Laboratory Press, Cold Spring Harbor, NY.
Massey, V., & Hemmerich, P. (1978) *Biochemistry 17*, 9–17.
Massey, V., Ghisla, S., & Moore, E. G. (1979) *J. Biol. Chem. 254*, 9640–9650.
Massey, V., Ghisla, S., & Kieschke, K. (1980) *J. Biol. Chem. 255*, 2796–2806.
McKnight, G. L., & McConaughy, B. L. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 4412–4416.
Nishimura, M., Akhmedov, Y. D., Strazalka, K., & Akazawa, T. (1983) *Arch. Biochem. Biophys. 222*, 397–402.
Richardson, K. E., & Tolbert, N. E. (1961) *J. Biol. Chem. 236*, 1280–1284.
Schuman, M., & Massey, V. (1971a) *Biochim. Biophys. Acta 227*, 500–520.
Schuman, M., & Massey, V. (1971b) *Biochim. Biophys. Acta 227*, 521–537.
Schwam, H., Michelson, S., Randall, W. C., Sondey, J. M., & Hirschmann, R. (1979) *Biochemistry 18*, 2828–2833.
Segel, I. H. (1975) *Enzyme Kinetics*, pp 161–166, Wiley, New York.
Sherman, F., Fink, G. R., & Hicks, J. B. (1983) *Methods in yeast genetics*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Strickland, S., Palmer, G., & Massey, V. (1975) *J. Biol. Chem. 250*, 4048–4052.
Sullivan, P. A., Choong, Y. S., Schreurs, W. J., Cutfield, J. F., & Shepherd, M. G. (1977) *Biochem. J. 165*, 357–383.
Thiele, D. J., Walling, M. J., & Hamer, D. H. (1986) *Science 231*, 854–856.
Tolbert, N. E., Clagett, C. O., & Burris, R. H. (1949) *J. Biol. Chem. 181*, 905–914.
Volokita, M., & Somerville, C. R. (1987) *J. Biol. Chem. 262*, 15825–15828.
Zelitch, I., & Ochoa, S. (1953) *J. Biol. Chem. 201*, 707–718.

Direct Measurement of Intramolecular Electron Transfer between Type I and Type III Copper Centers in the Multi-Copper Enzyme Ascorbate Oxidase and Its Type II Copper-Depleted and Cyanide-Inhibited Forms†

T. E. Meyer,‡ A. Marchesini,§ M. A. Cusanovich,‡ and G. Tollin*,‡

Department of Biochemistry, University of Arizona, Tucson, Arizona 85721, and Istituto Sperimentale per la Nutrizione Delle Piante, Sezione-Torino, 10125 Torino, Italy

Received November 14, 1990; Revised Manuscript Received February 8, 1991

ABSTRACT: Transient kinetics of reduction of zucchini squash ascorbate oxidase (AO) by lumiflavin semiquinone have been studied by using laser flash photolysis. Second-order kinetics were obtained for reduction of the type I copper with a rate constant of 2.7×10^7 M^{-1} s^{-1}, which is comparable to that obtained with other blue copper proteins such as plastocyanin. Following reduction, the type I copper was reoxidized in a protein concentration independent (i.e., intramolecular) reaction (k_{obs} = 160 s^{-1}). Comparison with literature values for limiting rate constants in transient single-turnover kinetic experiments suggests that intramolecular electron transfer probably is the rate-limiting step in enzyme catalysis. The extent of reoxidation of type I copper was approximately 55%, which is consistent with the approximately equal redox potentials of the type I and type III copper centers. Neither azide nor fluoride caused any significant changes in kinetics, although they are enzyme inhibitors and are thought to bind to the type II copper. In contrast, cyanide caused a concentration-dependent decrease in the extent of intramolecular electron transfer (with no change in rate constant), and decreased the rate constant for reduction of the type I copper by a factor of 2. The apparent dissociation constant for cyanide (0.2–0.4 mM) is similar to that reported for inhibition of enzyme activity. Removal of the type II copper from AO only marginally affected the kinetics of electron transfer to type I copper ($k = 3.2 \times 10^7$ M^{-1} s^{-1}) and slightly increased the extent but did not alter the rate constant of intramolecular electron transfer. This provides a direct confirmation that type III copper is the immediate electron acceptor from type I copper. Cyanide also inhibits intramolecular electron transfer in type II copper-depleted protein just as in the holoprotein, with a similar apparent dissociation constant. This suggests that cyanide binds to the type III copper center rather than to type II copper.

