The Ceruloplasmin Catalyzed Oxidation of Dimethyl-
p-phenylenediamine

I. Evidence that Wurster's Red Functions as a Substrate for the Enzyme

GÖSTA PETTERSSON and INGER PETTERSSON

Department of Biochemistry, University of Lund, Box 740, S-220 07 Lund, Sweden

The kinetics of the ceruloplasmin catalyzed oxidation of dimethyl-
p-phenylenediamine (DPD) have been investigated by analysis of
the time-dependence of the formation of the primary oxidation
product DPD$. In contrast to what has previously been assumed,
DPD$ was found to function as the main substrate for the enzyme
at DPD concentrations below 1 mM, and the reaction rate was shown
to be governed by a Michaelis-Menten type of equation with respect
to the concentration of DPD$. The method used for estimation of
reaction rates introduces corrections for the non-enzymatic dismutation
of DPD$, as well as for the non-enzymatic decomposition of
the second oxidation product DPD$+, and may be of great value
for reliable evaluation of further kinetic characteristics of the
oxidation process.

Dimethyl-p-phenylenediamine (DPD) is frequently used as a substrate in
standard determinations of ceruloplasmin (E. C. 1.12.3) activity, and a large
number of investigations concerning the kinetic properties of ceruloplasmin
have been based on spectrophotometric measurements of the rate of formation
of Wurster's red (DPD$), the primary oxidation product of DPD. In such
experiments DPD has been assumed to be the only substrate for ceruloplasmin,
the enzyme catalyzing reaction (1), exclusively, by rapid electron transfer to
oxygen. Walaas et al. have, however, observed that also the subsequent
oxidation step (2) is affected by the enzyme and suggested that both DPD
and DPD$ may function as substrates for ceruloplasmin. The final product
of the oxidation process (denoted compound D) has recently been shown to
be formed spontaneously by first-order decomposition of DPD$+, as indicated
in reaction (3):

\[
\text{DPD} \rightarrow \text{DPD}^+ + e^- \quad (1)
\]

\[
\text{DPD}^+ \rightarrow \text{DPD}^{2+} + e^- \quad (2)
\]

DPD$^2^+$ \[ \xrightarrow{k} \text{compound D} \] (3)

The inconsistency between the experimental data presented by Walaas et al. and simple models based on the action of one enzyme on two substrates was pointed out by one of the present authors, who showed that the characteristic kinetic features of the formation of DPD$^-$ in the presence of ceruloplasmin could be explained by taking proper regard to the fact that DPD is in rapid equilibrium with its first and second oxidation products:

\[
2 \text{DPD}^+ \xrightarrow{K} \text{DPD} + \text{DPD}^2^+ \] (4)

According to the latter model either DPD or DPD$^+$ or both can be the actual substrate(s) for ceruloplasmin, and when the enzyme operates under conditions of substrate saturation it will not be possible to distinguish between these cases. If, however, the enzymatic reaction velocity changes during the reaction such a change might be correlated to the corresponding variation of concentrations of reactants, and a careful analysis of experimental data (covering not only the initial reaction phase, but the larger part of the reaction) might reveal the nature of the enzymatic process. The results of such experiments are described and discussed in the present investigation. The equations derived and used for calculation of the enzymatic reaction rate introduce corrections not only for the dismutation equilibrium (4), but also for the non-enzymatic decomposition of DPD$^2^+$ indicated in reaction (3).

THEORETICAL

Let us consider concentrations of reactants in the reaction mechanism defined by (1)–(4) as functions of time $t$. With introduction of the notations $A=[\text{DPD}], B=[\text{DPD}^+], P=[\text{DPD}^2^+]$ and $D=[\text{compound D}]$ the equilibrium equation for reaction (4) becomes

\[ KB^2 = AP \] (5)

Under the usual assumption that concentrations of enzyme-containing intermediates in the enzymatically catalyzed oxidation process are negligibly small in comparison with the initial concentration $c_A$ of DPD, the following elementary stoichiometric relationships are obtained

\[ c_A = A + B + P + D \] (6)
\[ e = B + 2P + 2D \] (7)

The variable $e$ denotes the total amount of electrons enzymatically removed from the system, and the enzymatic reaction rate $v$ is consequently given by

\[ v = \frac{de}{dt} \] (8)

Similar reaction mechanisms have been treated in detail previously, and it may suffice here to mention that it can be shown that $B$ passes through

a maximum value obtained at \( t = t_m \). Solving eqn. (5) for \( A \) after elimination of \( P \) using eqn. (6) we get

\[
A = \frac{1}{2} \left\{ c_A - B - D_{(+)} \sqrt{(c_A - B - D)^2 - 4KB^2} \right\}
\]

whence according to eqn. (6)

\[
P = \frac{1}{2} \left\{ c_A - B - D_{(+)} \sqrt{(c_A - B - D)^2 - 4KB^2} \right\}
\]

(9)

(10)

Signs within brackets refer to the “negative” reaction phase, which is obtained for \( t > t_m \).

