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The Ceruloplasmin Catalyzed Oxidation of Dimethyl-*p*-phenylenediamine

II. Determination of the Steady-State Rate Equation

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The kinetics of the ceruloplasmin catalyzed oxidation of dimethyl-*p*-phenylenediamine (DPD) have been investigated with particular reference to previous reports that ceruloplasmin contains two different types of active sites. Statistical analysis of the experimental data gave evidence that both DPD and its primary oxidation product DPD^+ function as substrates for the enzyme, and the combined steady-state rate equation was determined. In contrast to previous reports the enzymatic reaction was found to conform to a first-degree reciprocal rate equation with respect to each of the two substrates, and the results are in quantitative consistence with a mechanism where the two substrates compete for one and the same enzymatic site.

Ceruloplasmin has been classified as a ferro:O_2 oxidoreductase (EC 1.7.3),[1] but has also oxidase activity against a variety of catecholamines and aromatic polyamines. Dimethyl-*p*-phenylenediamine (DPD) is frequently used as a standard substrate for ceruloplasmin,[2,3] enzyme activity being determined spectrophotometrically by observation of the rate of formation of Wurster's red (DPD^+). The kinetics of the ceruloplasmin catalyzed oxidation of DPD were studied by Curzon [3] and by Walaas *et al.*,[4] who showed that reciprocal plots of the enzymatic reaction rate against the reciprocal DPD concentration were non-linear. The results were interpreted in terms of two different types of substrate binding sites in the ceruloplasmin molecule, and kinetic constants as well as thermodynamic properties associated with the two sites were determined. Lövstad later stated that the two kinetically active sites may be located on the same or on different enzyme molecules, and presented evidence in favour of the latter alternative.[5]

Some objections may, however, be raised against the above interpretations of the kinetic properties of ceruloplasmin. Firstly, the rate equation which was fitted to the experimental data (eqn. (17)) has been derived for a

mechanism where two different enzymes act on the same substrate,[6,7] and cannot be applied to the bivalent case where the different sites are located on the same enzyme species.[7] Secondly, previous investigations have suggested that phenylenediamines and their first and second oxidation products are in rapid redox equilibrium.[8,9] This was recently confirmed in the case of DPD,[10] and it was shown that the dismutation equilibrium

$$2DPD^{+} \rightleftharpoons DPD + DPD^{2+} \quad (1)$$

has a considerable influence on the steady-state kinetics of the enzymatic oxidation of DPD.[11] For example, Curzon and Walaas *et al.* assumed that the enzymatic reaction rate was proportional to the rate of formation of DPD^{+}, but the functional relationship between these variables is much more complex and involves correction terms due to the dismutation equilibrium.[11] Corrections should also be made for the non-enzymatic decomposition of the second oxidation product DPD^{2+}.[12] Furthermore, it is not evident that DPD is the actual or the only substrate for the enzyme. A direct enzymatic oxidation of DPD^{+} is equally possible in view of the dismutation equilibrium, and recent kinetic studies provided clear evidence that the concentration of DPD^{+} has a major influence on the reaction velocity at low concentrations of DPD.[13] Since the variable [DPD^{+}] had not been controlled in the experiments of Curzon and Walaas *et al.* it was considered necessary to reinvestigate the kinetics of the ceruloplasmin catalyzed oxidation of DPD. The purpose of the present investigation is to study whether DPD or DPD^{+} or both function as substrates for ceruloplasmin, and to determine the corresponding empirical steady-state rate equation.