The blue copper oxidases are widespread in plants, animals, and fungi (Dawson, 1966; Reinhammar & Malmström, 1981; Kroneck et al., 1982). They are large molecular weight soluble proteins which couple the oxidation of small molecules such as phenols and ascorbate to reduction of molecular oxygen. They contain a minimum of four copper atoms which represent three copper environments per subunit. The three-dimensional structure of zucchini squash ascorbate oxidase has been determined (Messerschmidt et al., 1989) and serves as the prototype for all the blue oxidases, whose sequences have been aligned on the basis of folding patterns and residues necessary for binding the copper atoms (Messerschmidt & Hüber, 1990). Ascorbate oxidase exists as a dimer of 70-kDa subunits, each of which folds in three interacting domains. The type I or blue copper has an absorption maximum at about 610 nm, and its binding site is similar to that in the small copper proteins azurin, plastocyanin, pseudoazurin, and cucumber basic blue protein, for which there are also three-dimensional structures.

† This work was supported in part by grants from the National Institutes of Health (DK15057 to G.T. and GM21277 to M.A.C.).
* To whom correspondence should be addressed.
‡ University of Arizona.
§ Istituto Sperimentale per la Nutrizione Delle Piante.

0006-2960/91/0430-4619$02.50/0

Each of the three structural domains of ascorbate oxidase is homologous to the small copper proteins, but only domain 3 has a binding site for type I copper. The colorless type II or inorganic copper is part of a trinuclear center along with the EPR-invisible, spin-paired type III copper which absorbs light at 330 nm. The binding site for the type II, III center is shared between domains 1 and 3. The type III coppers are about 12 Å from the type I copper. The redox potentials of types I and III copper are the same, 344 mV, whereas type II copper has a lower redox potential (Kroneck et al., 1982).

We have previously measured transient kinetics of reduction of a variety of blue copper proteins using free flavin and flavodoxin semiquinones as probes (Tollin et al., 1986a). We found that the site of reduction was located in the region where the copper is nearest the surface (called the "hydrophobic patch" in plastocyanin). Furthermore, we found that there was severe steric hindrance (about 10-fold compared to plastocyanin and azurin) to reduction of the type I copper of *Rhus* laccase (a copper oxidase homologous to ascorbate oxidase). This is probably due to insertions of peptide chain near the site of reduction. We undertook the present study with ascorbate oxidase primarily because of the availability of a three-dimensional structure. To our knowledge, no direct kinetic measurement of intramolecular electron transfer has yet been reported for this enzyme. The laser flash photolysis technique [cf. Tollin et al. (1986b)] is particularly suitable for this purpose. As will be shown below, we have been able to directly observe the reduction of the type I copper center via a bimolecular reaction with a flavin semiquinone, with a rate constant comparable to that observed with plastocyanin (i.e., much larger than with laccase). This was followed by an intramolecular reoxidation of the type I center in a process whose properties are consistent with electron transfer to the type III copper site.

Materials and Methods

Ascorbate oxidase (AO) was prepared from zucchini squash (*Cucurbita pepo medullosa*) by the method of Marchesini and Kroneck (1979). The protein used for the kinetics experiments had a specific activity of 4100 Dawson units/mg (Dawson & Magee, 1957), and had a ratio of 330- to 610-nm absorbance of 0.7. Care was taken to completely remove peroxidase and pectin, which might interfere in the experiments to be performed. The copper content determined by atomic absorption was 8.6 Cu atoms per 140-kDa protein.

