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A Model for the Ceruloplasmin Catalyzed Oxidation of Dimethyl-*p*-phenylenediamine

GÖSTA PETTERSSON

Department of Biochemistry, University of Lund, Lund, Sweden

The paper shows that certain previously unexplained kinetic characteristics of the ceruloplasmin catalyzed oxidation of dimethyl-*p*-phenylenediamine are a probable consequence of the existence of a rapid dismutation equilibrium between dimethyl-*p*-phenylenediamine and its first and second oxidation products. A theoretical model for the enzymatic process is presented and discussed in view of experimental data.

The ceruloplasmin (EC 1.12.3) catalyzed oxidation of dimethyl-*p*-phenylenediamine (DPD) is usually followed by spectrophotometric determination of the primary oxidation product Wurster's red (DPD^+).[1,2] When DPD is used in large excess to the enzyme the concentration of DPD^+ almost linearly increases to a maximum value, which is independent of the amount of ceruloplasmin used.[2] After having reached its maximum value the concentration of DPD^+ almost linearly decreases to zero.

These remarkable kinetic characteristics of the process have not previously been satisfactorily explained. Walaas *et al.* have suggested that the oxidations of both DPD and DPD^+ are enzymatically catalyzed,[2] but steady-state rate equations derived for simple cases based on this mechanism cannot be brought into consistence with the experimental data.[3] On the other hand, both reaction steps must be influenced by enzyme action; otherwise the maximum concentration of DPD^+ obtained on enzymatic oxidation of DPD would show a significant dependence on the amount of ceruloplasmin used.[4]

The present paper takes the latter fact into consideration by introduction and discussion of a model for the enzymatic oxidation of DPD, which is based upon the recent observation [5] that DPD^+ is dismutable and in rapid equilibrium with both DPD and the final oxidation product DPD^{2+}.

THEORETICAL

Consider the reaction sequence

$$A \longrightarrow B^+ + e^- \longrightarrow P^{2+} + 2e^- \quad (1)$$

where B^+ is rapidly dismutable with the dismutation equilibrium constant K:

$$2B^+ \rightleftharpoons A + P^{2+} \quad (2)$$

With introduction of the abbreviations $A=[A]$, $B=[B^+]$, and $P=[P^{2+}]$ we have

$$K\ B^2 = AP \quad (3)$$

whence on differentiation with respect to time t

$$2KB \frac{dB}{dt} = A \frac{dP}{dt} + P \frac{dA}{dt} \quad (4)$$

Similarly, eqn. (6) is obtained on differentation of the stoichiometric relationship (5):

$$B + 2P = [e^-] \quad (5)$$

$$\frac{dB}{dt} + 2 \frac{dP}{dt} = \frac{d[e^-]}{dt} \quad (6)$$

Let us make the assumption that electrons continuously are enzymatically removed from the system at a rate $v > 0$. Eqn. (6) then gives

$$\frac{dB}{dt} + 2 \frac{dP}{dt} = v \quad (7)$$

If we, further, assume that the initial concentration of A (denoted c_A) is much higher than the total concentration of enzyme (*i.e.* that only negligible amounts of A and its oxidation products are bound to the enzyme) we may use the stoichiometric relationship

$$c_A = A + B + P \quad (8)$$

whence

$$\frac{dA}{dt} + \frac{dB}{dt} + \frac{dP}{dt} = 0 \quad (9)$$

Eqn. (9) combined to eqns. (4) and (7) yields

$$\frac{dB}{dt} = v \frac{A-P}{A+P+4KB} \quad (10)$$

Inspection of eqn. (10) shows that for $t=0$ (when $A=c_A$ and $B=P=0$) dB/dt equals the initial enzymatic oxidation rate v ($v>0$). For $A>P$ we have $dB/dt>0$ (this will be called the positive phase of the reaction), while $dB/dt<0$ for $A<P$ (the negative phase). B thus passes through a maximum value B_m, which is reached when $A=P$. Using the latter relationship B_m can be calculated from eqns. (3) and (8):

$$B_m = \frac{c_A}{1+2\sqrt{K}} \tag{11}$$

The equilibrium constant K can thus be experimentally determined by measurement of B_m as a function of c_A: a plot of B_m against c_A will give a straight line with the slope $1/(1+2\sqrt{K})$.

