Inhibition of Ascorbate Oxidase by Phenolic Compounds. Enzymatic and Spectroscopic Studies[†]

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ABSTRACT: Competitive inhibition by phenolic compounds of the ascorbic acid oxidation reaction catalyzed by ascorbate oxidase was investigated at pH 7.0 and 23.0 °C. Inhibition of *p*-nitrophenol is pH dependent over the range 5.0−8.0, with inhibitor binding favored at higher pH. Bulky substituents on the phenol nucleus reduce or prevent the inhibitory effect. The presence of phenol affects the binding characteristics of azide to the trinuclear cluster of the enzyme. In particular, binding of azide to type 2 copper is prevented, and the affinity of azide to type 3 copper is reduced. In addition, reduction of type 1 copper is observed upon prolonged incubation of ascorbate oxidase with excess phenol and azide, but not with phenol alone. It is proposed that binding of phenolic inhibitors occurs at or near the site where the substrate (ascorbate) binds. NMR relaxation measurements of the protons of phenols in the presence of ascorbate oxidase show paramagnetic effects due to the proximity of the bound inhibitor to a copper center, likely type 1 copper. Copper−proton distance estimates between this paramagnetic center and *p*-cresol or *p*-nitrophenol bound to ascorbate oxidase are between 4.4 and 5.9 Å.

Ascorbate oxidase (AO)[1] is a multicopper blue oxidase which is found in higher plants (Kroneck et al., 1982; Chichiriccò et al., 1989; Finazzi-Agrò & Rossi, 1992), and is most commonly purified from green zucchini squash (Lee & Dawson, 1973; Marchesini & Kroneck, 1979) or cucumber peelings (Aikazyan & Nalbandyan, 1979; Sakurai et al., 1987). Immunohistochemical localization of AO reveals that it is distributed over vegetative and reproductive organs, and linked with the cell wall and cytoplasm (Chichiriccò et al., 1989). AO is a dimeric enzyme containing two subunits of M_r 70 000. The X-ray crystal structure of AO from zucchini shows the presence of four copper atoms per monomer, one type 1 copper at 12 Å from a trinuclear cluster containing one type 2 and two type 3 coppers (Messerschmidt et al., 1989, 1992a). The type 1 copper has two histidines, a cysteine, and a methionine ligand, and is closely related to the copper center found in smaller electron transfer proteins such as plastocyanin and azurin (Guss & Freeman, 1983; Nar et al., 1991); this center is responsible for the strong optical absorption of AO at 610 nm and the EPR signal with small hyperfine constant ($A_{||} \sim 60 \times 10^{-4}$ cm^{-1}). The trinuclear cluster has eight histidine ligands and is composed of an antiferromagnetically coupled pair of copper atoms, each bound to three histidines and bridged by a hydroxyl group, which constitutes the putative type 3 copper, absorbing light at 330 nm and EPR silent, and a type 2 copper atom having two histidine ligands and a terminal hydroxide ligand, which absorbs weakly and is responsible for the normal EPR signal ($A_{||} \sim 190 \times 10^{-4}$ cm^{-1}).

Like the other blue oxidases, laccase and ceruloplasmin, AO catalyzes the four-electron reduction of dioxygen to water (Reinhammar, 1983, 1984; Reinhammar & Malmström, 1981); however, the *in vivo* role of AO has not yet been clarified. The enzyme presents a strong activity toward ascorbate (Kroneck et al., 1982) and is also active with a variety of catechol and aminophenol derivatives (Dayan & Dawson, 1976; Marchesini et al., 1977; Casella et al., 1985), which led to the proposal that AO might be involved in the ripening process of fruits (Kroneck et al., 1982).

A tentative catalytic mechanism of ascorbate oxidase has been proposed based on spectroscopic and mechanistic studies (Kroneck et al., 1982; Mondovì & Avigliano, 1984), and on the X-ray structures of AO (Messerschmidt et al., 1992a) and several of its derivatives (Messerschmidt et al., 1993). The structure of AO suggests that the putative binding site for the reducing organic substrate may be close to the type 1 copper, while the binding site of dioxygen is commonly accepted to occur at the trinuclear cluster (Messerschmidt et al., 1992a, 1993). The catalytic cycle is proposed to proceed by a sequential mechanism, where single electrons are provided by the substrate to type 1 copper. The electrons are then transferred through the protein to the type 3 copper pair. After four electrons have been transferred, and the hydroxyl bridge between the type 3 copper has been released, the fully reduced enzyme binds dioxygen. Transfer of two electrons from the copper pair to dioxygen leads to the formation of a hydroperoxide intermediate. A third electron is transferred from the type 2 copper to this

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[1] Abbreviations: AO, ascorbate oxidase; T2D AO, type 2 copper-depleted derivative of AO; NMR, nuclear magnetic resonance; EPR, electron paramagnetic resonance; CD, circular dichroism; LMCT, ligand to metal charge transfer.

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hydroperoxide intermediate and a fourth electron from type 1 copper to type 3 copper. The O−O bond is broken with formation of an oxygen radical intermediate and release of a water molecule. The reaction may then lead to the resting form, upon transfer of the fourth electron from type 3 copper to the oxygen radical intermediate, or proceed with reduction of the type 1 center by another molecule of substrate if turnover is continued (Messerschmidt et al., 1993).