Type II copper was removed from an aliquot of enzyme by overnight anaerobic dialysis against dimethylglyoxime and EDTA in the presence of ferrocyanide according to Avigliano et al. (1979). The chelating agents were then dialyzed away. The enzymic activity of the resulting sample was about 8% that of the native protein. Copper analysis gave a value of 6.5 Cu atoms per 140-kDa protein. Absorption spectra in the 330-nm region indicated that the type III center was oxidized, despite reports that depletion of type II copper results in reduction of type III copper (Sakurai et al., 1988).

Laser flash photolysis data collection and analysis were as previously described [cf. Tollin et al. (1986b) and references cited therein]. All experiments were performed in a buffer containing 20 mM phosphate, 2 mM EDTA, and 60 μM lumiflavin at pH 7.0. Pseudo-first-order decay of flavin semiquinone and appearance of reduced ascorbate oxidase were followed at 610 nm over 3–4 half-lives. Second-order rate constants for type I copper reduction were obtained by varying the concentration of the protein. All experiments were performed with a large molar excess of protein over the amount of flavin semiquinone produced by the laser flash (≤0.7 μM),

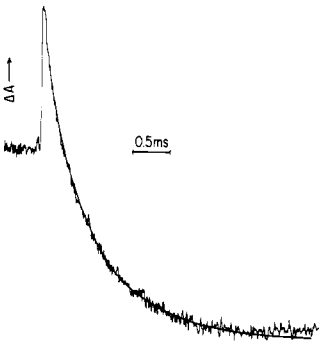

FIGURE 1: Transient formation (initial rise) and decay of lumiflavin semiquinone, generated by laser flash photolysis, concomitant with reduction of the blue or type I copper of ascorbate oxidase (110 μM) (decay to and below preflash base line), observed on a 2-ms time scale at 610 nm. Most of the absorbance decay data are nicely fit by a single-exponential curve, although some intramolecular electron transfer is apparent as a small increase at the end of the decay curve. Buffer was 20 mM phosphate, pH 7.0, containing 60 μM lumiflavin and 2 mM EDTA.

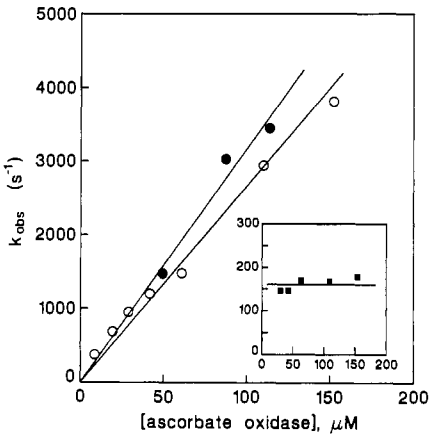

FIGURE 2: Second-order plot of the observed pseudo-first-order rate constants vs protein concentration for reduction of type I copper in native (open circles) and type II copper-depleted (closed circles) ascorbate oxidase. Conditions as in Figure 1. Inset: Plot of the observed rate constant for intramolecular reoxidation of type I copper vs protein concentration for native ascorbate oxidase.

which assured that no enzyme molecule was reduced by more than a single electron. Flavin semiquinone disproportionation, which can compete with protein reduction, was not a significant factor in obtaining rate constant values at the protein concentrations used. We estimate an error of ≤±10% in the determination of rate constants, both from analysis of single determinations and from multiple measurements.

