDS, sodium dodecyl sulfate.

cients, expressed per mole of protein, obtained in this work as well as values for cucumber ascorbate oxidase (Nakamura *et al.*, 1968) and *Polyporus* laccase (Malkin and Malmström, 1970).

Discussion

The Quaternary Structure of Ascorbate Oxidase. Table III shows the molecular weights obtained when ascorbate oxidase is subjected to various treatments. The data for 8 M urea indicate that ascorbate oxidase does not undergo dissociation in that solvent. As pointed out earlier, the presence of disulfide bonds in ascorbate oxidase very likely results in a smaller effective size as compared to a fully reduced protein of equal molecular weight. Thus, the apparent molecular weight (in 8 M urea) obtained by gel filtration (110,000) is considerably lower than the accepted value for the native enzyme from sedimentation and diffusion (140,000).

In 5 M GuCl, where reduced proteins behave as random coils (Tanford *et al.*, 1967), this same effect due to disulfide bonds may lead to an apparent molecular weight (*via* gel filtration) lower than the true value. This may explain the somewhat low value of 58,000 obtained in this solvent. Our results with sodium dodecyl sulfate electrophoresis (see Table II) indicate that electrophoretic mobility is not very sensitive to the presence or absence of disulfide bonds. Therefore, the molecular weight (68,000) of ascorbate oxidase measured by this technique is not subject to this uncertainty. We believe that the subunit of molecular weight 65,000 first observed in systems at pH 11 (Clark *et al.*, 1966) is the same species that we have observed in both sodium dodecyl sulfate and GuCl treated ascorbate oxidase. Neglecting the 58,000 value obtained in GuCl (for reasons indicated above), the data of Table III indicate a molecular weight of about 65,000 for this subunit. All of our data are consistent with a model for ascorbate oxidase of two identical subunits of molecular weight close to 65,000. Disc electrophoresis experiments have confirmed the homogeneity of the subunit.

Reduction of disulfide bonds in the subunit by thiol treatment in sodium dodecyl sulfate yields two components of molecular weights 38,000 and 28,000. This suggests that the subunit of ascorbate oxidase is composed of two different polypeptide chains cross-linked by one or more disulfide bonds. These two fragments are referred to as the A and B chains to distinguish them from the half molecular weight subunit.

2-Mercaptoethanol has been found in this study to be a poor reducing agent for the disulfides of ascorbate oxidase. We have no explanation for our observation that 2-mercaptoethylamine is markedly superior to 2-mercaptoethanol in ability to reduce the disulfides of ascorbate oxidase.

Cyanide treatment reduces all of the disulfides of ascorbate oxidase (Chang, 1970). After cyanide treatment, only the A and B chains are present. Under conditions of higher temperature cyanide treatment has been shown to also result in cleavage of peptide bonds of such polypeptide chains (Spande *et al.*, 1970). No evidence for such cleavage has been observed in this investigation. The fact that full activity and copper content can be restored to apoascorbate oxidase prepared by cyanide treatment under conditions which, except for the absence of sodium dodecyl sulfate, are the same as used in this investigation is strong evidence that peptide bonds are not cleaved. The molecular weights of the A and B chains of ascorbate oxidase, obtained by reduction with either of the thiols or with cyanide, are the same. This provides additional evidence for the lack of peptide bond cleavage by cyanide under these conditions. Treatment of bovine serum albumin with cyanide under these conditions resulted in the appearance of four new sulfhydryl groups per molecule with no change in molecular weight, as determined by sodium dodecyl sulfate electrophoresis.

The sum of the molecular weights of the A chain (38,000) and the B chain (28,000) is in excellent agreement with the molecular weight of the subunit (65,000). Therefore, the subunit must be composed of one A chain and one B chain. Since the native enzyme has four disulfide bonds and the evidence indicates two identical subunits, it follows that each subunit has two disulfide bonds. At least one of these disulfide bonds must cross-link the A and B chains. It appears therefore that the native enzyme is a tetramer, being composed of two identical subunits, each of which is made up of one A and one B chain.

Hydroxylamine Treatment. The treatment of ascorbate oxidase with hydroxylamine does not result in any change of the sodium dodecyl sulfate electrophoretic pattern. This result indicates that "ester-like" interchain bonds (Blumenfeld *et al.*, 1965) do not exist in ascorbate oxidase. Therefore, if the A or B chains contain covalent cross-links between smaller polypeptides, they are not sensitive to hydroxylamine. All available evidence indicates that the A and B chains represent single polypeptide chains.