Until now, only inorganic anions such as N_3^-, F^-, and SCN^- are known to induce competitive inhibition of AO as they can bind to the copper atoms of the trinuclear center (Strothkamp & Dawson, 1977; Sheline & Strothkamp, 1980). The binding of these ions to AO has been widely used to get insight into the structural features of the metal centers (Sakurai et al., 1987; Casella et al., 1988, 1991a; Cole et al., 1991). The search of organic compounds which can act as competitive inhibitors of AO with respect to ascorbate could provide information on the characteristics and localization of the binding site of the organic AO substrates. This information is of extreme importance for the understanding of the complex mechanism of electron transfers undergone by the enzyme, which is currently studied with a variety of fast techniques (Meyer et al., 1991; Farver & Pecht, 1994; Kyritsis et al., 1993; Tollin et al., 1993; Hazzard et al., 1994). In this paper, we address the problem of the AO substrate binding site showing that phenolic compounds are competitive inhibitors of AO with respect to ascorbate and, in addition, they prevent the binding of N_3^- to the type 2 copper and reduce type 1 copper when azide is present in the solution. ^{1}H NMR relaxation time measurements show that these inhibitors bind to the enzyme close to the paramagnetic copper centers. Competitive inhibitor binding studies of this type have never been performed before for any of the blue copper oxidases.

MATERIALS AND METHODS

Materials. All chemicals were of the highest grade commercially available and used as received. Ascorbate oxidase was purified from green zucchini squash (*Cucurbita pepo*) as previously described (Marchesini & Kroneck, 1979). The protein used for all experiments had optical indices $A_{280}/A_{610} = 24.0$ and $A_{330}/A_{610} = 0.8$ in 100 mM phosphate buffer, pH 7.0. Protein solutions were routinely concentrated as necessary by using Sartorius collodion bags, and protein concentration was determined assuming $M_r = 140\,000$ and $\epsilon_{610} = 9700\ M^{-1}\ cm^{-1}$. The type 2 copper-depleted (T2D) derivative of ascorbate oxidase was prepared according to the *N*,*N*-diethyldithiocarbamate method (Morpurgo et al., 1987; Casella et al., 1991a).

Physical Measurements. UV−Visible spectra were recorded on a diode array HP 4852 single-beam spectrophotometer equipped with a thermostated cell holder maintained at 23 ± 0.5 °C. Dioxygen consumption during enzymatic activity was measured on an Advanced Products Oxyan oxygraph fitted with a Clark electrode. ^{1}H NMR spectra were recorded with a Bruker AC-200 spectrometer operating at 200 MHz. Circular dichroism spectra were recorded at room temperature on a Jasco J-710 spectropolarimeter. Each spectrum was obtained as an average of 5−10 scans in order to increase the signal-to-noise ratio. EPR spectra were recorded in frozen solutions at −150 °C on a Varian E-109 spectrometer operating at X-band frequencies.

Oxidase Activity. Ascorbate oxidase activity was measured at 23 ± 0.5 °C by monitoring dioxygen consumption in a 1.2 mL incubation cell equipped with a Clark electrode. Enzyme inhibition was measured as a function of ascorbic acid concentration with several phenolic compounds. The experiments to determine the kinetic parameters K_m and K_i were carried out in 100 mM phosphate buffer at pH 7.0, with inhibitor concentration ranging from 0 to 30 mM and ascorbic acid concentration varying from 0 to 2 mM. The enzyme concentration was varied from 1 nM, in the experiments in the absence of inhibitor, to about 8 nM using the highest concentration of the inhibitor. The determination of the pH dependence of *p*-nitrophenol inhibitor activity was performed by studying the variation of the K_i value in the pH range from 5 to 8. The buffers used were 100 mM acetate at pH 5.0, and 100mM phosphate at pH 6.0, 6.5, 7.0, and 8.0. The *p*-nitrophenol and AO concentrations were varied as before in these experiments. The exact concentration of ascorbic acid solutions was determined before each experiment from its absorbance at 266 nm using $\epsilon_{266} = 15\,000\ M^{-1}\ cm^{-1}$. The reaction rate was determined from the maximum slope of the recorded O_2 consumption curve. Double-reciprocal plots of initial velocity *v*s initial ascorbic acid concentration were used, along with appropriate replots, to determine the inhibition constants (Segel et al., 1975). The oxidase activity of the AO sample determined with the above method was 2600 Dawson units at pH 7.0 [1 Dawson unit = 1 μmol·min^{-1}·(mg of enzyme)$^{-1}$]. When determined with the stopped-flow method, following the decrease in UV absorption of ascorbate, the activity corresponded to 3200 Dawson units, but this method is inapplicable here due to the strong UV absorption of the phenolic compounds.