Results and Discussion

Reduction of Type I Copper. The reduction of ascorbate oxidase type I copper by lumiflavin semiquinone was followed at 610 nm, and a typical result is shown in Figure 1. The initial rapid increase in absorbance is due to lumiflavin semiquinone formation by the laser flash. The subsequent absorbance decrease to below the preflash base line is due to type I copper reduction by the semiquinone. Note that this absorbance decay is well fit by a single-exponential curve, except at the very end of the trace. As will be demonstrated below, the poorer fit at longer times after the flash is due to intramolecular type I copper reoxidation. The observed pseudo-first-order rate constant was found to be dependent on protein concentration as shown in Figure 2. The calculated second-order rate constant (2.7×10^7 M^{-1} s^{-1}) is similar to those previously determined for other type I copper proteins such as plastocyanin and azurin [$k = (2–6) \times 10^7$ M^{-1} s^{-1};

Table I: Intramolecular Electron-Transfer Rate Constants for Multicenter Redox Enzymes

enzyme[a]	reaction	k (s^{-1})	ΔE^0 (mV)	distance (Å)	orientation (deg)	ref[c]
TMAD	FMNH• to 4Fe–S	62	60	5	*b*	1
XO	FADH• to 2Fe–S	97	40	?	*b*	2
PCMH	FADH• to heme *c*	220	≈400	8	65	3
SO	Mo to heme *b*	310	46	?	*b*	4
PR	FADH• to FMN	70	180	8–15	?	5
LD	FMNH• to heme *b*	400–1200[d]	5	10	20	6
AO	Cu I to Cu III	80	0	12	b	this work
NR	heme *c* to heme *d*	0.3–1	−7	?	≈90	7

[a] TMAD, trimethylamine dehydrogenase; XO, milk xanthine oxidase; PCMH, *Pseudomonas putida* *p*-cresol methylhydroxylase; SO, chicken liver sulfite oxidase; PR, rabbit NADPH–cytochrome P-450 reductase; LD, yeast lactate dehydrogenase; AO, zucchini ascorbate oxidase; NR, *Pseudomonas aeruginosa* nitrite reductase. [b] It is not clear how to define orientation in these cases, inasmuch as at least one of the redox centers is not planar. [c] (1) Hazzard et al. (1991); (2) Edmondson et al. (1987); (3) Bhattacharyya et al. (1985); (4) Kipke et al. (1988); (5) Bhattacharyya et al. (1991); (6) Walker and Tollin (1991); (7) Schichman and Gray (1981); Makinen et al. (1983). [d] Rate constant is ionic strength dependent.

Tollin et al., 1986a; Meyer et al., 1987]. Because the redox potentials of AO and these other copper proteins are similar, the results show that they all have a similar degree of steric hindrance to reduction of the type I copper by small molecules. This was an unexpected result based on the following observations. Laccase, which is also a copper oxidase, shows considerably more steric hindrance to flavin semiquinone reduction than do the simpler blue copper proteins (approximately 10-fold; Tollin et al., 1986a). Furthermore, the three-dimensional structure of AO shows that the protein folds in three domains. Domain 3 (residues 345–550), which is homologous to the small copper proteins, provides the binding site for type I copper at His-446, Cys-508, His-513, and Met-518. This binding site is similar to that of the small homologous copper proteins. It is apparent from superposition of the structures of plastocyanin and AO domain 3 that four-, six-, and two-residue insertions at positions 356–359, 437–442, and 510–511, respectively, near the expected site of reduction of AO should provide steric hindrance. However, as noted, we observed no such effect with lumiflavin semiquinone, although one might anticipate that large molecules would be hindered in their approach. It is of interest that this latter effect is precisely what was observed with stellacyanin (Tollin et al., 1986a), which reacts more rapidly with lumiflavin semiquinone than does plastocyanin and azurin, but considerably less rapidly with flavodoxin semiquinone. Ascorbate oxidase also dimerizes with the interface near the active site, which would further restrict access to other proteins. The results with laccase suggest that this protein may have similar or even larger insertions near the active site. Taken together, the present experiments indicate that the copper oxidases are designed for reaction with small molecules, rather than with other proteins for which there would be severe steric hindrance. This, of course, is consistent with their enzymology.

