INHIBITION MECHANISM OF *POLYPORUS* LACCASE BY FLUORIDE ION

Ali NAQUI and S. D. VARFOLOMEEV

Department of Biokinetics, A. N. Belozersky Laboratory of Molecular Biology and Bioorganic Chemistry, Moscow State University, Moscow 117234, USSR

Received 27 February 1980

1. Introduction

This paper is devoted to a copper-containing oxidase-laccase (*p*-diphenol: oxygen oxidoreductase, EC 1.10.3.2). It is one of the few enzymes that reduces O_2 to H_2O in a four-electron-transfer-process [1]. We have studied the inhibition mechanism of laccase by F^- and used the F^- as an agent to clarify certain aspects of the catalytic mechanism of *Polyporus* laccase in general.

Although the inhibition of laccase activity by F^- was discovered long ago [2] and this problem has been discussed [3–7], some aspects of inhibition are still unclear. It is well known that F^- binds with the type 2 Cu^{2+} of the laccase-active site [2]. We concentrated our attention on the following problems: (i) What is the nature of inhibition of laccase activity by F^-? Is it a competitive or a non-competitive inhibitor with respect to an electron donor and O_2? Answers to these questions could give us information about the role of type 2 Cu^{2+} in binding the electron donor or O_2. (ii) In what way does laccase activity and the effectiveness of laccase inhibition by F^- depend on pH?

2. Materials and methods

Fungal laccase from *Polyporus versicolor* was kindly donated by Prof. B. G. Malmström (Göteborg, Sweden). The protein sample is homogeneous and gives a single band in analytical electrophoresis test. All reagents were of analytical grade and used without further purification.

Kinetic measurements were carried out spectrophotometrically in a double-beam spectrophotometer (Hitachi-124, Japan) by measuring the initial oxidation rate of ferrocyanide-ion ($\epsilon_{420} = 1 \cdot 10^3\ cm^{-1}\ M^{-1}$) and polarographically by studying the integral kinetics of O_2 exhaustion in the system using a Clark's electrode (Polarograph LP7e, ČSSR). Integral curves were processed in a computer (PDP-8A, USA) as described in [8]. Errors in finding different parameters, shown here, are the mean quadratic deviation found in 4 to 7 independent experiments.

3. Results

3.1. *Competition between F^- and electron donor*

Steady state rate dependence of $[Fe(CN)_6]^{4-}$ oxidation by O_2 on concentrations of substrate, H^+ and F^- were studied. A typical form of initial reaction rate dependence on electron donor concentration at different F^- concentration is shown in fig.1. In the absence or at low concentrations of the inhibitor experimental curves are linear in Lineweaver-Burk's coordinate. But at high concentrations of substrate and inhibitor we found "non-linearity" in the kinetic behaviour of our system. In these conditions F^- acts as a relatively less effective inhibitor. This phenomenon of relative "increase" of enzyme reaction rate at high substrate concentration, when type 2 Cu^{2+} is "blocked" by F^- can be explained by assuming that there is an alternative electron pathway to the enzyme's active site (which acts at high concentrations of substrate and inhibitor). These experiments helped us to choose conditions, under which electron transfer through this alternative electron pathway is negligible. Under these conditions (inhibitor concentration upto $2 \cdot 10^{-3}$ M and substrate concentration from $2 \cdot 10^{-4}$ M to $5 \cdot 10^{-3}$ M) the F^- acts as a "classical" non-competitive inhibitor of laccase activity (fig.2).