EXPERIMENTAL

Materials. Human ceruloplasmin was obtained through the courtesy of Dr. H. Björling, AB Kabi, Stockholm. The preparation used was about 80 % pure, as determined from the ratio of extinctions at 610 nm and 280 nm.[14] Enzyme concentrations were calculated from the extinction at 610 nm using $\varepsilon_{610} = 1.09 \times 10^{4}$ M^{-1}.[14] *N,N*-Dimethyl-*p*-phenylenediamine dihydrochloride was obtained from Merck, Darmstadt. Immediately before use DPD,2HCl was dissolved in dilute NaOH containing 50 μM EDTA, and the free amine was extracted twice with ether at pH 6. After addition of calculated amounts of dilute acetic acid the ether was removed in vacuum to give a chloride free 0.05 M acetate buffer solution of DPD, the pH of which was adjusted to pH 4.8 by addition of NaOH. The concentration of this solution of DPD acetate was determined spectrophotometrically by measurement of the maximum extinction at 550 nm (due to DPD^{+}) obtained on rapid enzymatic oxidation.[10,13]

Assays. The oxidation of DPD acetate by ceruloplasmin was followed at 550 nm in a Zeiss PM Q II spectrophotometer. To avoid enhancement of activity by traces of iron, reactions were carried out in the presence of EDTA.[8,15] The reaction mixture was held in a 1 cm-path cell in a constant-temperature housing at 25°, and contained DPD, 50 mM acetate buffer, pH 4.8, 100 μM EDTA, and ceruloplasmin (0.6–0.8 μM) in a total volume of 3 ml. Reactions were started by addition of ceruloplasmin, and E_{550} was recorded at 5 sec intervals over the extinction range 0.05–0.80. All measurements were restricted to the positive phase of the reaction where the concentration of the second oxidation product DPD^{2+} is small;[11] possible inhibitory effects of DPD^{2+} could thus be eliminated.[13] Measurements of extinction *vs.* time curves were performed at several different initial levels of the DPD concentration (c_A), ranging from 5 mM to 0.1 mM. Due to the autoxidation of DPD reactions had to be started at initial concentrations of DPD^{+} in the order of 0.01 mM when c_A exceeded 1 mM.

Calculation methods. Methods for calculation of reaction velocities v and the corresponding concentrations of DPD and DPD^+ have previously been described in detail and will only be briefly specified here.[13] The experimentally determined E_{550}/time curves were partly used for calculation of $[DPD^+]$ (assuming that $\varepsilon_{550}=1.04\times10^4$ M^{-1}) and partly for determination of $d[DPD^+]/dt$ by numerical differentiation with respect to time t. Reaction velocities were then computed from eqn. (2), which includes corrections for the dismutation equilibrium (1) (K denotes the dismutation equilibrium constant) as well as for the non-enzymatic decomposition of DPD^{2+} (k stands for the corresponding first-order rate constant). At any particular value of $[DPD^+]$ and of c_A (the initial concentration of DPD) the corresponding actual concentration of DPD was computed using eqn. (3), which usually was found to give sufficiently precise results for the purpose of the present investigation.

$$v=\frac{\{c_A-(1-4K)[DPD^+]\}\frac{d[DPD^+]}{dt}+2kK[DPD^+]^2}{\sqrt{(c_A-[DPD^+])^2-4K[DPD^+]^2}} \quad (2)$$

$$[DPD]=\tfrac{1}{2}\{c_A-[DPD^+]+\sqrt{(c_A-[DPD^+])^2-4K[DPD^+]^2}\} \quad (3)$$

It may be observed that eqns. (2) and (3) can be considered as corrections of the simple relationships (4) and (5), respectively:

$$v=d[DPD^+]/dt \quad (4)$$

$$[DPD]=c_A-[DPD^+] \quad (5)$$

When c_A exceeded 2 mM, reaction velocities determined from eqn. (2) agreed well with the rate of formation of DPD^+, corrections to eqn. (4) generally being less than 1 %. At lower initial concentrations of DPD corrections became more significant (5—10 %). Experimental observations for which corrections exceeded 15 % were discarded.

The theory of a general method for processing experimental data in order to establish empirical rate equations has been described elsewhere.[16] All empirical rate equations and kinetic constants determined in the present investigation were obtained by this method.

