The Kinetics of Phenethylhydrazine Oxidation by Monoamine Oxidase

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1. In the presence of the substrate benzylamine, phenethylhydrazine has been shown to be a competitive inhibitor of monoamine oxidase from rat liver and pig brain. 2. Phenethylhydrazine is also a substrate for monoamine oxidase. Reciprocal plots for hydrazine oxidation give families of intersecting lines in contrast with the parallel lines previously reported for tyramine oxidation. 3. Two possible modifications of the mechanism obeyed by tyramine oxidation are suggested, but the product inhibition results are insufficient to distinguish between these two mechanisms.

The antidepressant drug phenethylhydrazine is believed to act by inhibiting the enzyme monoamine oxidase [monoamine-oxygen oxidoreductase (deaminating) EC 1.4.3.4] (Chessin, Dubnick, Leeson & Scott, 1959). Clineschmidt & Horita (1969*a,b*) have shown that this compound can function as a substrate as well as an inhibitor of monoamine oxidase, and the immediate product of this reaction has been shown to be the corresponding hydrazone, phenethylidenhydrazine (Tipton & Spires, 1971).

The kinetics of tyramine oxidation by pig brain monoamine oxidase have been studied (Tipton, 1968*b*) and it has been shown that the enzyme obeys a kinetic mechanism in which the reaction proceeds through a number of binary complexes without the formation of any kinetically significant ternary complex and in which a free modified form of the enzyme is produced as an intermediate. Recently Oi, Shimada, Inamasu & Yasunobu (1970) have shown that the enzyme from beef liver mitochondria obeys a similar kinetic mechanism and the enzyme from rat liver mitochondria probably also obeys such a mechanism (K. F. Tipton, unpublished observations).

In the present paper the kinetics of the oxidation of phenethylhydrazine by monoamine oxidase were studied and it is shown that the oxidation of this substrate proceeds by a different kinetic mechanism.

MATERIALS AND METHODS

Pig brain mitochondrial monoamine oxidase was purified by the method of Tipton (1968*a*). A partially purified preparation of rat liver monoamine oxidase was prepared by ammonium sulphate fractionation of rat liver mitochondria which had been sonicated in the

presence of benzylamine and then treated with Triton X-100. This procedure is an adaptation of the earlier stages of the more complete purification procedure of Youdim & Sourkes (1966). Before use, the enzyme prepared in this way was passed through a column of Sephadex G-25, equilibrated in 0.05M-tris-HCl buffer, pH 7.2.

The oxidation of benzylamine by monoamine oxidase was measured spectrophotometrically by the method of Tabor, Tabor & Rosenthal (1954), and the oxidation of phenethylhydrazine was determined by measuring the rate of increase of E_{235} (see Tipton, 1971*a*). Assays were carried out at 30°C in 0.2M-sodium phosphate buffer, pH 7.2, containing catalase. Extinctions were measured in a Hilger-Gilford recording spectrophotometer which permitted a scale expansion of 0.1 extinction units on a 10 in recorder.

The concentration of O_2 in the mixture was varied by the addition of solutions of sodium dithionite or H_2O_2 to the buffer mixture. The catalase was omitted from the buffer solution for experiments in which the inhibition by H_2O_2 was measured, and, since the dithionite addition would result in the formation of H_2O_2 (Dixon, 1971), O_2 concentration was adjusted in these experiments by gassing the mixture with O_2+N_2 mixtures. Phenethylhydrazine (Phenelzine, Nardil) was obtained from Fluka A. G. Chemische Fabrik, Buchs, Switzerland. Catalase was obtained from Boehringer Corp. (London) Ltd., London W.5, U.K. All other chemicals were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K., and were of the highest purity available.

RESULTS

The presence of benzylamine was found to protect monoamine oxidase against irreversible inhibition by phenethylhydrazine, and hence it was possible to investigate the reversible inhibition of the enzyme by this hydrazine in the presence of the

benzylamine substrate. The inhibition of rat liver monoamine oxidase by phenethylhydrazine is shown in Fig. 1. Inhibition was also found to be competitive in the case of the pig brain enzyme and the K_i values calculated for this inhibition were 23 and 42 μM for the rat liver and pig brain enzymes respectively.