Since reaction (3) is governed by first-order kinetics, the rate of formation of compound \( D \) is given by

\[
\frac{dD}{dt} = kP
\]

(11)

Hence integration yields

\[
D = k \int_0^t P \, dt
\]

(12)

and substituting eqn. (10) into eqn. (11) we get

\[
D = \frac{k}{2} \int_0^t \left\{ c_A - B - D_{(+)} \sqrt{(c_A - B - D)^2 - 4KB^2} \right\} dt
\]

(13)

which implicitly determines \( D \) as a function of \( c_A \) and \( B \) (and hence of time). The constant \( c_A \) may conveniently be determined from the experimental observations of \( B \) as a function of time using the approximate relationship

\[
c_A = (1 + 2\sqrt{K}) \cdot B(t_m) + D(t_m)
\]

(14)

Under the conditions used in the present investigation (or more precisely when the rate of decomposition of DP2\(^+\) is small in comparison with the enzymatic reaction velocity, i.e. \( kP \ll dP/dt \) at \( t \leq t_m \)) the error in eqn. (14) can be shown to be in the order of 0.01% of \( c_A \), and is thus completely negligible. Both \( k \) and \( K \) have previously been experimentally determined with sufficient precision; the values \( k = 0.05 \text{ min}^{-1} \) and \( K = 0.035 \) were used in the present investigation.

Analysis of an experimentally determined \( B(t) \)-curve (see Fig. 1) was carried out as follows. Starting by putting \( D = 0 \) \( c_A \) was calculated from eqn. (14), and preliminary estimates of \( D \) were determined by numerical evaluation of the integral in eqn. (13) using the well-known Simpson formula. About 30 equidistant points \( B(t_i) \), including the maximum point \( B(t_m) \), distributed over the larger part of the \( B(t) \)-curve were selected for these calculations. The resulting preliminary determinations of \( D(t_i) \) were then used for recalculation of \( c_A \) and \( D \) from eqns. (14) and (13), and the procedure was iterated until a self-consistent solution had been obtained. Three iterations were, in general, sufficient at the chosen level of precision (0.1%). When the functional dependence of \( D \) on time had been established, the corresponding values of \( A(t_i) \), \( P(t_i) \), and \( e(t_i) \) were computed using eqns. (9)–(11), and the enzymatic reaction velocity was determined from eqn. (8) by numerical derivation of \( e \); the conventional method of Milne and Thomson was used.

The above technique for determination of reaction velocities was found to be the most reliable one in studies of the total course of the reaction. If only the initial phase has to be considered reaction rates can be estimated in a simpler way. Differentiation of eqns. (5)—(7) yields

\[ 2KB \frac{dB}{dt} = A \frac{dP}{dt} + P \frac{dA}{dt} \]  
(15)

\[ \frac{dA}{dt} + \frac{dB}{dt} + \frac{dP}{dt} + \frac{dD}{dt} = 0 \]  
(16)

\[ \frac{de}{dt} = \frac{dB}{dt} + 2 \frac{dP}{dt} + 2 \frac{dD}{dt} \]  
(17)

Using eqns. (11) and (16) we may write eqn. (15) in the form

\[ \frac{dP}{dt} = \frac{(P+2KB)(dB/dt)+kP^2}{A-P} \]  
(18)

Substituting this into eqn. (17) and observing that the reaction velocity is defined by eqn. (8) we finally get

\[ v = \frac{A+P+4KB}{A-P} \cdot \frac{dB}{dt} + \frac{2kAP}{A-P} \]  
(19)

In the initial phase of the reaction we have \( D \approx 0 \) (see Fig. 4), and elimination of \( A \) and \( P \) in eqn. (19) under this restriction using eqns. (9) and (10) yields

\[ v = \frac{\{c_A-(1-4K)B\}(dB/dt)+2kKB^2}{\sqrt{(c_A-B)^2-4KB^2}} \]  
(20)

where \( B \) and \( dB/dt \) can be obtained from the initial part of the \( B(t) \)-curve. It may be observed that for \( B=0 \) eqn. (20) reduces to the simple relationship

\[ v = dB/dt \]  
(21)

**EXPERIMENTAL**

Ceruloplasmin was kindly provided by Dr. Björling, AB Kabi, Stockholm. The preparation used was about 65 %, pure; the \( E_{480}/E_{400} \) ratio was 0.028.