All calculations required for determination of reaction velocities and rate equations were carried out on a digital computer in units of the extinction of DPD^+. For construction of figures shown in this paper, and for the final evaluation of kinetic coefficients, the value $\varepsilon_{550}=1.04\times10^{-4}$ M^{-1} was used for the extinction of DPD^+ at 550 nm.[10] Throughout the paper enzymatic reaction velocities are given in moles of electrons transferred per min per mole of enzyme; the dimension is usually abbreviated to min^{-1}.

RESULTS

Fig. 1 shows the results of a typical experiment where the enzymatic reaction velocity was determined as a function of $[DPD^+]$ at various initial concentrations of DPD. Each curve was calculated from the experimentally measured E_{550}/time curves using eqn. (2), and for each experimental point in Fig. 1 the corresponding value of [DPD] is known according to eqn. (3). It may be observed that eqn. (5) gives a rough estimate of the decrease of [DPD] along each curve.

The previous finding that DPD^+ has a major influence on the reaction rate at low initial concentrations of DPD [13] was confirmed by the present investigation. For $c_A<1$ mM reaction velocities were found to increase with $[DPD]^+$ in a most significant way (see Fig. 1), in spite of the fact that there is a concomitant decrease in the concentration of DPD. On the other hand, examination of Fig. 1 also shows that DPD significantly affects the reaction

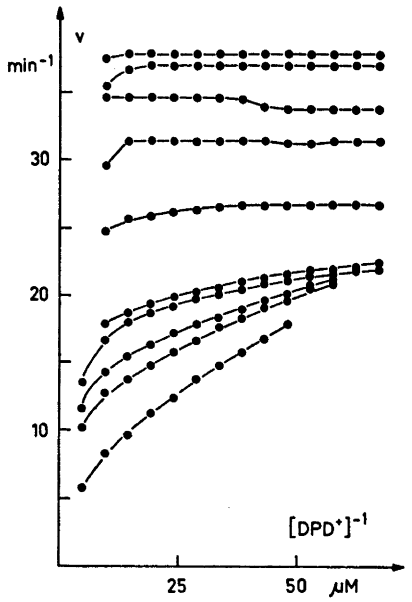

Fig. 1. Experimental determinations of the reaction velocity v as a function of [DPD$^+$] at ten different initial concentrations of DPD. The ceruloplasmin concentration used was 0.68 μM. The initial DPD concentrations were for the respective curves (starting with the highest one) 50, 40, 30, 20, 10, 5, 4, 3, 2, and 1 times 84.6 μM.

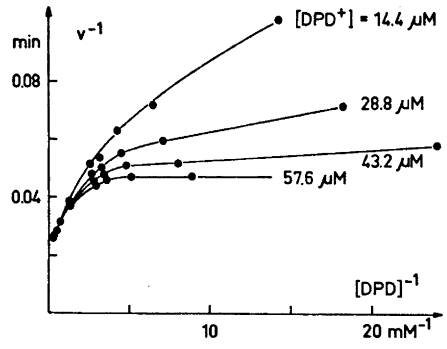

Fig. 2. Lineweaver-Burk plots with respect to DPD for the data shown in Fig. 1 at four different constant concentrations of DPD$^+$.

rate. At any constant value of [DPD$^+$] the reaction velocity steadily increases with c_A, and hence also with [DPD] (*cf.* eqns. (3) and (5)). These observations clearly indicate that both DPD and DPD$^+$ may function as substrates for the enzyme, and that the empirical rate equation should include both of the concentration variables [DPD] and [DPD$^+$].

It was considered convenient to start by examining the functional dependence of v on [DPD]. At any constant value of [DPD$^+$] each curve in Fig. 1 gives an experimental observation of v at the corresponding value of [DPD] as being calculated from eqn. (2). For preliminary analysis these paired values of v and [DPD] were plotted reciprocally according to the method of Lineweaver and Burk. Fig. 2 shows such plots for some different constant levels of [DPD$^+$], and it can be seen that the experimental points cannot be fitted to straight lines. Non-linear reciprocal rate plots may indicate the presence of higher-degree terms of the concentration variable in the rate equation, but will also be obtained for a first-degree rate equation if the reaction rate does not equal zero when the concentration variable does so.