Ligand Binding. Spectrophotometric titrations of azide binding to AO were carried out in a 1.0 cm quartz cuvette. The sample cuvette contained 0.5 mL of 44 μM AO in 100 mM phosphate buffer, pH 7.0; optical spectra were measured after addition of small volumes of more concentrated azide solutions in the same buffer. In the initial part of the titration, the azide concentration was 20 mM; then a 100 mM solution was used. The reference cuvette contained 0.5 mL of the buffer, and each addition of ligand to the AO sample was matched by an identical addition to the blank. For the experiments performed in the presence of phenol, 26.4 mM phenol was added to the AO sample and reference cuvettes, respectively. Differential spectra were obtained, and absorbance values were corrected for dilution by using a computer program that takes into account the volume changes of the solution following each addition of the titrant. The data were analyzed as described previously (Brown, 1979; Casella et al., 1991b) to deduce equilibrium constants for anion association.

EPR Measurements. For EPR investigations, a 150 μM AO solution in 100 mM phosphate buffer, pH 7.0, was employed. Spectra were recorded in frozen solutions after addition of increasing amounts of azide to final concentrations of 1.5, 15, and 75 mM to the enzyme solution. In the experiments in the presence of phenol, 90 mM phenol was added before the addition of azide.

Relaxation Rate Measurements. The enzyme samples for NMR studies were prepared by ultrafiltration and repeated exchanges with 0.1 M deuterated phosphate buffer, pH 7.0 (uncorrected for the small isotopic effect). The enzyme concentration of the initial solution was 109 μM in the

experiment with *p*-cresol and 309 μM in that with *p*-nitrophenol. Titrations of the inhibitors with the enzyme were carried out by adding small volumes of this AO solution in deuterated buffer to 400 μL of a 12.4 mM cresol or 50 mM *p*-nitrophenol solution in 0.1 M deuterated phosphate buffer, pH 7.0. The temperature was calibrated to 25 °C using Bruker standard solutions and controlled by the control unit of the instrument, with a precision of ±1 °C. Longitudinal relaxation times were determined at 200 MHz using the standard inversion recovery method. From the values of T_{1obs}, it is possible to calculate T_{1b}, which is the T_1 for the bound inhibitor when this is in fast exchange between the active site and the bulk solution, from the equation:

$$\frac{1}{T_{1obs}} = \left[\frac{1}{T_{1b}} - \frac{1}{T_{1f}}\right]\frac{E_0}{K_d + I_0} + \frac{1}{T_{1f}} \quad (1)$$

where T_{1f} is T_1 for the free inhibitor and T_{1b} that for the enzyme-bound inhibitor, E_0 and I_0 are the initial enzyme and inhibitor concentration, respectively, and K_d is the dissociation constant for the enzyme−inhibitor complex. T_{1b} is related to the paramagnetic component of the relaxation, T_{1M}, through the equation:

$$\frac{1}{T_{1b}} = \frac{1}{T_{1D}} + \frac{1}{T_{1M} + \tau_M} \quad (2)$$

where τ_M is the lifetime for chemical exchange and T_{1D} corresponds to the diamagnetic contribution to the relaxation for the bound inhibitor. In the presence of strong paramagnetic effects, the diamagnetic contribution can be neglected, and assuming that the exchange between the free and bound state is fast, eq 2 yields $T_{1b} = T_{1M}$.

The paramagnetic contribution to the relaxation rate, $(T_{1M})^{-1}$, of the protons of the bound inhibitor arising from the unpaired electrons on the copper center(s) is related to the copper−proton distance (r) by the Solomon−Bloemberger equation (Solomon & Bloemberger, 1956; Bloemberger, 1957; Dwek, 1973). In the present case, since the inhibitor is not bound to the metal center(s), only the dipolar term is retained in this equation, while the scalar term can be neglected (Dwek, 1973; Jardetzky & Roberts, 1981):

$$\frac{1}{T_{1M}} = \frac{2}{15}\frac{\gamma_I^2 g^2 S(S+1)\beta^2}{r^6}\left(\frac{3\tau_c}{1+\omega_I^2\tau_c^2} + \frac{7\tau_c}{1+\omega_S\tau_c^2}\right) \quad (3)$$

where ω_I and ω_S are nuclear and electronic Larmor frequencies, respectively, γ_I the magnetogyric ratio of the nucleus, g the electron g-factor (assumed isotropic), β the Bohr magneton, S the spin quantum number of the electron, and τ_c the effective correlation time of the dipolar interaction. In spite of the assumptions underlying the use of this equation (Jardetzky & Roberts, 1981), it proved to be extremely useful in our previous studies on substrate binding to heme peroxidases (Casella et al., 1991c, 1992, 1994). In the case of copper enzymes, it is generally assumed that the overall correlation time is determined by the electronic relaxation, and a τ_c value of 3.5×10^{-9} s has been estimated for a variety of copper enzymes (Bertini et al., 1985; Williams & Falk, 1986). The same τ_c value has been therefore employed here. The uncertainty in the value of τ_c has little impact on the distance estimates because of the r^{-6} dependence of $(T_{1M})^{-1}$.

Table 1: Competitive Inhibition of Ascorbate Oxidase by Phenolic Compounds[a]

inhibitor	pK_a[b]	pH	K_i (mM)[c]
phenol	10.0	7.0	6 (±1)
p-chlorophenol	9.4	7.0	1 (±0.5)
p-cresol	10.3	7.0	18 (±3)
p-cyanophenol	7.9	7.0	2 (±0.6)
p-nitrophenol	7.1	5.0	4.6 (±0.1)
		6.0	4.2 (±0.1)
		6.5	3.6 (±0.2)
		7.0	3.0 (±0.1)
		8.0	2.5 (±0.2)

[a] With respect to ascorbate. [b] In water solution [see Serjeant and Dempsey (1979)]. [c] Value in parentheses is the standard deviation.