Intramolecular Electron Transfer. Following reduction of type I copper in AO, we observed its slower reoxidation, as shown in Figure 3. Control experiments demonstrated that this was not due to residual dioxygen in the sample (also see below). The recoloring process is again well fit by a single-exponential curve (Figure 3). The observed rate constant for this reaction is concentration independent, as is shown by the inset in Figure 2 (k_{av} = 160 s^{-1}). The type I copper is not completely reoxidized back to the preflash base line, but returns only about 55%, presumably reflecting an equilibration between the type I copper and the type II, III trinuclear copper center. Thus, the observed rate constant is actually the sum of the forward and reverse rate constants for this equilibrium. The redox potentials of type I copper and type III copper are apparently the same (344 mV) at room temperature (25 °C), although they are different at 10 °C, where the type III center has a 30-mV higher potential (Kroneck et al., 1982). The type

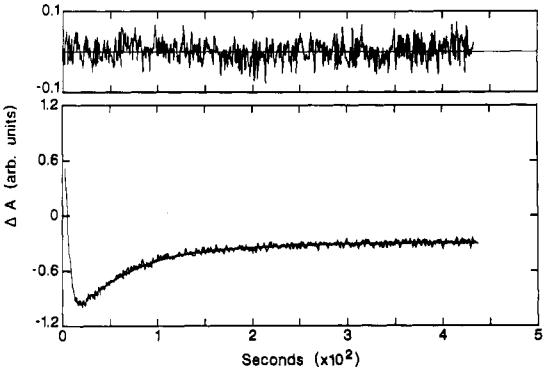

FIGURE 3: Rapid reduction of type I copper and slower intramolecular reoxidation followed on a 50-ms time scale. Conditions as in Figure 1. Note that the data can be accurately fit with two exponentials (solid curve), one corresponding to the initial reduction (decreasing absorbance; $k = 2.4 \times 10^3$ s^{-1}) and the other corresponding to the regeneration of the oxidized type I center (increasing absorbance; k = 168 s^{-1}). Calculated residuals for the two-exponential fit appear in the upper box. Note also that the type I copper is not completely reoxidized but approaches an equilibrium which is controlled by the difference in redox potentials of the two copper centers (see text).

III center behaves as a one-electron acceptor, in spite of the fact that no new EPR signal has yet been detected as a result of the apparent uncoupling of the two type III coppers. Our kinetic results are consistent with intramolecular electron transfer to an approximately equipotential one-electron acceptor, which suggests that it is the type III center that is functioning in the reoxidation of the type I copper (see below for further discussion). For such a system, $k_f = k_b$ = 80 s^{-1}. There is an interesting report that the type III center can act as both a one-electron or a two-electron acceptor in laccase, depending upon the potential of the donor (Farver et al., 1978). Whether this is also the case in AO remains to be determined.

Stopped-flow studies demonstrate that enzyme reduction by ascorbate ($k = 1.7 \times 10^4$ M^{-1} s^{-1}) (Kroneck et al., 1982) and reoxidation by dioxygen ($k = 4.8 \times 10^6$ M^{-1} s^{-1}) (Nakamura & Ogura, 1968) are not exceptionally rapid. In these studies, limiting rate constant values for reduction at high substrate concentrations were determined at 10 °C to be 80 s^{-1} at 610 nm and 100 s^{-1} at 330 nm. At 25 °C, a limiting rate constant in the vicinity of 120 s^{-1} has been observed (Baici et al., 1979). These values are comparable in magnitude to the rate constant which we have determined for intramolecular electron transfer from type I to type III copper (80 s^{-1} at 25 °C). Thus, these results strongly indicate that type I to type III copper intramolecular electron transfer is the rate-limiting step in enzyme catalysis.