In order to distinguish between these possibilities the empirical rate equation with respect to DPD was determined at each constant concentration of

[DPD^+] using statistical methods which have been described in detail elsewhere.[16] It was found that the estimated rate equations with respect to DPD at any level of [DPD^+] adhered to the general form

$$v = \frac{\alpha_0 + \alpha_1[DPD]}{1 + \beta_1[DPD]} \tag{6}$$

where the coefficients α_0, α_1, and β_1 are non-vanishing and independent of [DPD]. The presence of any higher-degree terms in the rate equation could not be established even at the 5 % level of significance, *i.e.* fits were not significantly improved by using the rate equation

$$v = \frac{\alpha_0 + \alpha_1[DPD] + \alpha_2[DPD]^2}{1 + \beta_1[DPD] + \beta_2[DPD]^2} \tag{7}$$

For these reasons it was concluded that the actual empirical rate equation was of the first degree with respect to DPD, and the results obtained on fitting eqn. (6) to the experimental material are listed in Table 1.

Table 1. Coefficient estimates (±standard deviations) obtained at different constant values of [DPD^+] on fitting eqn. (6) to the experimental data given in Fig. 1.

[DPD^+] μM	α_0 min^{-1}	α_1 $min^{-1}mM^{-1}$	β_1 mM^{-1}	α_1/β_1 min^{-1}
9.6	5.64±0.78	54.0±5.3	1.26±0.15	43
14.4	7.56±0.54	52.6±3.7	1.20±0.10	44
19.2	9.60±0.51	46.7±3.5	1.05±0.09	45
24.0	11.18±0.44	43.4±3.2	0.97±0.08	44
28.8	12.75±0.42	38.8±3.2	0.86±0.08	45
33.6	14.15±0.41	34.8±2.8	0.76±0.08	46
38.4	15.32±0.41	31.4±3.0	0.68±0.08	46
43.2	16.5±0.42	27.5±3.2	0.59±0.08	47
48.0	17.6±0.39	23.7±2.8	0.50±0.07	47
52.8	18.1±0.42	23.2±3.2	0.49±0.08	47
57.6	19.1±0.54	19.4±3.5	0.39±0.09	50

The functional dependence of v on [DPD^+] could not be similarly determined as the experimental material in Fig. 1 only occasionally gives more than one observation of the reaction rate at any particular concentration of DPD. However, examination of eqn. (6) shows that the coefficient α_0 represents the reaction velocity at [DPD] = 0, and the influence of DPD^+ on v in the absence of DPD can be studied using the paired values of α_0 and [DPD^+] given in Table 1. A preliminary analysis by means of Lineweaver-Burk plots (Fig. 3) indicated that the rate equation with respect to DPD^+ in absence of DPD was of the Michaelis-Menten type:

$$v_{([DPD]=0)} = \alpha_0 = \frac{\alpha_{01}[DPD^+]}{1 + \beta_{01}[DPD^+]} \tag{8}$$

This was confirmed statistically (see Ref. 16), and the coefficient estimates obtained are listed in Table 2. It may be observed that eqn. (8) is an alternate form of the well-known Michaelis-Menten equation, obtained by putting $V_{max}=\alpha_1/\beta_1$ and $K_m=1/\beta_1$; calculated values of the Michaelis-Menten coefficients are also shown in Table 2.

Table 2. Coefficient estimates obtained on fitting eqns. (8) and (11) to the data indicated in Figs. 3 and 4, respectively. Calculated values of the corresponding Michaelis-Menten coefficients are also given.

