Rat Liver Mitochondrial Monoamine Oxidase

A CHANGE IN THE REACTION MECHANISM ON SOLUBILIZATION

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1. The kinetics of benzylamine oxidation by a soluble preparation of rat liver mitochondrial monoamine oxidase were investigated and were shown to conform to a double-displacement (or Ping Pong) mechanism. 2. The pathway differs in detail from that followed by other amine oxidases, including the membrane-bound enzyme in rat liver mitochondrial outer membranes. 3. It is suggested that the conformation of the protein in the soluble state differs from that in the membrane-bound state. 4. The full rate equations for this mechanism have been deposited as Supplementary Publication SUP 50039 (5 pages) at the British Library (Lending Division) (formerly the National Lending Library for Science and Technology), Boston Spa, Yorks. LS23 7BQ, U.K., from whom copies can be obtained on the terms indicated in *Biochem. J.* (1975) 145, 5.

Monoamine oxidase (EC 1.4.3.4) catalyses the reaction:

Bernheim (1931) proposed that this reaction proceeded by way of an imine intermediate and indirect evidence has been provided to support this view (Tipton & Spires, 1971). The enzyme prepared from a number of sources, including rat liver, has been shown to be a flavoprotein, and it is believed that the flavin is reduced or partly reduced during the reaction (see, e.g., Gomes *et al.*, 1969; Tipton, 1968a; Sourkes, 1968).

The enzyme has been shown to be firmly bound to the mitochondrial outer membrane (Schnaitman *et al.*, 1967; Sottocasa *et al.*, 1967; Tipton, 1967) and we have reported that the reaction mechanism of benzylamine oxidation by membrane-bound rat liver mitochondrial monoamine oxidase (Houslay & Tipton, 1973c) obeys a double-displacement mechanism. The soluble preparations from ox thyroid (Fischer *et al.*, 1968), pig brain (Tipton, 1968b) and ox liver (Oi *et al.*, 1970) have also been shown to obey a double-displacement mechanism, although their formal reaction mechanisms differ in detail. Since lipid fragments have been shown to affect the temperature stability and inhibitor sensitivity of the enzyme (Houslay & Tipton, 1973a,b) we have studied the reactive mechanism of a soluble monoamine oxidase preparation from rat liver mitochondria in order to compare it with a previous study on the membrane-bound enzyme (Houslay & Tipton, 1973c).

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Methods

Preparation of the enzyme

A soluble partly purified preparation of the enzyme was obtained by using the method described in full elsewhere (Houslay & Tipton, 1973a), except that solubilization was achieved by sonication in the presence of a final concentration of 1.6% Triton X-100, and the enzyme was finally desalted into 0.02M-potassium phosphate buffer, pH 7.2. This preparation was then normally left for 2 days at 4°C before it (approx. 92ml at a final protein concentration of 8mg/ml) was loaded on to a column (4cm×3cm diam.) of DEAE-cellulose, equilibrated with the same buffer. This column was then washed through with 100ml of buffer, and activity was eluted with 150ml of the same buffer containing 0.075% Triton X-100. The eluate was then made to 60% saturation with (NH₄)₂SO₄ (at 4°C) while the pH was kept constant at pH 7.0 with 0.4M-NaOH. After stirring for 30min, centrifugation was carried out at 20000g for 20min (4°C). The pale-yellow aggregated material found floating on top of the fluid in the centrifuge tube was carefully removed and dissolved in 4ml of 0.05M-Tris-HCl buffer, pH 7.2, containing 0.1mM-dithiothreitol, and subsequently dialysed overnight against the same buffer (1 litre). This dialysed fraction (5ml) was then subjected to gel filtration on a column (55cm×3cm) of Sepharose 4B, equilibrated with 0.05M-Tris-HCl buffer, pH 7.2, containing 0.1mM-dithiothreitol. The fractions with the highest specific activity were pooled and concentrated by using an Amicon Ultrafilter with a PM10 or an XM100 membrane. For kinetic studies in the presence of H₂O₂ the enzyme was further fractionated by gel filtration on a Sepharose 6B

column (60 cm $\times$ 2 cm diam.), in an analogous manner to that described above. In some experiments the purified enzyme was treated with NaClO_4 in the manner described previously (Houslay & Tipton, 1973a).

Preparation of mitochondrial outer membranes

These were prepared by the method of Sottocasa *et al.* (1967) as previously described in full (Houslay & Tipton, 1973c).