RESULTS

Inhibition of AO by Phenolic Compounds. The rate of oxidation of ascorbic acid by AO decreased when AO was incubated with several phenolic compounds. In general, the presence of an electron-withdrawing, uncharged substituent strengthens the inhibitory effect of the phenol, but *p*-hydroxybenzoic acid is noninhibitory. The increase in the size of the substituents progressively decreases the inhibitor effect of the phenol, and, in fact, *p-tert*-butylphenol is noninhibitory. Clearly, the steric hindrance of the substituent may limit the access to the active site of AO. For several phenols, the type of inhibition was determined by measuring the initial rate of oxygen consumption over a range of ascorbate concentrations in the presence of fixed amounts of the inhibitors. In all cases, the double reciprocal plots of initial rates at different inhibitor concentrations were found to intersect the $1/v$ axis at the same point, and the replots of the slopes of the double-reciprocal plots vs the inhibitor concentration were linear (Segel, 1975). The phenolic compounds are thus competitive inhibitors for AO, and this enabled us to determine the inhibition constant K_i (Table 1), which represents the dissociation constant of the enzyme−inhibitor complex. These results suggest that binding of phenolic compounds may occur at or near the binding site of ascorbic acid.

Phenols can act as inhibitors of AO in their protonated or anionic forms. Therefore, we studied the pH dependence of the inhibition constant using *p*-nitrophenol, which is best suited for the investigation of this effect, since its pK_a is approximately 7 (Conrad et al., 1994) and in the pH range of 5−8 both its protonation states are sufficiently covered. The pH range below 5 and above 8 cannot be investigated for enzyme stability problems and, at pH >8, the easy transformation of ascorbic acid into its lactonic form. As shown by the data in Table 1, *p*-nitrophenol acts as an inhibitor both in the acidic and in the basic pH range, but the pH dependence of the inhibition can be due to a change in the ionization state of either the enzyme, or the inhibitor, or both. In any case, the observed K_i value is regulated by the pK_a of an ionizable group and depends on the protonated fraction of such a group. Indicating K_{ia} as the inhibition constant at acidic pH and K_{ib} as that at basic pH, the observed K_i is a weighted average that depends on the pK_a value and the pH according to the equation:

$$K_{i\,obs} = \frac{K_{ia}[H]^+ + K_{ib} \times K_a}{[H]^+ + K_a} \quad (4)$$

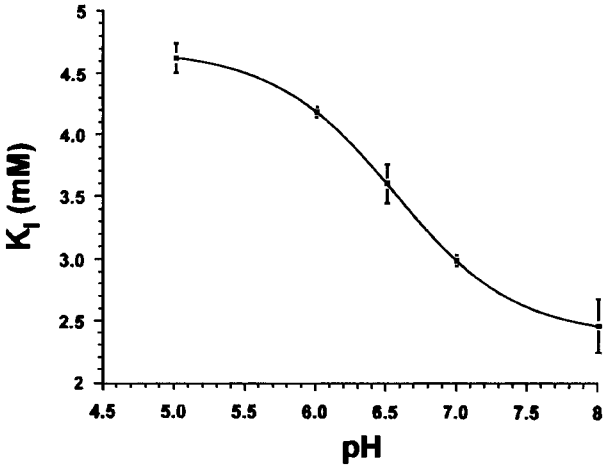


FIGURE 1: Inhibition of ascorbate oxidase by *p*-nitrophenol as a function of pH. The curve is the best fit to the data according to eq 4 (see text).

The best fit of the data reported in Table 1 according to eq 4 is shown in Figure 1 and gave the parameters $K_{ia} = 4.7 \pm 0.1$ mM, $K_{ib} = 2.4 \pm 0.1$ mM, and $pK_a = 6.55 \pm 0.04$. The last value agrees well with the pK_a value obtained for the ionization of *p*-nitrophenol from inhibition studies of tyrosinase, 6.82 ± 0.03 (Conrad et al., 1994), suggesting that the pH dependence of the inhibition constant is indeed associated with the protonation state of the phenol. The lower K_i value at basic pH thus suggests that the phenol prefers to bind to the AO active site in its phenoxide form.

UV−Visible Azide Titration. Binding of azide to the copper atoms of the trinuclear cluster of AO has been studied by several groups (Strothkamp & Dawson, 1977; Casella et al., 1988; Cole et al., 1991), and the results seem somewhat dependent on the method of preparation of the enzyme. In order to determine if the binding of phenol to AO could interfere with binding of azide to the trinuclear cluster of the enzyme, titration of AO by azide was performed, in parallel, in the absence and in the presence of phenol. As we found before, titration of AO with azide in 100 mM phosphate buffer, pH 7.0, is biphasic (Casella et al., 1988). The change observed in the UV−Visible spectrum on addition of small amounts of azide (≤10 equiv) consists of a broad increase of absorption between 400 and 500 nm. The differential spectrum reveals the presence of a maximum at 410 nm (Figure 2A). A high-affinity binding constant $K_1 = 4000 \pm 50$ M^{-1} can be calculated, in agreement with the value of 5000 M^{-1} estimated previously (Casella et al., 1988). At higher azide to enzyme molar ratios ($50 \leq N_3^- \text{ equiv} \leq 1000$), a well-defined band develops at 420 nm, accompanied by an isosbestic point at 360 nm and a decrease of absorption at 330 nm. A low-affinity binding constant $K_2 = 75 \pm 8$ M^{-1} can be calculated from these data. The total increase of absorbance of the LMCT band corresponds to $\Delta\epsilon \approx 6000$ M^{-1} cm^{-1}.