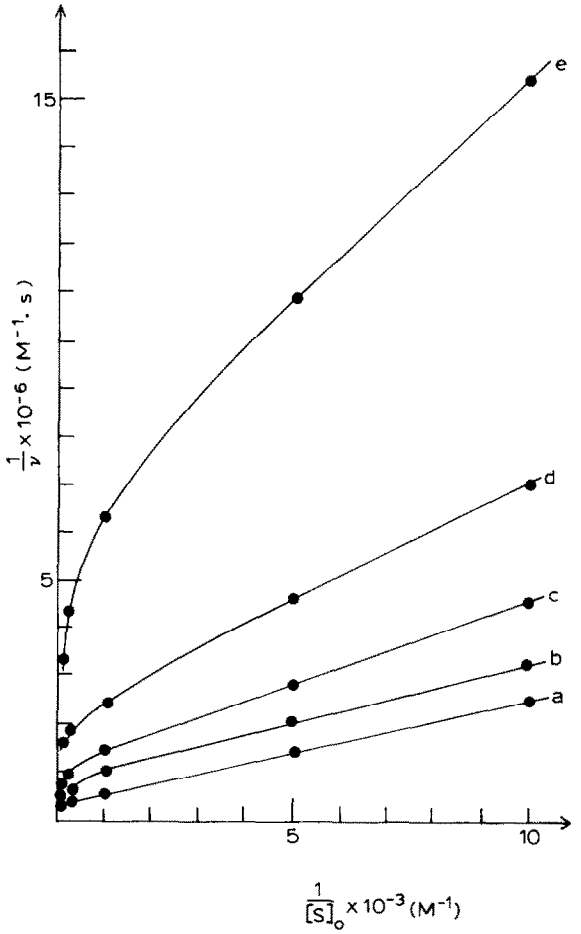

Fig.1. Dependence of ferrocyanide-ion oxidation rate on substrate concentration in Lineweaver-Burk's coordinate at different concentrations of F^- (M): (a) 0; (b) $2.5 \cdot 10^{-4}$; (c) $5 \cdot 10^{-4}$; (d) $1 \cdot 10^{-3}$; (e) $2.5 \cdot 10^{-3}$, acetate-phosphate buffer, μ = 0.1 M, pH 4.4; 25°C, enzyme concentration ($[E]_0$) = $2.7 \cdot 10^{-9}$ M.

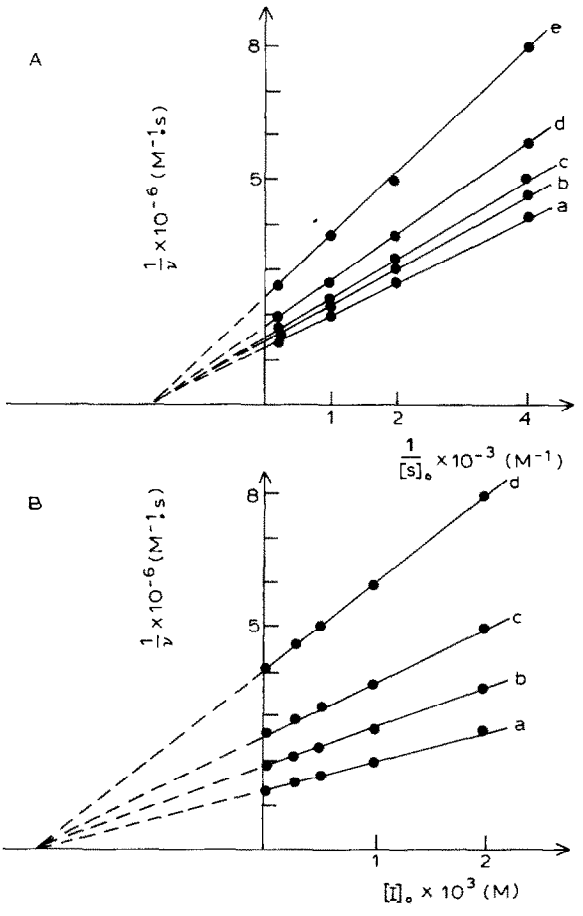

Fig.2. (A) Reaction rate dependence on substrate concentrations in Lineweaver-Burk's coordinate at different F^- concentrations (M): (a) 0; (b) $2.5 \cdot 10^{-4}$; (c) $5 \cdot 10^{-4}$; (d) $1 \cdot 10^{-3}$; (e) $2 \cdot 10^{-3}$, acetate-phosphate buffer; μ = 0.1 M; pH 6; 20°C, $[E]_0$ = $2.7 \cdot 10^{-9}$ M. (B) Data of (A) in Dixon's coordinate; $[Fe(CN)_6]^{4-}$ concentration (M): (a) $5 \cdot 10^{-3}$; (b) $1 \cdot 10^{-3}$; (c) $5 \cdot 10^{-4}$; (d) $2.5 \cdot 10^{-4}$.