Enzyme assays

All determinations of enzyme activity were carried out at 30°C in 0.12M-potassium phosphate buffer, pH 7.2, and specific activities are expressed as μmol of product formed/min per mg of enzyme protein. Enzyme assays were performed as described previously (Houslay & Tipton, 1973c), and the O_2 concentration was varied by using the apparatus described by Houslay & Tipton (1973c).

Polyacrylamide-gel electrophoresis

This was carried out by the method of Youdim (1972) by using 5% acrylamide gels, as described in full by Houslay & Tipton (1973a), or by using 3% gels (Davis, 1964). The gels were stained for protein with Amido Black [1% (w/v) in 7% (v/v) acetic acid] and the excess of stain was removed by soaking the gels in 7% (v/v) acetic acid. Monoamine oxidase activity was located either by the method of Glenner *et al.* (1957), with tyramine as the substrate, or by a novel method with benzylamine as substrate. This method utilizes the fact that the H_2O_2 produced during the reaction can form an insoluble yellow complex with diaminobenzidine. This reaction has been fully elucidated by Cohen (1973), who used it to assay sulphite oxidase and glucose oxidase, and has also been used by us to detect ox plasma monoamine oxidase on polyacrylamide gels (Houslay & Tipton, 1974b). Activity was located by incubating the gel for 30 min at 30°C in 0.03M-potassium phosphate buffer, pH 7.2, containing 0.25 mg of diaminobenzidine/ml and 5 mM-benzylamine. The colour could then be preserved by storing the stained gels in 7% acetic acid in the dark. No band was observed when benzylamine was left out of the reaction mixture, and the results obtained with the two staining methods appeared to agree well.

Lipid analysis

A sample of the enzyme was extracted for lipids (Folch *et al.*, 1951) and these were subsequently hydrolysed with 0.8M-KOH (90 min at 50°C), then acidified with HClO_4 and the free fatty acids were

extracted with hexane. These extracts were subsequently dried under a jet of O_2 -free N_2 , and methylated (Metcalf & Schmitz, 1961). The fatty acid methyl esters were extracted with hexane, and then dried with a stream of O_2 -free N_2 before g.l.c. analysis. G.l.c. was performed on a 5104 Pye-Unicam series 104 gas chromatograph with a 1.83 m (6 ft) column, internal diam. 0.25 m. The support was Gas-Chrom Q₁, 80–100 mesh, which had been acid-base-washed before coating with Apiezon L. All runs were isothermal at 250°C, with argon carrier gas (60 ml/min) and a detector temperature of 250°C. Fatty acid methyl esters were identified and quantified by using known standards.

Other methods

Protein was determined by the method of Goa (1953). Measurements of pH were made on a Radiometer PHM 22r pH-meter from Radiometer A/S, Copenhagen, Denmark. Water was double-glass-distilled before use.

Benzylamine free base was converted into its hydrochloride before use, and benzaldehyde was redistilled [see Houslay & Tipton (1973c) for details]. The H_2O_2 solutions were calibrated by adding small amounts to 120 mM-potassium phosphate buffer, pH 7.2, at 30°C in the presence of 1 unit of catalase in a calibrated oxygen electrode (Tipton & Spire, 1971).

All chemicals were of the highest grade available and obtained from BDH Chemicals Ltd., Poole, Dorset, U.K., except for diaminobenzidine, benzylamine free base and benzaldehyde, which were obtained from Ralph N. Emanuel Ltd., Wembley, Middx., U.K., and catalase, which was from Boehringer (U.K.) Ltd., London W.5, U.K.

Throughout, initial velocities (v) are expressed in arbitrary units, one arbitrary unit being defined as a change in extinction of 0.001 E_{250} /unit/min (i.e. production of 724 pmol of benzaldehyde/min).

Results

Enzyme preparation

The enzyme preparation obtained from the Sepharose 4B gel-filtration column had a specific activity of 29 munits/mg of protein when assayed in 0.12M-potassium phosphate buffer, pH 7.2, at 30°C in the presence of 1 mM-benzylamine. Such a preparation was free of kinetically contaminating enzyme species such as aldehyde dehydrogenase and alcohol dehydrogenase, and indeed the presence of the enzyme preparation did not alter the value of the extinction coefficient that could be obtained for benzaldehyde, indicating that the production of

benzaldehyde was truly reflected by the increase in absorption at 250nm under assay conditions. Velocity of reaction was directly proportional to enzyme concentration, and rates were linear with time for at least 5min under assay conditions.