When titration of AO in 100 mM phosphate buffer, pH 7.0, by azide was carried out in the presence of 600 equiv of phenol, at low concentrations of azide (≤10 equiv) the differential spectra showed a broad absorption centered at 392 nm (Figure 2B), from which a high affinity binding constant $K_1' = 3600 \pm 30$ M^{-1}, lower than that obtained in the absence of phenol, could be calculated. At higher azide to enzyme molar ratios ($50 \leq N_3^- \text{ equiv} \leq 1000$), a spectrum with a maximum at 415 nm develops. In spite of some

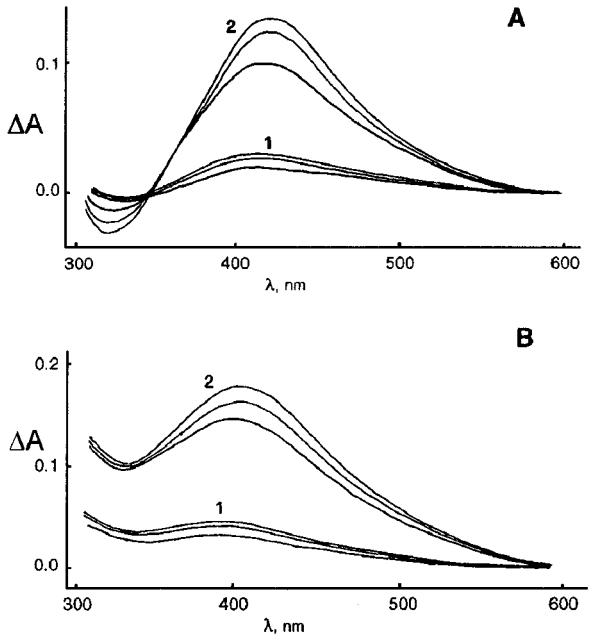


FIGURE 2: Representative UV−Vis difference spectra obtained upon titration of AO (about 40 μM) with azide in the absence (A) and in the presence (B) of 600 equiv of phenol in 100 mM phosphate buffer, pH 7.0: (1) initial phase, with $N_3^- < 10$ equiv; (2) final phase, with $200 < N_3^- < 1000$ equiv.

interference by the strong absorption of phenol in the near-UV, no decrease of absorption at 330 nm seems to occur in this case. A much reduced value for the low-affinity constant $K_2' = 10 \pm 4$ M^{-1} was estimated from the optical data. The total increase of absorption due to the LMCT band now corresponds to $\Delta\epsilon \approx 4500$ M^{-1} cm^{-1}. Thus, the spectral characteristics of the AO−azide complex are perturbed in the presence of phenol, and the affinity of this ligand for AO is markedly lowered.

EPR Studies. The modifications of the EPR spectrum of AO induced by binding of excess azide to a 150 μM solution of AO in 100 mM phosphate buffer, pH 7.0, were investigated in the absence and in the presence of phenol. The EPR changes produced by addition of increasing amounts of azide to the present preparation of AO were the same as those described previously (Casella et al., 1988), and basically consist of a reduction in the hyperfine splitting constant of type 2 copper. This spectrum remains unchanged after incubation for 24 h at 4 °C. When 90 mM phenol is incubated with 150 μM AO, the EPR spectrum of the enzyme is not modified even after prolonged incubation (72 h) at 4 °C. The addition of increasing amounts of azide to AO previously incubated with 600 equiv of phenol produces remarkable changes in comparison with the experiment in the absence of phenol (Figure 3). No shift of the low-field $M_I = -3/2$ hyperfine line of type 2 copper to higher field is observed, indicating that binding of azide to this center is prevented in the presence of phenol, and, in addition, the intensity of the type 1 copper signal *decreases* with time upon addition of azide in the EPR tube. After 48 h incubation of AO with 600 equiv of phenol and 500 equiv of azide at 4 °C, only the EPR spectral features associated with type 2 copper were present in the spectrum. The EPR parameters calculated from the spectrum ($g_{\|} = 2.250$, $g_{\perp} = 2.057$, $A_{\|} = 178 \times 10^{-4}$ cm^{-1}) are similar, though not identical, to those estimated from computer simulation of the spectrum of the native enzyme (Marchesini & Kroneck,

