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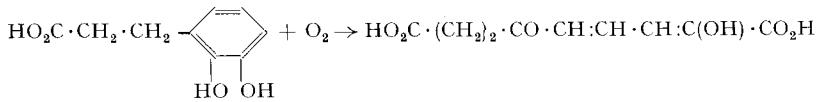
The time sequence of interactions of a dioxygenase with its substrates

When aerobic bacteria are grown at the expense of cinnamic acid[1,2] or hydrocinnamic acid[2,3] the growth substrate is first metabolized to give 2,3-dihydroxy-β-phenylpropionate (DHP) and the benzene nucleus is then cleaved by 'meta fission' as follows:



Extracts of Achromobacter grown with hydrocinnamic acid were heat-treated (55°, 5 min) to remove inactive material and brought to 70% satn. with $(NH_4)_2SO_4$ as previously described[3]. Solutions of the enzyme (DHP dioxygenase) obtained by this procedure showed an absolute requirement for Fe^{2+} and catalysed a stoicheiometric oxidation of DHP in accordance with the above equation. Reactions were followed by measuring O_2 utilized. The apparatus, from Rank Bros., Bottisham, Cambs., England, consisted of a water-jacketed, air-tight Perspex vessel with a reaction volume of 6.5 ml. The contents, at 20°, were stirred by a rotating magnet and concentrations of O_2 were measured by a Clark oxygen electrode fitted with a microammeter and recorder. The instrument was calibrated by measuring the electrode current, first for a known concentration of O_2 and then after adding dithionite to exhaust O_2, when a small reading was given. A linear relationship was obtained between concentrations of O_2 and currents. The concentration of O_2 in water exposed to water-saturated air at 20° was taken to be 0.287 mM, and in water saturated with pure O_2, 1.25 mM. The concentrations of O_2 required in reactions were obtained by gassing the buffer *in situ* with O_2–N_2 mixtures to attain given microammeter readings just before experiments were performed.

The enzyme-catalyzed uptake of O_2 depended strongly upon the order in which reactants were added. When this sequence was: DHP dioxygenase, Fe^{2+}, DHP at intervals of 10 sec the reaction was immediate and rapid; but when DHP was added before Fe^{2+} there was a lag of 5 min preceding a much slower reaction. Accordingly, in all subsequent experiments the enzyme was activated by preincubation with ferrous ammonium sulphate at 0°. Even with this procedure, however, DHP at high concentrations partially reversed the activation due to Fe^{2+}. Thus, with 0.5 and 2.0 mM Fe^{2+} in the preincubation mixture, initial rates of reaction increased through the range 31–154 μM DHP (Table I) whereas with 0.1 and 0.2 mM Fe^{2+}, rates decreased at concentrations > 92 μM DHP.

Uptakes of O_2 were measured at various concentrations of DHP and O_2 after preincubating the enzyme with a fixed amount of Fe^{2+} in all experiments. When DHP was varied at a series of fixed concentrations of O_2 there was no inhibition by DHP, and each curve of the family was approximately hyperbolic (Fig. 1B). In contrast, when O_2 was varied initial reaction rates were maximal at 4.8 mM O_2 and higher concentrations were inhibitory, particularly at the lower concentrations of DHP (Fig. 1A). This inhibition was reversed by increasing the concentration of DHP.

Abbreviation: DHP, 2,3-dihydroxy-β-phenylpropionate.

TABLE I

DEPENDENCE OF RATES OF UPTAKE OF OXYGEN ON DHP AND Fe^{2+}

DHP dioxygenase was activated at 0° by ferrous ammonium sulphate. Reaction mixtures in 6.5 ml of phosphate buffer (3.3 g KH_2PO_4 per l, pH 7.3) each contained 0.95 mg of cell extract protein. Reaction rates are in μmoles O_2 per min. Initial concentration of O_2, 0.287 mM.

Fe^{2+} (mM) during incubation at 0°	*Reaction rates*				
DHP (μM):	*31*	*62*	*92*	*123*	*154*
2.0	0.70	0.93	1.24	1.40	1.55
0.5	0.39	0.52	0.62	0.72	0.76
0.2	0.21	0.41	0.47	0.45	0.43
0.1	0.17	0.31	0.35	0.31	0.31

FERDINAND[4] and others have discussed the kinetics of an enzyme that catalyzes a reaction between two substrates X and Y, forming a ternary complex which rapidly decomposes to give the overall products of reaction. If the enzyme binds the two substrates at one and the same site, events may occur by two sequences, neither of which excludes the other. Thus X may bind first to give a binary complex that reacts with Y to give the ternary complex. If the rate constants of these reactions are substantially greater than those for the alternative sequence in which Y binds first, then the curve relating reaction rates with concentrations of Y (that of X being fixed) will show a maximum. This arises because high concentrations of Y favour the second and slower pathway in which Y is bound first. No such inhibition is to be expected when the concentration of Y is fixed and that of X is raised, although the curve may become sigmoid if the various rate constants are assigned certain values[4]. The observed kinetics of the DHP dioxygenase system are in general agreement with these con-