This preparation yielded a single band of activity when either of the polyacrylamide-gel-electrophoresis methods were used, and appeared to be independent of the assay substrate. Protein staining indicated that the preparation was not homogeneous.

G.l.c. analysis showed that the preparation contained 22.2ng of lipid/mg of protein. The acyl side chains comprising this were 16:0 (5.4ng), 18:0 (7.0ng), 18:1 (5.6ng), 20:4 ω 6 (3.0ng) 22:6 ω 3 (1.2ng).

After further gel filtration on Sepharose 6B, preparations of specific activity in excess of 55 munits/mg of protein were obtained, and these possessed no significant catalase activity (<0.01 munit/mg) and thus were suitable for use in the presence of H₂O₂ for product-inhibition studies. The presence of 2mM-KCN, a reversible inhibitor of the enzyme (Houslay & Tipton, 1973c), and also a catalase inhibitor, had no significant effect on the inhibition given by H₂O₂.

The purified preparation could be treated with NaClO₄ in the manner previously described (Houslay & Tipton, 1973a) with no loss in activity. This treated preparation could then be readily assayed in the standard kinetic buffer system when the final protein concentration was in the range 50–100 μ g/ml.

Kinetic investigation

Double-reciprocal plots obtained when the initial rates of benzylamine oxidation were determined at different benzylamine concentrations and a series of O₂ concentrations exhibited a series of parallel lines (Fig. 1a). Similar parallel lines were obtained when the O₂ concentration was varied at a series of fixed benzylamine concentrations (Fig. 1b). Plots of apparent K_m values against apparent maximum velocities for both varied substrates (Slater, 1955) were linear and passed through the origin. Secondary intercept plots were linear and yielded values of 475 μ M as K_m for benzylamine and 156 μ M as K_m for oxygen.

Such reciprocal plots, exhibiting lines of equal slope, which are independent of the concentration of the second substrate, are indicative of a mechanism proceeding through a free modified form of the enzyme in which a series of binary complexes are formed, without the occurrence of any kinetically significant amounts of a ternary complex. This type of mechanism is known as a double-displacement or Ping Pong mechanism. However, as we have discussed previously in connexion with the kinetics of the membrane-bound enzyme (Houslay & Tipton, 1973c), the occurrence of such parallel plots, although indicative of a double-displacement mechanism, is not conclusive. Also it is difficult to tell whether the lines in such plots are truly parallel or weakly intersecting, that is, whether in the general kinetic equation for two-substrate reactions:

Fig. 1. Kinetics of oxidation of benzylamine

(a) Double-reciprocal plot of the initial velocities of benzylamine oxidation against benzylamine concentration at a series of fixed O₂ concentrations, which were 0.13 mM (■), 0.23 mM (○), 0.498 mM (△) and 0.795 mM (□). (b) Double-reciprocal plot of the initial velocities of benzylamine oxidation against O₂ concentration at a series of fixed benzylamine concentrations, which were 0.2 mM (■), 0.25 mM (●), 0.4 mM (▲), 0.5 mM (□), 1.0 mM (○) and 2.0 mM (△).

$$v = \frac{V_{\max.}}{1 + \frac{K_s^A}{[A]} + \frac{K_m^B}{[B]} + \frac{K_s^A K_m^B}{[A][B]}}$$

(in which K_s^A is the apparent dissociation constant of the EA complex, and K_m^A and K_m^B are Michaelis constants) the term $K_s^A \cdot K_m^B/[A][B]$ is zero or is simply very small.

Evidence for the absence of a $K_s^A \cdot K_m^B/[A][B]$ term is supplied by the form of the Slater (1955) plots, and can also be obtained if the two substrates A (benzylamine) and B (O_2) are varied in a constant ratio. In such a situation double-reciprocal plots of the initial velocities of benzylamine oxidation against either of the substrate concentrations will be linear unless $K_m^A \cdot K_m^B/[A][B]$ is finite, in which case it will be non-linear (see, e.g. Houslay & Tipton, 1973c). Fig. 2 demonstrates linear plots at three different ratios (n) of $[A]/[B]$, and these plots intersect on the ordinate axis irrespective of whether the reciprocal of oxygen or of benzylamine concentration is plotted on the abscissa. Such linear plots intersecting in this fashion are consistent with a double-displacement mechanism with no evidence of high-substrate inhibition (Middleton, 1974), and indeed we have never observed high-substrate inhibition in our kinetic studies with this enzyme.