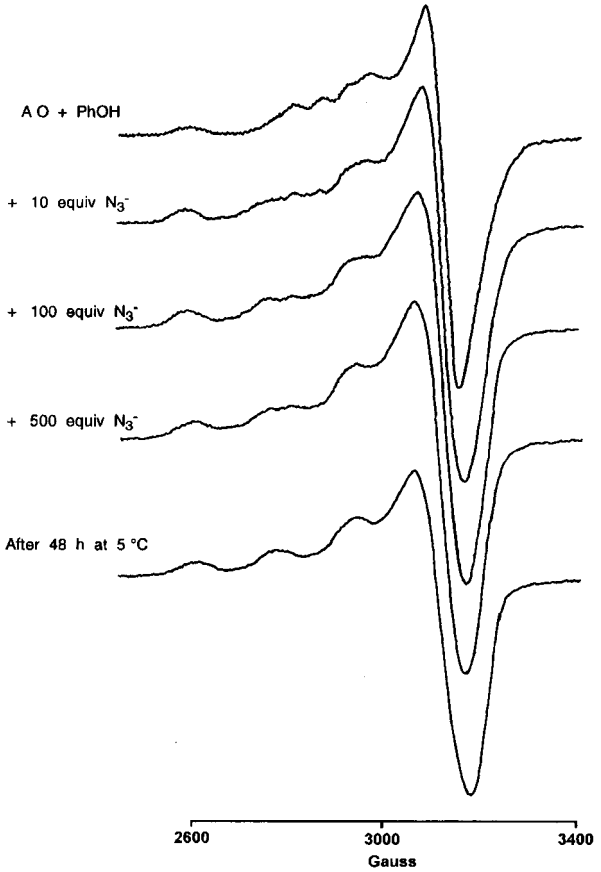


FIGURE 3: EPR spectra of AO (150 μM) recorded in the presence of 600 equiv of phenol and with increasing amounts of azide (10, 100, and 500 equiv) in 100 mM phosphate buffer, pH 7.0. The final spectrum was recorded after incubation of the last sample for further 48 h at 4 °C. Conditions: temperature −150 °C; microwave frequency 8.98 GHz; modulation amplitude 8.0 G; modulation frequency 100 kHz; microwave power 10 mW.

1979); in particular, the value of the hyperfine constant is slightly smaller. The disappearance of the type 1 copper EPR signal indicates that reduction of this copper by phenol is related with azide binding to type 3 copper. After the experiments described above were performed, the AO sample containing azide and phenol was dialyzed extensively against phosphate buffer. All the azide + phenol induced spectral features were reversed by dialysis, and, furthermore, 80% activity of the enzyme was recovered after dialysis.

Circular Dichroism Studies. The addition of excess azide produces changes in the near-UV CD spectrum of AO (Casella et al., 1989, 1991a). Relatively high azide concentration (500 equiv) causes a broad decrease of CD activity between 400 and 470 nm; the resulting spectrum is almost flat between 300 and 350 nm and contains a very broad negative band extending from 370 to 500 nm comprising several absorptions (Figure 4). The CD difference spectrum has a negative maximum near 420 nm, corresponding to the λ_{max} of the azide−Cu(II) LMCT band developed in the optical spectrum. While phenol (600 equiv) alone does not affect the CD spectrum of AO, incubation of AO with phenol (600 equiv) and azide (500 equiv) produces less CD change than that caused by azide binding (500 equiv) to native AO (Figure 4). This effect is likely due to the lack of azide binding to the type 2 copper in the presence of phenol. When incubation was continued for 48 h at 4 °C, the absorption and CD intensity decreased throughout the visible spectral

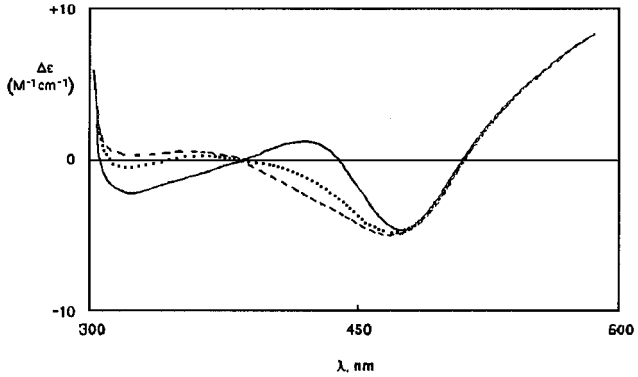
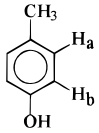
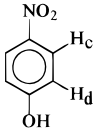


FIGURE 4: Circular dichroism spectra of native AO (44 μM) (—), AO in the presence of 500 equiv of azide (- - -), and AO in the presence of 500 equiv of azide and 600 equiv of phenol (···).

Table 2: Longitudinal Relaxation Time of the Protons for the AO-Bound Inhibitors and Copper−Proton Distances Evaluated with Equation 3

Inhibitor	Proton	δ (ppm)	T_{1b} (ms)	r_{Cu-H} (Å)
	CH_3	2.26	0.89	4.5
	H_a	6.80	2.25	5.3
	H_b	7.12	4.22	5.9
	H_c	8.10	0.80	4.4
	H_d	6.86	0.87	4.5

range, dominated by CT features of the type 1 copper, confirming the reduction process occurring at this site of the enzyme, in the presence of excess phenol and azide, indicated by the EPR measurements (data not shown).