3.2. *pH-dependence of catalytic activity and inhibition by F^- of laccase*

The effectiveness of laccase inhibition (inhibition constant K_i) by F^- was found to be dependent on H^+ concentration (fig.3). pH-dependence of maximum rate of laccase catalysed reaction is shown in fig.3. At neutral pH laccase activity decreases. Slope of this curve increases from 0 (pH 3–4.5) to 2 (pH > 6). It is clear that the catalytic activity of the enzyme is controlled by ionogenic groups with pK_{a_1} = 5 and pK_{a_2} = 6.3. pH-dependence of inhibition constant, too, is shown in fig.3. Inhibition constant is controlled at least by one ionogenic group having pK_a = 5.

3.3. *Competition between F^- and O_2*

The integral kinetics of O_2 exhaustion in the presence of laccase at different F^- concentration were studied. In these experiments the concentration of electron donor was far more than that of O_2 and the Michaelis constant with respect to donor. In this case O_2 concentration was variable and using the integral form of Michaelis–Menten's equation the maximum reaction rate ($V_M{}^{O_2}$) and Michaelis constant ($K_M{}^{O_2}$) with respect to O_2 can be determined.

Experimentally determined parameters ($V_M{}^{O_2}$ and $K_M{}^{O_2}$) at different F^- concentrations are shown in fig.4. It is clear that the inhibition mechanism by

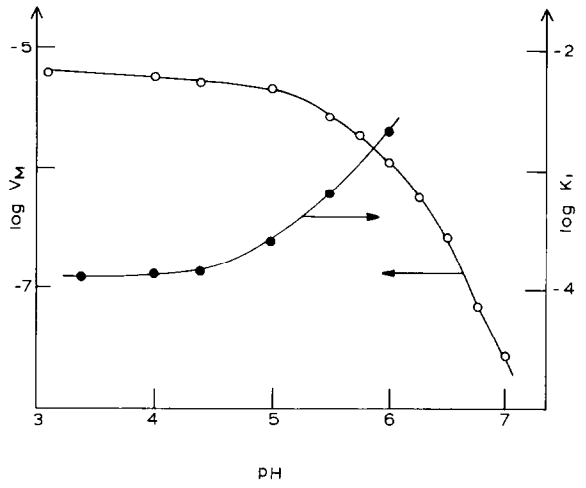

Fig.3. pH dependence of maximum reaction rate and laccase inhibition constant by F^-; acetate-phosphate buffer; $\mu = 0.1$ M; 25°C; $[E]_0 = 2.7 \cdot 10^{-9}$ M.

F^- with respect to O_2 does not correspond to "classical" types of inhibition (competitive and non-competitive) and it has a complex character.

4. Discussion

The above-mentioned results give us interesting information on the mechanism of the laccase-active site.

(i) pH-dependence of maximum reaction rate shows that in the laccase-active site there are at least two ionizable groups, having close pK_a. Deprotonization of these groups (on binding of OH^- to the enzyme's active site) results in a loss of the enzyme's catalytic activity. Probable reaction active (1–3) and reaction non-active (4–6) forms of the enzyme's active site which can be realised at different pH are given in Scheme 1, where Cu_I^{2+}, Cu_{II}^{2+}, Cu_{III}^{2+} are type 1, type 2 and type 3 copper-ions, respectively. Each of these equilibriums can explain the observed pH-dependence of catalytic activity.

$$Cu_I^{2+}\left[\begin{matrix}Cu_{III}^{2+}\ HA\\ Cu_{III}^{2+}\ HA\end{matrix}\right] \quad Cu_{II}^{2+} \rightleftharpoons Cu_I^{2+}\left[\begin{matrix}Cu_{III}^{2+}\ A^-\\ Cu_{III}^{2+}\ A^-\end{matrix}\right] \quad Cu_{II}^{2+}$$

(1) (4)

$$Cu_I^{2+}\left[\begin{matrix}Cu_{III}^{2+}\ HA\\ Cu_{III}^{2+}\end{matrix}\right] \quad Cu_{II}^{2+} \rightleftharpoons Cu_I^{2+}\left[\begin{matrix}Cu_{III}^{2+}\ A^-\\ Cu_{III}^{2+}\end{matrix}\right] \quad OH^- \; Cu_{II}^{2+}$$