The intramolecular electron-transfer rate constant in AO is compared with those obtained for other multicenter redox enzymes in Table I. At present, no clear correlation can be

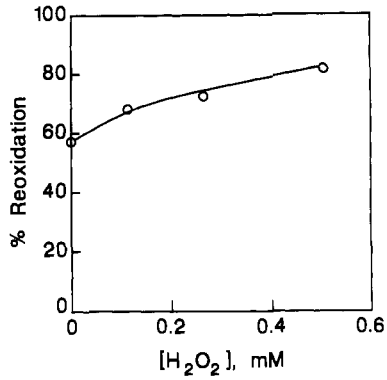

FIGURE 4: Plot of percent reoxidation of type I copper vs peroxide concentration for native ascorbate oxidase. Conditions as in Figure 1.

made between electron-transfer rate constants and any one factor, but on theoretical grounds [cf. Marcus and Sutin (1985)], one would expect that the differences in redox potentials and intercenter distances, the relative orientation of the redox centers, and the nature of the intervening medium between redox centers should be the principal factors controlling electron-transfer rates. On the basis of the three cytochrome examples in Table I, there is an apparent effect of parallel vs nonparallel orientation of the aromatic rings of heme and flavin or of two hemes, but whether this is a real correlation or just coincidental remains to be established. There are currently no clear examples of enzymes having large differences in distance between redox centers which could provide an evaluation of the importance of this parameter, and there is no clear correlation with thermodynamic driving force among the examples listed in Table I. Furthermore, we have no way at present to assess the effect of intervening media in these systems. Site-directed mutagenesis techniques may eventually allow us to separately evaluate the contribution of these factors to intramolecular electron-transfer rates. In this respect, ascorbate oxidase would be an ideal candidate.

Effects of Inhibitors. We have examined the effect of various enzyme inhibitors on the kinetics of reduction and intramolecular electron transfer in AO. There was no effect of azide or fluoride up to 1 mM concentration on either reaction, although both compounds are known to be inhibitors of the steady-state oxidation of ascorbate (Sheline & Strothkamp, 1980). We also observed no effect of carrying out the flash experiment under aerobic conditions on AO transient kinetics, although, as expected, there was a marked decrease in the extent of protein reduction due to quenching of the flavin triplet state and oxidation of flavin semiquinone by dioxygen. Hydrogen peroxide changed the extent of intramolecular electron transfer in a concentration-dependent manner, as shown in Figure 4, although the kinetic rate constants were unaffected. The extent of reoxidation of type I copper was increased from 55% to about 75% at 0.5 mM peroxide. This result may be attributable to the report that a fraction of type III copper is reduced in the native enzyme and that hydrogen peroxide is able to reoxidize these reduced centers (Casella et al., 1988).

The effect of cyanide was much more pronounced as is shown in Figure 5. The observed rate constant for reduction of type I copper was changed slightly (approximately 2-fold slower; cf. Figure 5), but the rate constant for reoxidation was not at all modified (data not shown). The extent of intramolecular electron transfer was decreased from 55% to about 8% at 1.5 mM total cyanide concentration. The dissociation constant for cyanide ($HCN + CN^-$) calculated from Scatchard

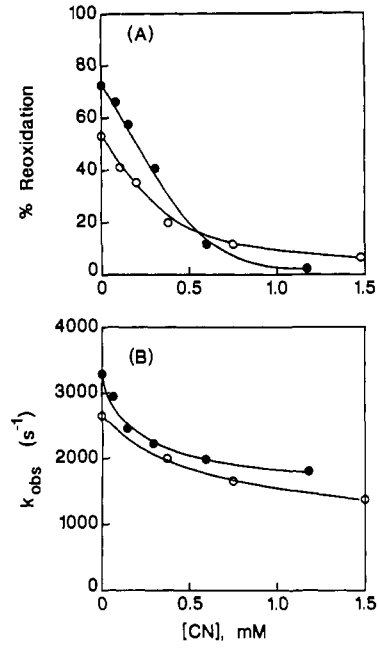

FIGURE 5: Effect of cyanide concentration on extent of intramolecular electron transfer (A) and kinetics of reduction of type I copper (B) for native (open circles) and type II copper-depleted (closed circles) ascorbate oxidase. Conditions as in Figure 1.