N-Terminal Determination. Because of the usefulness of an N-terminal determination in investigating the quaternary structure of proteins, an attempt has now been made to detect N-terminal amino groups in ascorbate oxidase denatured with urea or sodium dodecyl sulfate. Both denaturants dissociate the copper from ascorbate oxidase and increase the reactivity of the side-chain groups (Stark and Dawson, 1962). It has been observed that dansylation of the oxidase in both 8 M urea and 1.0% sodium dodecyl sulfate fails to yield any α-dansyl derivatives. Therefore, it has been confirmed that no

free α-amino groups exist in ascorbate oxidase. As suggested earlier (Kirschenbaum and Dawson, 1963), it appears that the terminal amino group of each polypeptide chain is covalently derivatized.

Removal of Sodium Dodecyl Sulfate. If one compares the five bands obtained on electrophoresis of ascorbate oxidase after the removal of sodium dodecyl sulfate, a regular pattern is seen. In going from X_1 to X_5, the relative intensity of each band and the distance between successive bands decreases, an indication of aggregation. This aggregation, occurring in the absence of sodium dodecyl sulfate, cannot be the result of intermolecular disulfide bond formation because, as pointed out earlier, the aggregated material gives only the subunit on sodium dodecyl sulfate electrophoresis.

The relationship between mobility and gel concentration was studied over a range of gel concentrations. Figure 1 clearly shows that the mobilities are converging. When extrapolated, the lines intersect at a gel concentration of about 1.7%. This is to be expected for an oligomeric series and has been observed for bovine serum albumin (Hendrick and Smith, 1968) and β-tyrosinase (Jolley *et al.*, 1969).

A comparison of retardation coefficients (Table III) for each species with that of "native" ascorbate oxidase in 6 M urea indicates that either component X_1 or X_2 could correspond to the native molecular weight. Urea has been shown not to cause dissociation of ascorbate oxidase. Whether X_1 is the subunit of ascorbate oxidase or a dimer of the subunit, the fact that each successive degree of aggregation gives a single electrophoretic band, under conditions where charge differences would be detected, argues in favor of subunit homogeneity.

Spectral Properties. Absorption spectra have been published for *Polyporus* laccase (four copper atoms per molecule), and the 600- and 330-nm bands have been assigned to specific copper sites (Malkin *et al.*, 1969). A single, esr detectable cupric ion, referred to as a type 1 or "blue," is responsible for the absorption at 600 nm. The 330-nm chromophore is believed to result from two cupric ions which are esr nondetectable. The fourth cupric ion in laccase, referred to as type 2 or "non-blue," makes no detectable contribution to the visible absorption spectrum.

The oxidized minus reduced spectrum of ascorbate oxidase clearly shows the near ultraviolet band at 335 nm. The entire spectrum from 300 to 700 nm is virtually identical with that of *Polyporus* laccase. The comparison of extinction coefficients given in Table II would suggest that, per mole of protein, ascorbate oxidase contains twice as much type 1 copper and also twice as much esr nondetectable cupric copper as does laccase. This is consistent with the experimental observation that the number of copper atoms per molecule of ascorbate oxidase is about double that of *Polyporus* laccase.

In view of the similarity of the reactions catalyzed by laccase and ascorbate oxidase (Mason, 1965), one might infer that the two enzymes possess very similar active sites, at least in respect to the number and types of copper atoms present at the site. The spectral properties of laccase and ascorbate oxidase are consistent with this inference.

The esr spectra of ascorbate oxidase (Lee and Dawson, 1973b) indicate the presence of both type 1 and type 2 cupric ions, and also account for the presence of copper in the esr nondetectable state. Studies concerning the type 2 copper are currently in progress in this laboratory.

An ascorbate oxidase protein having two "laccase-type" active sites per molecule of molecular weight 140,000 would be consistent with all of these observations. It would also be consistent with the model of the quaternary structure of ascorbate oxidase herein proposed, since each of the two identical subunits could contain one active site. It would, of course, be of considerable interest to compare the structure of the subunit of ascorbate oxidase (molecular weight 65,000) with *Polyporus* laccase (molecular weight 62,000).

Considerable disagreement exists at the present time concerning the molecular weight, copper content, and quaternary structure of ceruloplasmin (Kasper and Deutsch, 1963; Magdoff-Fairchild *et al.*, 1969; Ryden, 1972). In this connection, it is of interest that the same three types of copper found in ascorbate oxidase and laccase are also present in ceruloplasmin (Carrico *et al.*, 1971).