Substrate	Coefficient estimates	V_{max} min^{-1}	K_m μM
DPD^+	$\alpha_{01}=670$ $min^{-1}mM^{-1}$ $\beta_{01}=17.8$ mM^{-1}	38	56
DPD	$\alpha_{10}=58.5$ $min^{-1}mM^{-1}$ $\beta_{10}=1.30$ mM^{-1}	45	770

As the reaction mechanism apparently is of the first degree with respect to both DPD and DPD^+, it follows from the theory of Wong and Hanes [17] that the combined rate equation would be given by

$$v=\frac{\alpha_{01}[DPD^+]+\alpha_{10}[DPD]}{1+\beta_{01}[DPD^+]+\beta_{10}[DPD]} \tag{9}$$

Substitution of eqn. (5) into eqn. (9) shows that the functional dependence of v on $[DPD^+]$ can be approximated as

$$v=\frac{a_0+a_1[DPD^+]}{1+b_1[DPD^+]} \tag{10}$$

where

$$a_0=v_{([DPD^+]=0)}=\frac{\alpha_{10}c_A}{1+\beta_{10}c_A} \tag{11}$$

$$a_1=\frac{\alpha_{01}-\alpha_{10}}{1+\beta_{10}c_A} \tag{12}$$

$$b_1=\frac{\beta_{01}-\beta_{10}}{1+\beta_{10}c_A} \tag{13}$$

Eqn. (10) is exactly analogous to eqn. (6), and can be analogously fitted statistically to the experimental data shown in Fig. 1 in order to obtain a_0 which represents the reaction velocity in absence of DPD^+. Such a method of extrapolation was used for each curve in Fig. 1, and yielded values of a_0 (the intercept on the v-axis) at the corresponding values of c_A. These results are given in Fig. 4 in the form of a Lineweaver-Burk plot which shows that the

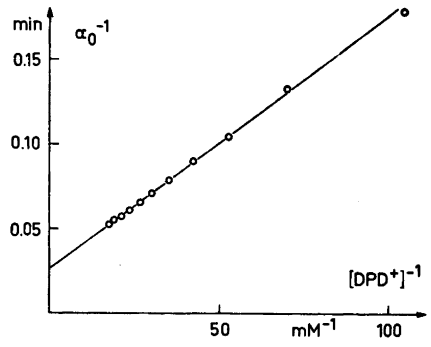

Fig. 3. Lineweaver-Burk plots with respect to DPD^+ at zero concentration of DPD, using the paired values of $[DPD^+]$ and $\alpha_0 = v_{([DPD]=0)}$ listed in Table 1.

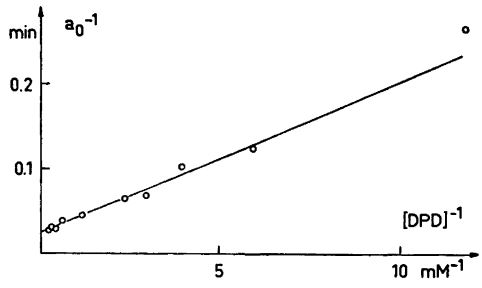

Fig. 4. Lineweaver-Burk plots with respect to DPD at zero concentration of DPD^+, using the paired values of $[DPD] = c_A$ and $a_0 = v_{([DPD^+]=0)}$ obtained on fitting eqn. (11) to the experimental data shown in Fig. 1.

points fall well along a straight line, and hence provides evidence for the validity of eqn. (11). The coefficients α_{10} and β_{10} may, consequently, be estimated graphically from the straight line in Fig. 4, or by statistical analysis. The coefficient estimates obtained statistically are given in Table 2.

A final test of the validity of the combined rate equation (9) was carried out in the following way. A comparison between eqns. (6) and (9) shows that

$$\alpha_1 = \frac{\alpha_{10}}{1 + \beta_{01}[DPD^+]} \tag{14}$$

$$\beta_1 = \frac{\beta_{10}}{1 + \beta_{01}[DPD^+]} \tag{15}$$

Both α_1 and β_1 should, consequently, decrease with $[DPD^+]$, and inspection of Table 1 confirms that this is the case. Furthermore, one obtains

$$\frac{\alpha_1}{\beta_1} = \frac{\alpha_{10}}{\beta_{10}} \tag{16}$$

and as shown in Table 1 the quotient α_1/β_1 exhibits a remarkable constancy in view of the precision of the coefficient estimates indicated by the corresponding standard deviations. The mean value of the quotient α_1/β_1 (46 min^{-1}) agrees excellently with the quotient $\alpha_{10}\beta_{10}$ as calculated by the extrapolation method (45 min^{-1}; see Table 2). It can be concluded that eqn. (9) may be accepted as the empirical rate equation with respect to the substrates involved in the ceruloplasmin catalyzed oxidation of DPD.