When monoamine oxidase was incubated with phenethylhydrazine the initial rate of change of E_{235} was found to be proportional to the enzyme concentration. The kinetics of oxidation of phenethylhydrazine are shown as plots of reciprocal initial velocity against reciprocal substrate concentration at a series of fixed oxygen concentrations (Fig. 2). The reciprocal plots obtained when the oxygen concentration was varied at a series of fixed phenethylhydrazine concentrations are shown in Fig. 3.

Fig. 1. Effect of phenethylhydrazine on the initial rate of benzylamine oxidation by rat liver monoamine oxidase. Rates were measured at 30°C by following the increase in E_{250} . The phenethylhydrazine concentrations used were: 0 μM (●), 5 μM (○), 12.5 μM (△) and 25 μM (▲).

Fig. 2. Kinetics of phenethylhydrazine oxidation by pig brain monoamine oxidase. Reciprocal plots of initial velocities against phenethylhydrazine concentration at a series of fixed oxygen concentrations. Rates were measured at 30°C by following the increase in E_{235} . The oxygen concentrations used were: 88 μM (□), 198 μM (△), 230 μM (●) and 300 μM (○).

Secondary plots of the intercept on the velocity axis against the reciprocal concentration of the second substrate were linear in all cases and from these plots K_m values for oxygen of 250 and 312 μM were calculated for the pig brain and rat liver enzymes respectively, whereas the respective K_m values for phenethylhydrazine were 48 and 22 μM .

In the case of the pig brain enzyme the inhibition of the reaction by hydrogen peroxide was studied. It has already been shown that hydrogen peroxide

Fig. 3. Kinetics of phenethylhydrazine oxidation by pig brain monoamine oxidase. Reciprocal plots of initial velocities against oxygen concentration at a series of fixed phenethylhydrazine concentrations. Rates were measured at 30°C by following the increase in E_{235} . The phenethylhydrazine concentrations used were: 6.6 μM (■), 10 μM (▲), 16.5 μM (△), 25 μM (●) and 50 μM (○).

Fig. 4. Inhibition of pig brain monoamine oxidase by hydrogen peroxide. Reciprocal plots of initial velocity against phenethylhydrazine concentration at a series of fixed inhibitor concentrations. The hydrogen peroxide concentrations used were: 0 μM (○), 90 μM (△), 360 μM (●) and 540 μM (▲). The oxygen concentration was 230 μM .

Fig. 5. Inhibition of pig brain monoamine oxidase by hydrogen peroxide. Reciprocal plots of initial velocity against oxygen concentration at a series of fixed inhibitor concentrations. The hydrogen peroxide concentrations used were: $0 \mu\text{M}$ (●), $90 \mu\text{M}$ (▲), $360 \mu\text{M}$ (△) and $540 \mu\text{M}$ (○). The phenethylhydrazine concentration was $50 \mu\text{M}$.

will not irreversibly inhibit the enzyme under the conditions used in these experiments (Tipton, 1968b). Fig. 4 shows that the inhibition with respect to phenethylhydrazine is competitive at an oxygen concentration of $230 \mu\text{M}$ and the K_i calculated was $180 \mu\text{M}$. At saturating concentrations of oxygen (1.3 mm) the form of inhibition was unchanged. The inhibition by hydrogen peroxide with respect to oxygen concentration is shown in Fig. 5. At concentrations of phenethylhydrazine that are close to the K_m for that substrate, inhibition is of the mixed type. However, at very high concentrations of phenethylhydrazine (5 mm) negligible inhibition was produced by $150 \mu\text{M}$ -hydrogen peroxide at an oxygen concentration of $230 \mu\text{M}$.