analysis of the kinetic data was 0.42 mM, and from the data on the extent of reoxidation was 0.24 mM. These numbers are in reasonable agreement with each other considering the errors involved in the measurement. Values for cyanide inhibition constants for AO measured at pH 5.6 have been reported to be in the range of 0.13–0.46 mM (Strothkamp & Dawson, 1977). Cyanide is expected to bind at the type II, III copper center as is the case for the other inhibitors. Despite this, there was a change in the rate constant for reduction of type I copper, which suggests a lowered redox potential or decreased solvent exposure, possibly brought about by conformational change rather than by direct binding of cyanide to type I copper. The change in the extent of intramolecular electron transfer suggests that the redox potential of the type II, III center was lowered 60 mV or more relative to the type I center upon binding cyanide. This will be discussed further below.

Effect of Removal of Type II Copper. Type II copper is less tightly bound than are the other copper centers in AO, and it can be specifically removed by anaerobic dialysis against appropriate copper-ligating agents such as EDTA, dimethylglyoxime, and diethyldithiocarbamate (Avigliano et al., 1979; Morpugo et al., 1987). Enzyme which had the type II copper removed by using the method of Avigliano et al. (1979) had only 8% of the activity of the native enzyme. The reduction of type I copper by lumiflavin semiquinone was slightly faster than for native enzyme ($k = 3.2 \times 10^7\ M^{-1}\ s^{-1}$; cf. Figure 2), which may reflect a small conformational change. Surprisingly, the rate constant for intramolecular electron transfer was unaffected (data not shown), although the extent of reoxidation was significantly larger than in native enzyme (73% vs 55%). This is strong evidence in support of type III copper serving as the electron acceptor from type I copper. The change in extent of reaction suggests a larger difference in the redox potentials of types I and III copper as a result of removal of type II copper.

Cyanide had a similar effect on type II copper-depleted enzyme as on the native enzyme (cf. Figure 5); i.e., the rate constant for reduction of type I copper was decreased by a

factor of about 2, and the extent of reoxidation dropped to less than 5%. The cyanide dissociation constant calculated from the kinetic data (K = 0.1 mM) is similar to that obtained from the equilibrium measurements (K = 0.4 mM). These numbers are within experimental error of each other and of the values obtained with the native enzyme. The similarity in the effects of cyanide on both native and type II copper-depleted AO strongly indicates that cyanide directly binds to type III copper in both forms of the enzyme. Recent results with tyrosinase demonstrate that cyanide can indeed bind to a type III copper center (Beltramini et al., 1990).

In conclusion, we have measured the intramolecular rate constant for a single electron transfer between type I and type II, III copper sites in AO and find that it is similar to the limiting rate constant for the enzyme-catalyzed reaction in a single-turnover experiment, and thus probably constitutes the rate-limiting step in catalysis. The same intramolecular electron transfer occurs in native as well as in type II copper-depleted enzyme, and thus we conclude that the type III center is the immediate acceptor of the first electron from type I copper. Cyanide binding studies confirm this assignment. This interpretation is also consistent with the relative locations of the copper sites as determined from the X-ray structural analysis (Messerschmidt et al., 1989), and with the relative redox potentials of the copper centers. Our experiments do not provide any information concerning the fate of additional electrons in AO, noting that four electrons are required in order to reduce dioxygen to water. However, additional intramolecular electron-transfer steps are evidently not any slower than the initial one. Cyanide appears to bind to type III copper and lowers its redox potential significantly. The other inhibitors appear to bind to type II copper with no effects on intramolecular electron transfer. We believe that these results have provided important new insights into the enzymatic mechanism of AO, and by analogy other copper oxidases as well.

References

Avigliano, L., Desideri, A., Urbanelli, S., Mondovi, B., & Marchesini, A. (1979) *FEBS Lett. 100*, 318–320.

Baici, A., Luisi, P. L., Palmieri, S., & Marchesini, A. (1979) *J. Mol. Catal. 6*, 135–143.

Beltramini, M., Salvato, B., Santamaria, M., & Lerch, K. (1990) *Biochim. Biophys. Acta 1040*, 365–372.