DISCUSSION

Several investigations have demonstrated the presence of two different kinds of valence-changing copper atoms in ceruloplasmin. For this reason, Curzon [3] and Walaas *et al.*,[4] observing that non-linear Lineweaver-Burk plots with respect to DPD were obtained, suggested that two different catalytic sites are present in the enzyme and evaluated their steady-state kinetic data in view of the postulated rate equation

$$v = \frac{V_1[\mathrm{DPD}]}{K_{m1}+[\mathrm{DPD}]} + \frac{V_2[\mathrm{DPD}]}{K_{m2}+[\mathrm{DPD}]} \tag{17}$$

where V_i and K_{mi}, $i = 1, 2$, are the usual Michaelis-Menten coefficients for the two different sites. Eqn. (17) may be written as

$$v = \frac{(V_1K_{m2}+V_2K_{m1})[\mathrm{DPD}]+(V_1+V_2)[\mathrm{DPD}]^2}{K_{m1}K_{m2}+(K_{m1}+K_{m2})[\mathrm{DPD}]+[\mathrm{DPD}]^2} \tag{18}$$

and is thus a second-degree rate equation of the Wong-Hanes type.[17] The present investigation confirmed that reciprocal rate plots with respect to DPD are non-linear (Fig. 2), but the statistical analysis gave no evidence for the presence of any second-degree terms in the rate equation. The experimental results were found to be properly described by the first-degree rate eqn. (6), which immediately indicates the presence of a competing substrate; according to eqn. (6) the reaction rate does not equal zero at zero concentration of DPD. Such an impartial choice between rate equations of different type cannot be easily made by graphical methods, and due to the fact that the postulated rate equation was not adequately chosen in previous investigations the possibility that DPD^+ affects the reaction rate was overlooked. The present paper thus illustrates the great advantage of statistical analysis over the commonly used graphical methods for evaluation of enzyme kinetic data.

Another likely reason for the previous failure to detect the influence of DPD^+ is that the reaction rate appears to be almost independent of $[DPD^+]$ at high initial concentrations of DPD. Differentiation of eqn. (10) with respect to $[DPD^+]$, using the relationships (11) – (13), yields

$$\frac{\mathrm{d}v}{\mathrm{d}[\mathrm{DPD}^+]} = \frac{\alpha_{01}-\alpha_{10}-c_A(\alpha_{10}\beta_{01}-\alpha_{01}\beta_{10})}{(1+\beta_{10}c_A+(\beta_{01}-\beta_{10})[\mathrm{DPD}^+])^2} \tag{19}$$

which shows that the effect of $[DPD^+]$ on the reaction rate in general decreases with increasing values of c_A and $[DPD^+]$. It can further be seen from eqn. (19) that $\mathrm{d}v/\mathrm{d}[DPD^+]$ equals zero for

$$c_A = \frac{\alpha_{01}-\alpha_{10}}{\alpha_{10}\beta_{01}-\alpha_{01}\beta_{10}} \tag{20}$$

when the reaction rate becomes completely independent of $[DPD^+]$:

$$v = \frac{\alpha_{01}-\alpha_{10}}{\beta_{01}-\beta_{10}} \tag{21}$$

Insertion of the coefficient estimates listed in Table 2 shows that such a situation of complete independence would be expected to arise for $c_A = 3.6$ mM when the constant reaction rate would equal 38 min^{-1}, which is in excellent agreement with the experimental data given in Fig. 1. Eqn. (19) also implies that the reaction rate would decrease as $[DPD^+]$ increases when c_A exceeds the value indicated by eqn. (20). Such effects will not be significant within the concentration ranges indicated in Fig. 1, but might be seen at higher c_A-values. It must be emphasized, however, that in view of the precision of the coefficient estimates obtained (Table 2) the observed difference between maximum activities for DPD and DPD^+ may be insignificant. If such is the case we have $\alpha_{10}\beta_{01} = \alpha_{01}\beta_{10}$ and $dv/d[DPD^+]$ will steadily approach zero with increasing values of c_A and $[DPD^+]$. Anyhow, the apparent independence of v on $[DPD^+]$ at high initial concentrations of DPD cannot be taken as evidence that DPD^+ does not function as a substrate for the enzyme; the kinetic properties of the ceruloplasmin system may be brought into quantitative consistence with rate eqn. (9) also in this respect.

The mere fact that the radical DPD^+ affects the reaction rate does not prove that it functions as a substrate for ceruloplasmin, but the observation that a Michaelis-Menten relationship between v and $[DPD^+]$ is obtained in the absence of DPD (Fig. 3) provides clear evidence that DPD^+ is enzymatically oxidized. This finding is of interest in view of previous reports on the mechanism of action of ceruloplasmin and related enzymes. Semiquinone radicals have been shown to be immediate products in the peroxidase [18] and laccase [19] catalyzed oxidation of quinol, but these radicals appeared to be poor substrates for the enzymes; the experiments provided strong evidence that the radicals disappeared by non-enzymatic reaction with one another. Free radicals have also been identified as initial products in the ceruloplasmin catalyzed oxidation of catecholamines [20] and of *p*-phenylenediamine (PPD) and its *N*-methylated derivatives.[4,9] The possibility that the radical cation PPD^+ formed from PPD functions as a substrate for ceruloplasmin was considered by Malmström *et al.*,[9] but mathematical model experiments appeared to favour that radical decay took place by the formal dismutation reaction

$$2PPD^+ \longrightarrow PPD + PPD^{2+} \qquad (22)$$

The experimental data could not be brought into consistence with models based upon enzymatic formation of PPD^{2+} by direct two-electron transfer, or by two successive steps of one-electron transfer. However, the possibility of successive one-electron transfers (*i.e.* enzymatic oxidation of the radical PPD^+) combined to a dismutation process was not considered, and even though the studies carried out by Malmström *et al.* provide evidence that phenylenediamine radicals disappear by dismutation they do not exclude a direct enzymatic oxidation of radicals in addition to the dismutation process.

On the other hand, the kinetic results obtained in the present investigation eliminate the possibility that the radical cation DPD^+ disappears exclusively by dismutation in presence of ceruloplasmin. Phenylenediamine derivatives are exceptional in exhibiting very low equilibrium constants for dismutation of the corresponding radicals, *i.e.* the radicals are exceptionally

stable.[10] This might explain the apparent differences between reaction mechanisms for quinols and phenylenediamines in the reactions discussed.

There is strong evidence that only the completely reduced form of ceruloplasmin reacts with oxygen,[2] and it follows that four substrate molecules probably have to combine to and react with the enzyme before reoxidation completes the reaction cycle. According to the kinetic theory of Wong and Hanes the degree of a steady-state rate equation usually equals the number of enzyme-containing species (including free enzyme) in the reaction cycle which react with the substrate,[17] and the theoretical rate equation for the ceruloplasmin-DPD system might thus be expected to be of a higher degree than unity. This is particularly true if the enzyme is assumed to contain several catalytic sites with different characteristics.[7] In view of these facts, the established empirical first-degree rate eqn. (9) appears to be unexpectedly simple; eqn. (9) is known to be obtained for a mechanism where two substrates compete for one and the same enzymatic site, and where product formation is associated with direct regeneration of the enzyme.[7] There are several possible explanations for the observed first-degree behaviour, but the present experiments do not distinguish between such possibilities. For the present time it may only be concluded that there is no kinetic evidence for the operation of a higher-degree mechanism at pH 4.8.

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