These results indicate that the reaction catalysed by the soluble enzyme from rat liver mitochondria proceeds via a double-displacement mechanism, as does the membrane-bound enzyme from the same

source, and the solubilized enzymes from other sources.

Product inhibition

Double-reciprocal plots indicated that benzaldehyde was a mixed inhibitor with respect to benzylamine (Fig. 3a) and to O_2 (Fig. 3b), when the non-varied substrate was non-saturating (approx. $2 \times K_m$). This can be reconciled to the double-displacement mechanism if it is assumed that benzaldehyde can bind to both the reduced and the oxidized forms of the enzyme. As observed with the membrane-bound enzyme (Houslay & Tipton, 1973c) the Dixon (1953) plots of these data were non-linear (Figs. 4a and 4b) and such a result could be expected if benzaldehyde were capable of reversing the reaction:

This probably indicates that NH_3 remains bound to the enzyme either until after aldehyde dissociates from the enzyme or until after O_2 binds to the enzyme (cf. Houslay & Tipton, 1973c). Slope replots of the inhibition data for both varied substrates were non-linear; however, the intercept replot when benzylamine was the varied substrate yielded a non-linear result (Fig. 5a). This is in contrast with the intercept replots for the membrane-bound enzyme, which were both linear (Fig. 5b), although both the slope replots were non-linear.

Fig. 2. Mixed-substrate experiments

(a) Double-reciprocal plot of initial velocities of benzylamine oxidation against benzylamine concentration at several different ratios (n) of [benzylamine]/ $[O_2]$, which were $\frac{1}{2}$ ($\circ$), 1 ($\square$) and 2 ($\triangle$). (b) Double-reciprocal plot of initial velocities of benzylamine oxidation against O_2 concentration at several fixed values of n , which were $\frac{1}{2}$ ($\circ$), 1 ($\square$) and 2 ($\triangle$).

Fig. 3. Reciprocal plots of the inhibition of monoamine oxidase by benzaldehyde

(a) Double-reciprocal plot of the initial rates of benzylamine oxidation against varied benzylamine concentration at several fixed benzaldehyde concentrations, which were zero (○), 0.008 mm (□), 0.016 mm (△), 0.02 mm (●) and 0.028 mm (■). The O_2 concentration was kept constant at 0.23 mm. (b) Double-reciprocal plot of the initial velocities of benzylamine oxidation against O_2 concentration at various fixed benzaldehyde concentrations, which were zero (○), 0.008 mm (△), 0.012 mm (□), 0.016 mm (▲) and 0.02 mm (●). The benzylamine concentration was kept constant at 1 mm.

Fig. 4. Inhibition of monoamine oxidase by benzaldehyde

Dixon (1953) replots of the data in Fig. 3. (a) Reciprocals of the initial velocities of benzylamine oxidation against inhibitor concentration at a series of benzylamine concentrations. The oxygen concentration was 0.23 mm throughout. Benzylamine concentrations were 4 mm (○), 1 mm (△), 0.5 mm (□), 0.4 mm (●) and 0.2 mm (▲). (b) Reciprocals of the initial velocities of benzylamine oxidation against inhibitor concentration at a series of O_2 concentrations. The benzylamine concentration was 1 mm throughout. O_2 concentrations were 0.628 mm (○), 0.391 mm (△), 0.23 mm (□) and 0.135 mm (●).

Fig. 5. Secondary replots of product inhibition by benzaldehyde

(a) Intercept replots for the soluble enzyme when oxygen (■) and benzylamine (●) were the varied substrates. Data are taken from Figs. 3(a) and (b). (b) Intercept replots for the membrane-bound enzyme when oxygen (■) and benzylamine (●) were the varied substrates. Adapted from Houslay & Tipton (1973c) with additional data.

Double-reciprocal plots showed that NH_3 was a mixed inhibitor towards both benzylamine and O_2 (Figs. 6a and 6b) when the non-varied substrate was non-saturating (approx. $2 \times K_m$), the Dixon (1953) plots obtained being linear. However, when the benzylamine concentration was kept constant at a saturating value ($10 \times K_m$) a double-reciprocal plot of inhibition by NH_3 with O_2 as the varied substrate yielded a mixed inhibition pattern (Fig. 7).