Bhattacharyya, A., Tollin, G., McIntire, W., & Singer, T. P. (1985) *Biochem. J. 228*, 337–345.

Bhattacharyya, A. K., Lipka, J. J., Waskell, L., & Tollin, G. (1991) *Biochemistry 30*, 759–765.

Casella, L., Gullotti, M., Pallanza, G., Pintar, A., & Marchesini, A. (1988) *Biochem. J. 251*, 441–446.

Dawson, C. R. (1966) in *The Biochemistry of Copper* (Peisach, J., Aisen, P., & Blumberg, W. E., Eds.) pp 305–334, Academic Press, New York.

Dawson, C. R., & Magee, R. J. (1957) *Methods Enzymol. 2*, 831–835.

Edmondson, D. E., Hazzard, J. T., & Tollin, G. (1987) in *Flavins and Flavoproteins* (Edmondson, D. E., & McCormick, D. B., Eds.) pp 403–408, de Gruyter, Berlin and New York.

Farver, O., Goldberg, M., Wherland, S., & Pecht, I. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75* 5245–5249.

Hazzard, J. T., McIntire, W. S., & Tollin, G. (1991) *Biochemistry* (in press).

Kipke, C. A., Cusanovich, M. A., Tollin, G., Sunde, R. A., & Enemark, J. H. (1988) *Biochemistry 27*, 2918–2926.

Kroneck, P. M. H., Armstrong, F. A., Merkle, H., & Marchesini, A. (1982) in *Advances in Chemistry, Ascorbic Acid: Chemistry, Metabolism, and Uses* (Seib, P. A., & Tolbert, B. M., Eds.) pp 223–248, American Chemical Society, Washington, D.C.

Makinen, M. W., Schichman, S. A., Hill, S. C., & Gray, H. B. (1983) *Science 222*, 929–931.

Marchesini, A., & Kroneck, P. M. H. (1979) *Eur. J. Biochem. 101*, 65–76.

Marcus, R. A., & Sutin, N. (1985) *Biochim. Biophys. Acta 811*, 265–322.

Messerschmidt, A., & Hüber, R. (1990) *Eur. J. Biochem. 187*, 341–352.

Messerschmidt, A., Rossi, A., Ladenstein, R., Hüber, R., Bolognesi, M., Gatti, G., Marchesini, A., Petruzzelli, R., & Finazzi-agro, A. (1989) *J. Mol. Biol. 206*, 513–529.

Meyer, T. E., Cusanovich, M. A., Krogmann, D. W., Bartsch, R. G., & Tollin, G. (1987) *Arch. Biochem. Biophys. 258*, 307–314.

Morpurgo, L., Savini, I., Mondovi, B., & Avigliano, L. (1987) *J. Inorg. Biochem. 29*, 25–31.

Nakamura, T., & Ogura, Y. (1968) *J. Biochem. 64*, 267–270.

Reinhammar, B., & Malmström, B. G. (1981) in *Copper Proteins* (Spiro, T. G., Ed.) pp 111–149, Wiley, New York.

Sakurai, T., Suzuki, S., & Sano, M. (1988) *Inorg. Chim. Acta 152*, 3–4.

Schichman, S. A., & Gray, H. B. (1981) *J. Am. Chem. Soc. 103*,

Sheline, R. R., & Strothkamp, K. G. (1980) *Biochem. Biophys. Res. Commun. 96*, 1343–1348.

Strothkamp. R. E., & Dawson, C. R. (1977) *Biochemistry 16*, 1926–1929.

Tollin, G., Meyer, T. E., Cheddar, G., Getzoff, E. D., & Cusanovich, M. A. (1986a) *Biochemistry 25*, 3363–3370.

Tollin, G., Meyer, T. E., & Cusanovich, M. A. (1986b) *Biochim. Biophys. Acta 853*, 29–41.

Walker, M. C., & Tollin, G. (1991) *Biochemistry* (in press).