The general functional dependence of A and P on B can be obtained by combination of eqns. (3) and (8):

$$A = \tfrac{1}{2}\{c_A - B \left(\stackrel{+}{-}\right) \sqrt{(c_A-B)^2-4KB^2}\} \tag{12}$$

$$P = \tfrac{1}{2}\{c_A - B \left(\stackrel{-}{+}\right) \sqrt{(c_A-B)^2-4KB^2}\} \tag{13}$$

where signs within brackets refer to the negative reaction phase. Insertion of this into eqn. (10) gives

$$\left(\stackrel{+}{-}\right) \frac{dB}{dt} = v \frac{\sqrt{(c_A-B)^2-4KB^2}}{c_A-(1-4K)B} \tag{14}$$

The rate of change of B is thus partly dependent upon a function relating to the dismutation process, and partly upon the enzymatic reaction rate v which, in general, is a function of both B and t. Let us, however, consider the case that v is independent of both B and t.

Assuming that v is constant, B as a function of time becomes symmetrical about its maximum value, and only the positive reaction phase will be treated in detail. Integration of eqn. (14) by separation of variables then yields ($B=0$ at $t=0$):

$$c_A - \sqrt{(c_A-B)^2-4KB^2} = vt \tag{16}$$

Eqn. (16) may be solved for B to give ($1-4K \neq 0$):

$$B = \frac{c_A}{1-4K} - \sqrt{\frac{c_A^2}{(1-4K)^2} + \frac{vt(vt-2c_A)}{1-4K}} \tag{17}$$

For $1-4K=0$ we get

$$B = vt\left(1 - \frac{2c_A}{vt}\right) \tag{18}$$

which may be regarded as a limiting case of eqn. (17).

With introduction of the notation $\tau = vt/c_A$, which may be considered as a standardization of the time scale, eqn. (17) becomes

$$\frac{B}{c_A} = \frac{1-\sqrt{(1-4K)(\tau-1)^2+4K}}{1-4K} \tag{19}$$

This eqn., in fact, describes the general dependence of B on time; the positive reaction phase is obtained for $0<\tau<1$ and the negative phase for $1<\tau<2$, maximum occurring for $\tau=1$.

The shape of the B/time curves obtained for different values of the dismutation equilibrium constant K are indicated in Fig. 1. Similar curves

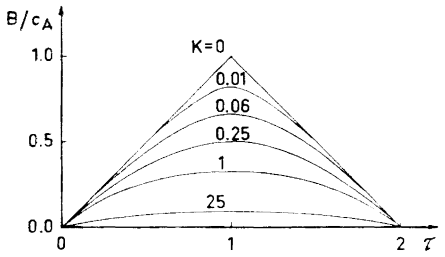

Fig. 1. Variation of the B/time curves with the value of the dismutation equilibrium constant K. The curves have been calculated according to eqn. (19).

have been described and discussed in relation to studies on the non-enzymatic oxidation of DPD.[5] It may, therefore, suffice to mention that approximately linear relationship are obtained in the early ($\tau \approx 0$) and the late ($\tau \approx 2$) phase of the reaction, and that these linear parts of the curves become more extended the smaller the value of K is.

EXPERIMENTAL

Ceruloplasmin was kindly provided by Dr. Björling, AB Kabi, Stockholm. The preparation used was about 65 % pure; the E_{605}/E_{280} ratio was 0.028.