(2) (5)

$$Cu_I^{2+}\left[\begin{matrix}Cu_{III}^{2+}\\ Cu_{III}^{2+}\end{matrix}\right] \quad Cu_{II}^{2+} \rightleftharpoons Cu_I^{2+}\left[\begin{matrix}Cu_{III}^{2+}\\ Cu_{III}^{2+}\end{matrix}\right] \quad OH^- \; OH^-Cu_{II}^{2+}$$

(3) (6)

(1)

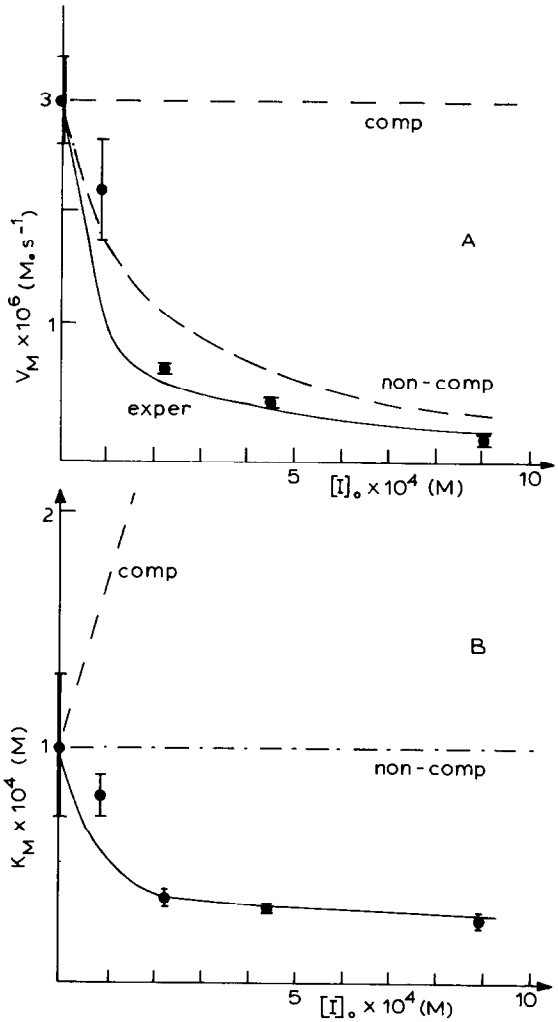

Fig.4. Dependence of maximum reaction rate (A) and effective Michaelis constant (B) with respect to O_2 on F^- concentration; acetate-phosphate buffer; $\mu = 0.1$ M; 30.5°C; $[E]_0 = 8.1 \cdot 10^{-9}$ M. (– – –), competitive and (— · —), non-competitive mechanisms of inhibition; theoretical curve assuming that $\alpha = 0.27, \beta = 0.07, K_i = 1.5 \cdot 10^{-4}$ M (Scheme 2)

Inhibition of laccase activity by F^- is controlled by at least one of these ionizable groups. This shows that deprotonization of an acidic group belonging to the active site (and accordingly the appearance of a non-compensated negative charge in the active site) or binding of an OH^- (for example to type 2 Cu^{2+}) forces out F^- from the enzyme's active site.

(ii) Fluoride-ion is a non-competitive inhibitor with regard to the electron donor in ferrocyanide oxidation reaction. This indicates that F^- being bound to type 2 Cu^{2+} has no influence on complex formation between electron donor and enzyme. In other words type 2 Cu^{2+} does not take part in binding of electron donor.

(iii) The relation between type 2 Cu^{2+} and O_2 in the inhibition mechanism is interesting, as illustrated in Scheme 2, where E is the enzyme's active site, (EO_2) the intermediate complex between enzyme and oxygen formed before the rate-limiting step, EI the enzyme-inhibitor complex, and (IEO_2) the inhibitor–enzyme-oxygen complex.

$$\begin{array}{ccccc} E + O_2 & \overset{K_M^{O_2}}{\rightleftharpoons} & (EO_2) & \xrightarrow{k_{cat}^{O_2}} & E + P \\ K_i \Big\downarrow \Big\uparrow & & \Big\downarrow \Big\uparrow \alpha K_i & & \\ IE + O_2 & \underset{\alpha K_M^{O_2}}{\rightleftharpoons} & (IEO_2) & \xrightarrow{\beta k_{cat}^{O_2}} & IE + P \end{array} \qquad (2)$$