Oxidations of DPD (5—100 \( \mu \text{M} \)) were carried out at 25° in 0.05 M sodium acetate buffer solutions, pH 5.5, containing 10 \( \mu \text{M} \) EDTA and 0.2 mM chloride ion (added partly as DPD, 2HCl and partly as NaCl) in a final volume of 3 ml. Reactions were started by the addition of catalytic amounts (0.67 \( \mu \text{M} \)) of ceruloplasmin, the variation in the concentration of DPD\(^+\) being followed continuously over a period of 10—20 min by measurement of the extinction of the reaction solution at 550 mp in a Zeiss PM Q H spectrophotometer.

In order to avoid errors due to uncertainty in the experimental value of the extinction coefficient \( \varepsilon_{480} \) of DPD\(^+\), and to uncertainty in concentration of prepared solutions of DPD (\( c_A \)) all calculations required for analysis of the experimental data were carried out on a digital computer in units of \( \varepsilon_{480} \). For construction of figures shown in this paper, and for the final calculation of kinetic coefficients, the value \( \varepsilon_{480} = 1.04 \times 10^4 \text{ M}^{-1} \) was used.\(^5\)

RESULTS AND DISCUSSION

Typical curves showing the time-dependence of the concentration of DPD$^+$ during the ceruloplasmin catalyzed oxidation of DPD are given in Fig. 1 for three different initial concentrations of DPD (expts. I—III). As was shown in the theoretical section (eqn. (21)) the initial rate of oxidation can be approximately estimated from the initial rate of formation of DPD$^+$, but in order to determine velocities over a larger part of the reaction the rates of formation of all of the oxidation products must be considered. Analysing the curves in Fig. 1 by the method described in the theoretical section, the

![Graph showing time dependence of extinction and calculated amounts of electrons removed](image)

**Fig. 1.** Experimental determination of the time dependence of the extinction (E) at 550 nm during the oxidation of DPD (initial concentrations were in expt. I 161 μM, expt. II 110 μM, and expt. III 50 μM) in presence of catalytic amounts (0.67 μM) of ceruloplasmin.

**Fig. 2.** Calculated amounts of electrons removed from the system (e) as a function of time in expts. I—III.

The total amounts (e) of electrons removed from the system were calculated as a function of time (Fig. 2), and hence the reaction velocities could be obtained by derivation (Fig. 3). The analysis also gave the time dependence of the concentrations of DPD, DPD$^{2+}$, and compound D, as shown in Fig. 4 for expt. II.

Inspection of Figs. 2 and 3 immediately shows that DPD cannot be the only substrate for ceruloplasmin. The concentration of DPD steadily decreases during the oxidation process, but velocity curves exhibit pronounced maxima; a comparison of the curves obtained in the different experiments eliminates the possibility that this rate behaviour is due to substrate inhibition. The shape of the curves, in fact, indicates that there is an excellent correlation

*Acta Chem. Scand. 23 (1969) No. 9*
between the reaction rate and the concentration of DPD⁺ (see Fig. 4), which thus appears to be the main substrate for the enzyme under the conditions used in the present investigation.

A more detailed examination of the experimental results reveals that reaction velocities (v) at equal concentrations of DPD⁺ are higher in the positive phase of the reaction (vₗ denotes rates for t < tₘ) than in the negative phase (v₋). This can easily be seen from Fig. 4, where v₊ and v₋ have been given for corresponding concentrations of DPD⁺. It follows that also other reactants than DPD⁺ must affect the enzymatic reaction rate. One possible explanation is that DPD functions as a second substrate for the enzyme, the corresponding (minor) contribution to the reaction rate decreasing with [DPD] and thus being most pronounced in the initial phase of the reaction. It is, however, also possible that the observed difference between v₊ and v₋ is due to product inhibition caused by the appearance of DPD²⁺ and compound D in the late phase of the process.

In order to discriminate between these possibilities use can be made of the fact that expts. I—III were carried out at three different initial concentrations of DPD (c₃(I) > c₃(II) > c₃(III)). If DPD significantly contributes to the reaction rate a plot of v against [DPD⁺] would yield three initially separate curves characterized by v₊(I) > v₊(II) > v₊(III), which would tend to become identical in the late reaction phase where [DPD] can be neglected (v₋(I) ≈ v₋(II) ≈ v₋(III)). The reverse situation would arise in the case of product inhibition. Product concentrations, which initially are equal in the sense that they are all negligibly small (v₋(I) ≈ v₋(II) ≈ v₋(III)), will finally reach entirely different levels in expts. I—III with a corresponding influence on the reaction rates (v₋(I) < v₋(II) < v₋(III)).