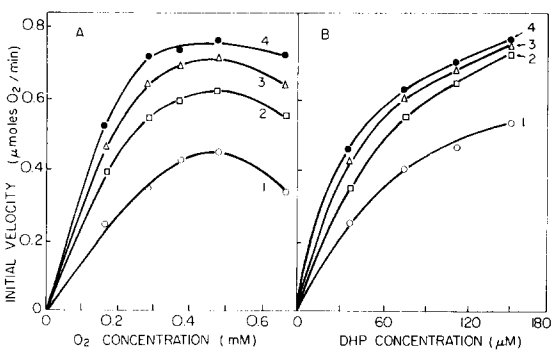


Fig. 1. Dependence of reaction velocity on the concentration of each substrate of DHP oxygenase. The concentration of the second substrate was held constant at a series of values. Reactions, in 6.5 ml of phosphate buffer (3.3 g KH_2PO_4 per l, pH 7.3) at 20°, were started by adding 0.2 ml of cell extract (2.7 mg of protein per ml) which had been preincubated at 0° with 1 mM ferrous ammonium sulphate. A. O_2 varied at the following concentrations of DHP (μM): 1, 37; 2, 75; 3, 112; 4, 149. B. DHP varied at the following concentrations of O_2 (mM): 1, 1.7; 2, 2.8; 3, 3.7; 4, 4.8. A curve for 6.7 mM O_2 was coincident with 2.

siderations, X corresponding to DHP and Y to O_2. We therefore propose the following as the 'preferred' sequence for this reaction:

$$E + Fe^{2+} \rightleftarrows Fe^{2+}\text{–}E \xrightarrow{DHP} Fe^{2+}\text{–}E(DHP) \xrightarrow{O_2} O_2 \cdot Fe^{2+}\text{–}E(DHP) \rightarrow \text{ring fission product}$$

The scheme implies that Fe^{2+}, DHP and O_2 are brought together within a small area on the enzyme. Fe^{2+} and DHP would be bound separately at closely adjacent sites so that DHP might partly impede the binding of Fe^{2+}. The quaternary complex would be formed when O_2 attached to bound Fe^{2+}; and the overall reaction rate would be faster with DHP already bound than would be the case when, at concentrations of $O_2 > 0.48$ mM, increasing numbers of sites for DHP were rendered less accessible by the adjacent Fe^{2+}–O_2 complexes. Direct binding of O_2 to bound Fe^{2+} has been suggested for metapyrocatechase[5]. By absorption on DEAE-cellulose followed by passage through Sephadex G-200 we purified DHP dioxygenase a further 18-fold and determined a molecular weight of about 70 000 by the method of ANDREWS[6]. It is of interest that a molecular weight of 140 000 has been found for both metapyrocatachase[5] and protocatechuate 4,5-oxygenase[7].

The last-named enzyme catalyzes a reaction analogous to that of DHP dioxygenase, and its activity is increased by adding Fe^{2+}; but in this case the activation is not reversed by the carbon substrate. Non-enzymic oxidation of free Fe^{2+} is rapid under the conditions that obtained in these experiments, and Fe^{3+} is not an effective activator. However, it has been shown for protocatechuate 4,5-oxygenase[7] that when bound to protein, Fe^{2+} is much less readily oxidized than when it is free in solution. Accordingly, our procedure of preincubating the enzyme with Fe $(NH_4SO_4)_2$ minimized the non-enzymic oxidation of Fe^{2+} to inactive Fe^{3+}.

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1 C. B. COULSON AND W. C. EVANS, *Chem. Ind. London*, (1959) 1543.
2 E. R. BLAKLEY AND F. J. SIMPSON, *Can. J. Microbiol.*, 10 (1964) 175.
3 S. DAGLEY, P. J. CHAPMAN AND D. T. GIBSON, *Biochem. J.*, 97 (1965) 643.
4 W. FERDINAND, *Biochem. J.*, 98 (1966) 278.
5 M. NOZAKI, K. ONO, T. NAKAZAWA, S. KOTANI AND O. HAYAISHI, *J. Biol. Chem.*, 243 (1968) 2682.
6 P. ANDREWS, *Biochem. J.*, 96 (1965) 595.
7 S. DAGLEY, P. J. GEARY AND J. M. WOOD, *Biochem. J.*, in the press.

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