According to Scheme (2):

$$V_M^{O_2}(I) = \frac{V_M^{O_2}\left(1 + \frac{\beta\,[I]}{\alpha K_i}\right)}{1 + \frac{[I]}{\alpha K_i}} \qquad (3)$$

$$K_M^{O_2}(I) = \frac{K_M^{O_2}\left(1 + \frac{[I]}{K_i}\right)}{1 + \frac{[I]}{\alpha K_i}} \qquad (4)$$

From the experimental data of fig.4 α,β and K_i were found by the method described in [8]. These parameters were found to be $\alpha = 0.27$, $\beta = 0.07$, $K_i = 1.5 \cdot 10^{-4}$ M. Then using these constants two theoretical curves were drawn from Eqns. (3) and (4) and represented in fig.4 by the continuous lines. These theoretical curves accord closely with the experimental points (also shown in fig.4).

It is interesting to note that the dissociation constants of the F^--enzyme's active site found by oxidation of electron donor and from integral kinetics of O_2 uptake are practically the same ($K_i^D = 1.4 \cdot 10^{-4}$ M, pH 4.0 and $K_i^{O_2} = 1.5 \cdot 10^{-4}$ M, pH 4.0).

Thus being bound to the type 2 Cu^{2+} of the enzyme's active site, the F^- blocks the electron transfer to O_2 ($\beta = 0.07 \ll 1$). However, as a result the ability of the enzyme's active site to bind O_2 far from decreasing, actually increases ($\alpha = 0.27 < 1$).

There are indications in the literature on this subject that type 3 copper ions take part in oxygen binding [9]. Probable states for the enzyme's active site are shown in Scheme (5).

$$Cu_I^{2+}\begin{bmatrix} Cu_{III}^{2+} \cdots O^{\cdot} \\ | \\ Cu_{III}^{2+} \cdots O^{\cdot} \end{bmatrix} Cu_{II}^{2+} \rightleftharpoons Cu_I^{2+}\begin{bmatrix} Cu_{III}^{2+} \cdots O^{\cdot} \\ | \\ Cu_{III}^{2+} \cdots O^{\cdot} \end{bmatrix} \cdots F^- \cdots Cu_{II}^{2+} \qquad (5)$$

The fluoride-ion after binding to the type 2 Cu^{2+} stabilizes the enzyme's active site–oxygen complex, however, the possibility of further reduction of O_2 is practically nil.

Acknowledgements

The authors are indebted to Prof B. G. Malmström (Göteborg, Sweden) for kindly presenting the protein and for arousing our interest in laccase and to Prof I. V. Berezin (Moscow State University, USSR) for fruitful discussions of the results.

References

[1] Malmström, B. G., Andréasson, L. E. and Reinhammar, B. (1975) The Enzymes (Boyer, P. D. ed) vol. 12 B, pp. 507–579, Academic Press, New York.
[2] Malkin, R., Malmström, B. G. and Vänngård, T. (1968) FEBS Lett. 89, 50–54.
[3] Brändén, R., Malmström, B. G. and Vänngård, T. (1971) Eur. J. Biochem. 18, 238–241.
[4] Brändén, R., Malmström, B. G. and Vänngård, T. (1973) Eur. J. Biochem. 36, 195–200.
[5] Andréasson, L. E., Malmström, B. G., Strömberg, C. and Vänngård, T. (1973) Eur. J. Biochem. 34, 434–439.
[6] Holwerda, R. A. and Gray, H. B. (1975) J. Am. Chem. Soc. 97, 6036–6041.
[7] Farver, O., Goldberg, M., Lancet, D. and Pecht, I. (1976) Biochem. Biophys. Res. Commun. 73, 494–500.
[8] Berezin, I. V. and Klyosov, A. A. (1976) Practical course of chemical and enzyme kinetics (in Russ.), pp. 77–186, Moscow Univ. Publ., Moscow.
[9] Malkin, R. and Malmström, B. G. (1970) Adv. Enzymol. 33, 177–244.