The results of the present investigation provide clear evidence for the latter alternative. As shown in Fig. 5, plots of v₋ against [DPD⁺] yield three entirely different curves for expts. I—III, located in relation to each others as would be expected in the case of product inhibition. Furthermore, experi-

mental points fall well along one single curve in similar plots for $v_+$ (Fig. 5), which definitely establishes that the influence of DPD on the reaction velocity is negligible over the tested range of concentrations.

Consequently, the reaction rate should be dependent exclusively upon $[\text{DPD}^+]$ during the larger part of the positive phase (cf. Fig. 4), and investigation of the corresponding functional dependence using a Lineweaver-Burk plot gave the results shown in Fig. 6. The experimental points can be excellently fitted to a straight line, and it follows that the reaction appears to be governed by a Michaelis-Menten type of rate equation with respect to the concentration of DPD$^+$. Calculation of the corresponding kinetic coefficients from the reciprocal rate plot in Fig. 6 gave $K_m = 35 \mu\text{M}$ and $V = 48 \mu\text{M}$ electrons transferred per min per $\mu\text{M}$ ceruloplasmin.

The decrease in reaction rates due to product inhibition could not be sufficiently precisely determined from expts. I—III for reliable evaluation of the kinetic characteristics of the inhibition mechanism. Even though it seems most likely that competitive inhibition by DPD$^{2+}$ is a main effect, the experimental results did not exclude that also compound D acts as an inhibitor, or that the inhibitory effect of DPD$^{2+}$ is not purely competitive.

The importance of the dismutation process (4) has not been recognized in previous investigations of the kinetics of the ceruloplasmin catalyzed oxidation of DPD. Reaction rates have previously been assumed to be proportional to the rate of formation of DPD$^+$ in accordance with eqn. (21), but as was shown in the theoretical section this relationship will be valid only when the concentration of DPD$^+$ is zero. Since the formation of significant initial amounts of DPD$^+$ produced by non-enzymatic oxidation of DPD cannot be avoided in kinetic experiments, particularly not at high concentrations of DPD$^+$, initial reaction velocities should preferably be calculated from eqn. (20).

The present investigation appears to establish that DPD\(^+\) functions as the main substrate for ceruloplasmin at DPD concentrations below 1 mM. This fact has not previously been recognized, and kinetic data concerning ceruloplasmin may have been misinterpreted in several respects. Walaas et al.\(^2\) and Curzon\(^1\) have, for example, independently studied the effect of DPD concentrations on the reaction rate, assuming that DPD is the only substrate for the enzyme. Reciprocal rate plots with respect to DPD were found to show significant curvature, which was interpreted as being due to the presence of two DPD-binding enzymatic sites. Kinetic coefficients were determined for both sites by analysis of the reciprocal rate plots but, in view of the present results, these determinations cannot be valid as the effect of the concentration of DPD\(^+\) (which was not controlled or measured during the experiments) on the reaction rate was neglected. It seems, in fact, likely that the observed curvature in the reciprocal rate plots with respect to DPD can be partly or entirely ascribed to the fact that DPD\(^+\) has a major influence on reaction velocities at low DPD concentrations; it may be noted that the kinetic coefficients obtained by Walaas et al. (\(K_m = 40 \, \mu\text{M}, V = 3 \, \text{min}^{-1}\)) and Curzon (\(K_m = 60 \, \mu\text{M}, V = 24 \, \text{min}^{-1}\)) for the “low \(K_m\)-site” of DPD fairly well agree with the values obtained for DPD\(^+\) in the present investigation (\(K_m = 35 \, \mu\text{M}, V = 48 \, \text{min}^{-1}\)).

The excellent precision of the present method for determination of reaction rates is indicated by the low variance of the experimental points in the reciprocal rate plot shown in Fig. 6. Even though the technique of analysing the total course of the oxidation process only can be used at fairly low concentrations of DPD (below 1 mM), the method can be applied to the initial reaction phase under any chosen conditions, and the results may be reliably evaluated in view of the above determination of the empirical rate equation with respect to DPD\(^+\). Such investigations, concerning further kinetic characteristics of the DPD\(^+\)-ceruloplasmin interaction, are in progress.

REFERENCES


Received April 19, 1969.