H_2O_2 was shown to be a competitive inhibitor when benzylamine was the varied substrate (Fig. 8a) and an uncompetitive inhibitor with respect to oxygen as the varied substrate (Fig. 8b) when the second substrate was non-saturating ($2 \times K_m$). Dixon (1953) replots yielded linear results. The inhibition caused by H_2O_2 was freely reversible and showed no time-dependence, being easily relieved by dialysis, gel filtration or addition of catalase followed by re-equilibration of the O_2 concentration.

The reaction pathway shown in Scheme 1 appears to be the simplest that accounts for the data obtained. Such a mechanism is similar in outline to

that obtained for the membrane-bound enzyme. In Scheme 1 the free enzyme (F_{ox}) reacts with the amine to give a free modified form of the enzyme ($\text{F}_{\text{red}}^{\text{NH}_3}$) in an analogous fashion to that proposed for the membrane-bound enzyme, but then an isomerization step, $\text{F}_{\text{red}}^{\text{NH}_3} \rightleftharpoons \text{E}_{\text{red}}^{\text{NH}_3}$, is postulated before O_2 binding, where only $\text{E}_{\text{red}}^{\text{NH}_3}$ can bind O_2 .

In the absence of products, Scheme 1 can be defined by the steady-state rate equation:

$$v = \frac{V_{\text{max.}}}{1 + \frac{K_m^{\text{amine}}}{[\text{amine}]} + \frac{K_m^{\text{O}_2}}{[\text{O}_2]}}$$

During product inhibition by aldehyde, a step

is postulated; thus a steady-state rate equation can be derived of the form:

$$\begin{aligned} \frac{V_{\text{max.}}}{v} = & 1 + \frac{K_m^{\text{amine}}}{[\text{amine}]} + \frac{[\text{aldehyde}]}{[\text{amine}]} \cdot \left(\frac{K_s^{\text{amine}} K_{st}^{\text{iv aldehyde}}}{K_{st}^{\text{aldehyde}}} + \frac{K_s^{\text{amine}}}{K_{st}^{\text{aldehyde}}} + \frac{K_{st}^{\text{v aldehyde}}}{K_{st}^{\text{aldehyde}}} \right) + \frac{[\text{aldehyde}]^2 K_s^{\text{amine}}}{[\text{amine}] K_{st}^{\text{aldehyde}} K_{st}^{\text{aldehyde}}} \\ & + \frac{K_m^{\text{O}_2}}{[\text{O}_2]} + \frac{[\text{aldehyde}]}{[\text{O}_2]} K_{mt}^{\text{aldehyde}} \cdot \left(\frac{1}{K_{st}^{\text{aldehyde}}} + \frac{1}{K_{st}^{\text{aldehyde}}} \right) + \frac{[\text{aldehyde}] K_{mt}^{\text{aldehyde}} K_s^{\text{amine}}}{[\text{O}_2] [\text{amine}] K_{st}^{\text{aldehyde}}} \cdot \left(1 + \frac{[\text{aldehyde}]}{K_{st}^{\text{aldehyde}}} \right) \\ & + [\text{aldehyde}] \cdot \left(\frac{1}{K_{st}^{\text{aldehyde}}} + \frac{1}{K_{st}^{\text{aldehyde}}} \right) \end{aligned}$$

Fig. 6. Inhibition of monoamine oxidase by NH_3

(a) Double-reciprocal plot of the initial velocities of benzylamine oxidation against benzylamine concentration at several fixed NH_3 concentrations, which were zero ($\square$), 100 mM ($\circ$), 150 mM ($\triangle$), 200 mM ($\blacksquare$) and 250 mM ($\bullet$). The O_2 concentration was kept constant at 0.23 mM. (b) Double-reciprocal plot of the initial rates of benzylamine oxidation against O_2 concentration at several fixed NH_3 concentrations, which were zero ($\square$), 50 mM ($\circ$), 100 mM ($\triangle$), 150 mM ($\blacksquare$) and 200 mM ($\bullet$). The benzylamine concentration was kept constant at 1 mM. Variations in ionic strength over the range used here did not appear to have any significant effect on the form of the double-reciprocal plots, where NH_3 was used as inhibitor. The NH_3 source was NH_4Cl adjusted to pH 7.2 with KOH .