DISCUSSION

The ping-pong kinetic mechanism has been established to apply to tyramine oxidation by pig brain monoamine oxidase by the form of the reciprocal plots for this oxidation, the pattern of inhibition given by the products of this reaction and by the observation that stoichiometric quantities of aldehyde were released when the enzyme was incubated with tyramine in the absence of oxygen (Tipton, 1968b). The families of intersecting lines given by the reciprocal plots in the case of phenethylhydrazine oxidation are inconsistent with such a kinetic mechanism occurring in this case. Intersecting reciprocal plots are given by mechanisms involving the formation of a ternary complex between the enzyme and its two substrates or by mechanisms of the Theorell-Chance type (Theorell & Chance, 1951). The oxidation of amines by monoamine oxidase is believed to proceed by way of an

imine, which is then spontaneously hydrolysed by water (Bernheim, 1931):

where the reduced enzyme probably represents a form of the enzyme in which the FAD component is reduced (Erwin & Hellerman, 1967; Igaue, Gomes & Yasunobu, 1967; Tipton, 1968c). The spontaneous hydrolysis of the imine could occur either on the enzyme surface, or after it had dissociated from the enzyme.

The oxidation of hydrazines by monoamine oxidase has been shown to proceed as far as the corresponding hydrazone (Tipton & Spires, 1971)

If hydrolysis of the imine, produced by amine oxidation, occurred while it was still bound to the enzyme surface, this could facilitate release of the products by weakening the chelate effect. In the case of hydrazine oxidation, the relative stability of the hydrazone to hydrolysis could result in the product being less rapidly released from the enzyme, and this could account for the difference in kinetics observed. Thus the second substrate might bind to the enzyme before the release of the hydrazone as shown in Scheme 1. Alternatively the second substrate could displace the hydrazone from the enzyme without the formation of a ternary complex in a Theorell-Chance mechanism (Scheme 2). In

both these mechanisms the reduced form of the enzyme is designated as E'.

Both these steady-state mechanisms would give a kinetic equation of the general form:

$$v = \frac{V_{\max.}}{1 + \frac{K_A}{[\text{Hydrazine}]} + \frac{K_B}{[\text{O}_2]} + \frac{K_{AB}}{[\text{Hydrazine}][\text{O}_2]}}$$

where K_A and K_B are the Michaelis constants for the hydrazine and oxygen respectively.

These two mechanisms could be distinguished by the patterns of inhibition given by the products of the reaction. Unfortunately, since the hydrazone product has been found to be a potent irreversible inhibitor of the enzyme (Tipton 1971b), product inhibition studies had to be restricted to using the second product, hydrogen peroxide. In the presence of hydrogen peroxide, both the above kinetic mechanisms would give rate equations of the following general form:

$$v = \frac{V_{\max.}}{1 + K_A \left(1 + \frac{[\text{H}_2\text{O}_2]}{K_I}\right) + \frac{K_B}{[\text{O}_2]} + \frac{K_{AB} \left(1 + \frac{[\text{H}_2\text{O}_2]}{K_I'}\right)}{[\text{Hydrazine}][\text{O}_2]}}$$

where K_I and K_I' represent the apparent inhibitor constants for hydrogen peroxide.

Thus the competitive inhibition by hydrogen peroxide with respect to phenethylhydrazine over a wide range of oxygen concentrations, and the mixed inhibition with respect to oxygen, which becomes negligible at saturating concentrations of phenethylhydrazine, would be consistent with either of the above kinetic mechanisms. The possibility that the hydrazine is oxidized by a mechanism totally different from that obeyed by tyramine oxidase, such as mechanisms involving a random order of

substrate binding, or oxygen binding preceding hydrazine binding, are not, however, consistent with the inhibitory pattern given by hydrogen peroxide.

Steady-state kinetic studies are, in this case, unable to distinguish between the two proposed mechanisms, and, since the hydrazone product is an irreversible inhibitor, comparison of forward and backward reactions (see e.g. Dalziel, 1957) cannot be used to resolve the problem.

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