It seems likely that these three "blue" oxidases may have evolved from a common ancestor. Ascorbate oxidase and ceruloplasmin may have an evolutionary relationship to *Polyporus* laccase not unlike the proposed relationship of hemoglobin to myoglobin.

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Structure and Activity of Methylated Horse Liver Alcohol Dehydrogenase†

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ABSTRACT: Horse liver alcohol dehydrogenase is modified by formaldehyde in the presence of sodium borohydride. This reagent methylates 26 of its 60 lysine residues. Methylation of the enzyme causes increased activity as well as conformational changes. The modified liver alcohol dehydrogenase has a larger Stokes radius and exhibits a slight blue shift with an increased absorbance in the ultraviolet spectrum, an increased trough at 233 nm in the optical rotatory dispersion spectrum, and an increased stability toward heat and mercurial inactivations. Methylation partially desensitizes liver alcohol dehydrogenase to substrate inhibition by ethanol. NADH protects 4 of the 26 lysine residues from modification. Possible functions of lysine residues are discussed.

Kosower (1962) postulated that the positively charged ϵ-amino group of lysine found in dehydrogenases was involved in the binding of coenzyme. Chemical modifications of horse liver alcohol dehydrogenase (EC 1.1.1.1) with methyl picolinimidate (Plapp, 1970) and pyridoxal phosphate (McKinley-McKee and Morris, 1972) indicate that one or more lysine residues are at or near the site where the coenzyme is bound. Methyl picolinimidate modifies 50 of the 60 lysine residues causing an overall increase in enzyme activity. Pyridoxal phosphate, in the presence of sodium borohydride, modifies 11 residues causing an overall decrease in activity. The lack of the selectivity of these modifications and the difference in their effect on liver alcohol dehydrogenase raise the question concerning the specific function of the lysine residue in the dehydrogenase.

Formaldehyde, in the presence of sodium borohydride, was used as a specific reagent for amino groups (Means and Feeney, 1968; Rice and Means, 1971). In modifying liver alcohol dehydrogenase, this reagent may minimize structural perturbation due to the electronic neutrality and relatively small bulk of the methyl group introduced. Methylation of the dehydrogenase increases the activity[1] with concurrent conformational changes. Chemical and physical studies of the methylated dehydrogenase were carried out to assess the function of lysine residues in the dehydrogenase and the factors which are responsible for the enhancement of enzyme activity.

Materials and Methods

Chemicals. Horse liver alcohol dehydrogenase, NAD^+, NADH, and *p*-ClHgBzO were obtained from Sigma Chemical Co. Marker proteins were purchased from Worthington Biochemical Corp. Other chemicals were products of Fisher Chemical Co. Absolute ethanol and formaldehyde were redistilled prior to use.

Methylation of Liver Alcohol Dehydrogenase. Methylation of the dehydrogenase with formaldehyde in the presence of sodium borohydride was carried out as described by Means and Feeney (1968). One milliliter of freshly prepared sodium borohydride solution, 2.5 mg/ml, was mixed with a solution containing 25 mg of the dehydrogenase in 0.1 M sodium pyrophosphate buffer (pH 9.0). Portions (10 μl) of 9.25% formaldehyde solution were added every 10 min for 60 min. After the final addition of formaldehyde, the reaction mixture was kept in ice for an additional 30 min and then dialyzed for 18 hr against three changes of distilled water and lyophilized. The NADH protected sample was prepared by methylating liver alcohol dehydrogenase in the presence of 1.0 mM NADH. The control was prepared in an identical manner by replacing formaldehyde solution with water. The treatment of the dehydrogenase with formaldehyde alone by an identical procedure did not affect the enzyme activity.

Assay of Liver Alcohol Dehydrogenase. The assay mixture contained 1.0 mmol of sodium pyrophosphate buffer (pH 9.0), 1.0 μmol (low concentration assay) or 1.0 mmol (high concentration assay) of ethanol, 1.0 μmol of NAD^+, and 2.4 nmol of dehydrogenase or methylated dehydrogenase in a total volume of 3.0 ml. The rate of NADH formation was followed at 340 nm using a Beckman DB spectrophotometer

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[1] When this manuscript was in preparation, a communication reporting methylation of horse liver alcohol dehydrogenase appeared (Jörnvall, 1973).

[2] Abbreviations used are: NAD^+, nicotinamide adenosine dinucleotide; NADH, reduced nicotinamide adenosine dinucleotide; *p*-ClHgBzO, *p*-chloromercuribenzoate.