NMR Measurements. The ^{1}H NMR spectra of *p*-cresol and *p*-nitrophenol in 100 mM deuterated phosphate buffer, pH 7.0, were recorded in the presence of increasing amounts of AO in the same buffer. Broadening of the proton signals of the inhibitors was observed upon addition of AO. A similar effect was observed using phenol, but the longer relaxation rates of its aromatic protons make measurements on this compound less convenient. The proton longitudinal relaxation times (T_{1obs}) of the inhibitors were found to decrease with the increase in enzyme concentration, indicating that the bound inhibitor undergoes a paramagnetic effect by the enzyme copper centers. The NMR signals of the two inhibitors used for T_{1b} calculations are indicated in Table 2. For all the protons, the plots of $(T_{1obs})^{-1}$ versus $E_0/(K_d + I_0)$, the fraction of enzyme-bound inhibitor, were linear. For K_d in this expression, we used the inhibition constant derived from competitive inhibition studies. From the slopes of the plots, the T_{1b} values for protons of bound cresol or *p*-nitrophenol were calculated, and the data are reported in Table 2. For *p*-cresol, the methyl protons undergo the strongest paramagnetic effect and, therefore, must be closer to the paramagnetic center(s), followed by the meta and ortho protons, respectively. The paramagnetic effect undergone by the protons in *p*-nitrophenol is stronger than those

observed for the *p*-cresol. This indicates that the former inhibitor binds closer to the copper center(s).

Since type 3 copper is essentially diamagnetic and type 1 and type 2 coppers are very far apart in the AO structure, it can be assumed that only one of the latter two centers is responsible for the paramagnetic effect. Taking T_{1b} as the T_{1M} value for the corresponding proton, it is possible to derive structural information on the binding of the phenols to AO from eq 3. The distance estimates obtained from the T_{1M} values are reported in Table 2. The shorter distance from the paramagnetic center of bound *p*-nitrophenol, with respect to *p*-cresol, may be due to the larger fraction of ionized species present at neutral pH. Both the protonated and the anionic forms of the phenol inhibit competitively the enzyme, but they bind to AO with different binding constants. Thus, while the binding site of the two forms is probably almost the same, the copper−proton distances obtained using eq 3 are an average of those of the bound protonated and ionized forms (with the phenoxide form approaching more closely the copper center). On the other hand, as shown by the inhibition studies, the AO binding site is rather closed, and increasing the size of the phenol substituent may keep the bound inhibitor molecule further away from the copper center.

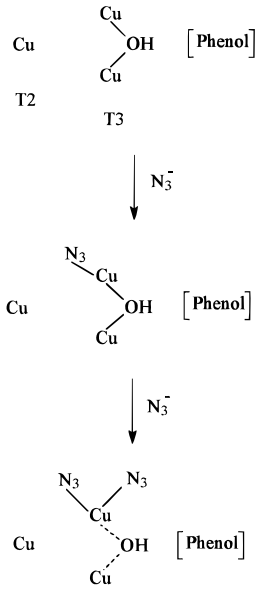
Similar NMR experiments were carried out using the T2D form of AO, but unfortunately this form is much less stable than the native form in the presence of the phenols and undergoes partial denaturation, with formation of precipitate, during the experiment.

DISCUSSION

Simple phenolic compounds are a class of competitive inhibitors of AO and, therefore, are expected to bind to the enzyme at the ascorbate binding site, or very close to this site, so that they can induce a conformational modification in the protein and thereby inhibit substrate binding. The best inhibitors contain an uncharged, electron-withdrawing substituent on the phenol ring; space limitation prevents the access to phenols carrying large substituent groups. The inhibitor effect is favored at higher pH, indicating that the anionic form of the phenol is a stronger inhibitor of AO. This is reasonable in view of the fact that at neutral pH ascorbic acid (pK_a 4.1) is ionized. However, the data in Table 1 show that the inhibitory effect is not simply related to the acidity of the phenol, but appears to be controlled by steric effects at the para substituent.

Although phenols do not interact directly with the copper centers, their presence affects the properties of the trinuclear cluster. In particular, the spectral features of the azide−AO complex are slightly changed in the presence of phenol, and the affinity of this exogenous ligand for AO is reduced. As is evident from the EPR experiments, the type 2 copper becomes inaccessible when phenol is bound to the enzyme, and this indicates that the inhibitor induces some protein modification that switches off the access channel to this site from the protein surface that is shown by the AO crystal structure (Messerschmidt et al., 1992a). The slight change in EPR parameters of type 2 copper reflects such a modification. The access to type 3 copper also seems affected, based on the decrease of affinity for azide. Thus, it is proposed that binding of azide to the phenol-bound form of AO occurs according to Scheme 1. Azide binds to type 3 copper in the terminal mode, since the spectral features of the azide−Cu(II) LMCT band lack the multicomponent structure characteristic of bridging azide (Pate et al., 1989; Casella et al., 1993), particularly in the CD spectra, where the low-energy component of the LMCT band, between 450 and 500 nm, which is weaker in absorption, becomes dominant (Beltramini et al., 1995). Both azide molecules are supposed to bind to the same (upper) type 3 copper atom of the couple, since this is the one accessible from the solvent. The picture emerging for the azide−AO−phenol adduct is thus similar to the structure of the azide−AO adduct resulting from the X-ray investigation (Messerschmidt et al., 1993), except for the possible presence of the hydroxide bridge between the two coppers, which is impossible to assess here with the present data. The bridge is absent in the crystal structure of the azide−AO complex, and we have evidence for its cleavage in solution at high azide concentration through NMR studies.[2]