The ceruloplasmin catalyzed oxidation of DPD (DPD, 2HCl was obtained from Merck, Darmstadt) was followed spectrophotometrically at 550 mμ, and experiments were carried out at 25° in 0.05 M sodium acetate buffer, pH 5.5, as described previously.[1,2] DPD^+ concentrations were calculated from the extinction at 550 mμ, using a value of 1.04×10^4 M^{-1} for the extinction coefficient.[5]

RESULTS AND DISCUSSION

Walaas *et al.* reported [2] that the maximum concentration of DPD^+ was constantly equal to about 75 % of the initial concentration of DPD, in experiments where the ceruloplasmin concentration was varied by a factor

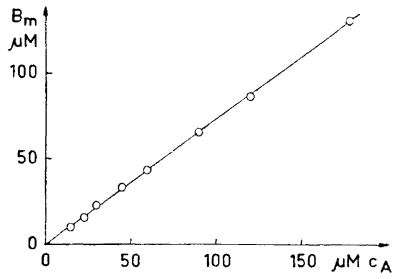

Fig. 2. Determination of the maximum concentration of DPD^+ (B_m) as a function of the initial concentration of DPD (c_A). The ceruloplasmin concentration was kept constant at 0.81 μM (calculated from E_{605}).

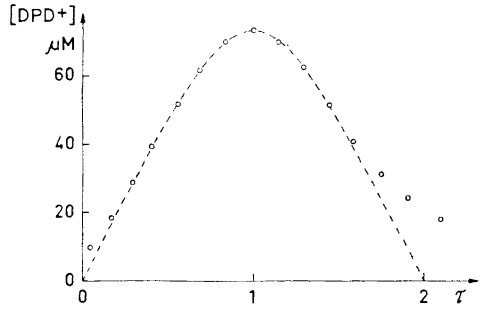

Fig. 3. Ceruloplasmin (1.35 μM) catalyzed oxidation of DPD (101 μM). Circles show experimental determinations of $[DPD^+]$ as a function of time. The dashed curve shows the theoretical relationship according to eqn. (19) for $K = 0.035$.

of 30. These results, which have been confirmed by the present author, would be expected according to the model described in the theoretical section. B_m should be given by eqn. (11) independently of the enzymatic reaction rate, and hence independently of the enzyme concentration. Fig. 2 shows the results of experiments in which the maximum concentration of DPD^+ (B_m) was determined at different values of the initial concentration of DPD (c_A). In consistence with the thoretical model B_m was found to vary linearly with c_A, and statistical calculation of the regression coefficient gave a value of 0.73 for the slope of the line in Fig. 2. The value of K corresponding to this slope (according to eqn. (11) the slope equals $1/(1+2\sqrt{K})$ is 0.0342, in excellent agreement with the previously reported [5] value of 0.035 ± 0.002.

A comparison between the B/time curve calculated from eqn. (19) for $K = 0.035$ and typical experimental observations of the variation of $[DPD^+]$ with time is made in Fig. 3. It is seen that the theoretical curve gives a good general description of the characteristic features of the experimental curve. Significant deviations from the theoretical relationship are only observed in the initial and the late phase of the reaction, where the assumption of a constant reaction rate v, on which eqn. (19) is based, may be false.

The model described in the present paper thus appears to explain the above remarkable characteristics of the ceruloplasmin catalyzed reaction sequence

$$DPD \longrightarrow DPD^+ \longrightarrow DPD^{2+}$$

and it may be concluded that the dismutation equilibrium

$$2\ DPD^+ \rightleftharpoons DPD + DPD^{2+}$$

is of great importance for interpretation of the kinetics of the enzymatic process. A consequence of this fact is that it may be very difficult to decide whether DPD or DPD^+ (or possibly both compounds) is the actual substrate for the enzyme. The latter problem might possibly be attacked by detailed examination of the discrepancies between theoretical and empirical curves in experiments analogous to the one shown in Fig. 3. Such experiments are in progress.

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