Scheme 1



Another peculiar aspect of the phenol-bound form of AO is the observation of type 1 copper reduction when azide is present. Reduction of type 1 Cu has been observed by reaction of the enzyme with nitric oxide (Dawson et al., 1980; Gorren et al., 1987) or, as the primary event, with radicals produced by pulse radiolysis (O'Neill et al., 1983), or photochemically (Meyer et al., 1991; Farver & Pecht, 1994; Tollin et al., 1993; Hazzard et al., 1994). Type 1 copper is also the primary electron acceptor from the reducing substrate (Kroneck et al., 1982; Mondovì & Avigliano, 1984; Sakurai et al., 1986). In general, it is accepted that small ligands such as fluoride and azide do not affect the reduction of type 1 copper and, when present at relatively low concentration (up to 1 mM), also the rate of intramolecular electron transfer from type 1 copper to type 3 copper of AO under anaerobic conditions (Meyer et al., 1991). In the azide−AO−phenol system studied here, we suggest that phenol can reduce type 1 copper upon prolonged incubation, being stoichiometrically oxidized to phenol radical, and that intramolecular electron transfer to the fully bound azide complex of type 3 copper obtained at high azide concentration is inhibited. The type

[2] Unpublished observation.

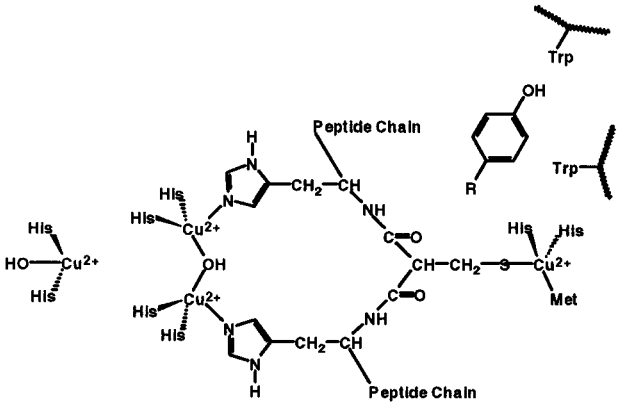


FIGURE 5: Schematic structure of the proposed phenol binding site near the type 1 copper center of AO.

3 coppers are likely to remain in the oxidized state in the presence of excess azide since, as we found (Casella et al., 1989, 1991), this ion induces reoxidation of reduced type 3 copper in T2D AO. In the absence of azide, reoxidation of the coppers after intramolecular electron transfer may occur, but the phenol oxidation reaction is too slow to be detected in the conventional kinetic experiments, where very small concentrations of enzyme are used. It is interesting to note that in laccase the intramolecular electron transfer is inhibited even at low fluoride concentrations (Tollin et al., 1993), probably because the affinity of this ligand for type 3 copper is higher than in ascorbate oxidase. In any case, the type 1 copper reduction process observed in the azide−AO−phenol complex supports the view that the site of phenol binding to the enzyme lies close to the type 1 center. The aromatic ring of these inhibitors seems an ideal candidate for positive interaction with the two tryptophan residues (Trp-163 and Trp-362) in the pocket of the putative substrate binding site, communicating with the type 1 center through the His-512 ligand (Figure 5) (Messerschmidt et al., 1992a). The paramagnetic NMR relaxation rate enhancement undergone by the phenol protons in the presence of the enzyme is in complete agreement with this view.

The relatively short (4.4−5.9 Å) copper−proton distances found for the AO−inhibitor adduct indicate that, indeed, when the substrate is arranged in a similar disposition within the enzyme, an extremely fast electron transfer rate between ascorbate and type 1 copper can readily occur (Kroneck et al., 1982). Although it was not possible to obtain an independent confirmation for the proposed location of the inhibitor through NMR relaxation measurements using T2D AO, due to the instability of this enzyme form, the alternative interpretation involving phenol binding at the other paramagnetic center, type 2 copper, is ruled out by the absence of any effect of the phenols on the spectroscopic properties of this site. A reverse electron flow from the phenol to the trinuclear site to type 1 copper would be necessary to explain the observed reduction of type 1 copper in the presence of excess phenol and azide. But if type 2 copper is the primary electron acceptor, the process would leave type 2 copper reduced, whereas the EPR experiments clearly show it remains in the oxidized state. In any case, the three-dimensional structure of AO shows that the steric accessibility of type 2 copper is very limited and the close approach to this center by a relatively large molecule such as *p*-cresol would be virtually impossible (Messerschmidt et al., 1992a, 1993). By contrast, the steric approach to the type 1 center of AO is much less restricted, and even a large molecule such as a flavin can apparently come close to this center (Hazzard et al., 1994). The present investigation further shows that occupation of the substrate pocket of AO has marked influence on the accessibility of the type 2 and type 3 centers. The possibility that this effect can, in turn, produce some change in the redox reactivity of the trinuclear cluster is very high and deserves further study.

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