Fig. 7. Inhibition by NH_3 at a high benzylamine concentration

A double-reciprocal plot of the initial rates of benzylamine oxidation against O_2 concentration at several fixed NH_3 concentrations, which were zero ($\square$), 100 mM ($\circ$), 150 mM ($\triangle$), 200 mM ($\blacksquare$) and 250 mM ($\bullet$). The benzylamine concentration was constant at approx. $10 \times K_m$ (benzylamine.)

This equation shows that Scheme 1 accounts for the nature of the slope and intercept replots obtained for the aldehyde product-inhibition studies. All slope replots will be non-linear irrespective of which is the varied substrate; the intercept plot when amine is the

varied substrate will be linear, as no term in aldehyde other than power 1 will exist in the equation. However, when O_2 is the varied substrate, intercept replots will be non-linear, as a power-2 term in aldehyde is included, namely:

Fig. 8. Inhibition of monoamine oxidase by H₂O₂

(a) Double-reciprocal plot of the initial velocities of benzylamine oxidation against benzylamine concentration at several fixed H₂O₂ concentrations, which were zero (□), 1.8 mM (○), 3.0 mM (△), 4.2 mM (■) and 6.0 mM (●), the O₂ concentration being kept constant at 0.23 mM. (b) Double-reciprocal plot of the initial velocities of benzylamine oxidation against O₂ concentration at several fixed H₂O₂ concentrations, which were zero (△), 3.0 mM (○), 4.2 mM (□), 6.0 mM (▲) and 9.0 mM (●), the benzylamine concentration being constant at 1.0 mM.

$$\frac{[\text{aldehyde}]^2 K_s^{\text{amine}}}{[\text{amine}] K_{si}^{\text{aldehyde}} K_{si}^{\text{aldehyde}}}$$

During inhibition by NH₃ we postulate the existence of the step:

Steady-state treatment yields a rate equation of the form:

$$\frac{V_{max}}{v} = 1 + \frac{K_m^{\text{amine}}}{[\text{amine}]} \left(1 + \frac{[\text{NH}_3]}{K_{si}^{\text{NH}_3}} \right) + \frac{1}{[O_2]} \left(K_m^{O_2} + \frac{[\text{NH}_3] K_s^{O_2} K_{ml}^{\text{NH}_3}}{K_{si}^{\text{NH}_3}} \right) + \frac{[\text{NH}_3] K_{ml}^{\text{NH}_3}}{K_{si}^{\text{NH}_3}}$$

This equation defines inhibition by NH₃ as being mixed with respect to both varied substrates, as has

been observed. It also predicts that saturation with either substrate will still result in a mixed-inhibition pattern with the other substrate varied. Indeed such an observation was noted with O₂ as the varied substrate and with benzylamine at a saturating concentration (10 × K_m), when a plot of intersecting lines meeting below the abscissa was obtained. Such a plot indicates that

$$K_m^{O_2} > [\text{NH}_3] \cdot K_s^{O_2} \cdot \frac{K_{ml}^{\text{NH}_3}}{K_{si}^{\text{NH}_3}}$$

cf. the membrane-bound enzyme (Houslay & Tipton, 1973c). Product inhibition at 'saturating' O₂ concentration was not carried out, as it is impossible to dissolve O₂ to a sufficient concentration at 30°C.

H₂O₂ appears not to form a dead-end complex with the free reduced forms of the enzyme, but only to

Scheme 1. Proposed scheme for benzylamine oxidation by soluble rat liver mitochondrial monoamine oxidase

The steady-state rate equations that may be derived for such a model are discussed in the text, and the full equations in terms of the individual rate constants have been deposited as Supplementary Publication SUP 50039. The abbreviations ox. and red. refer to the forms of the enzyme in which the flavin moiety is in an oxidized or reduced state respectively.

Table 1. Some kinetic parameters of the soluble and membrane-bound forms of the enzyme

All these K_i values were obtained with the second (non-varied) substrate non-saturating (i.e. approx. $2 \times K_m$). N.L., Non-linear.

		Membrane-bound enzyme	Purified soluble enzyme	Perchlorate-treated soluble enzyme
$K_m^{\text{benzylamine}}$		475 μM	571 μM	—
$K_m^{\text{O}_2}$		156 μM	113 μM	—
$K_i^{\text{benzaldehyde}}$	(slope) Benzylamine varied	N.L.	N.L.	N.L.
$K_i^{\text{benzaldehyde}}$	(intercept) Benzylamine varied	22 μM	82 μM	106 μM
$K_i^{\text{benzaldehyde}}$	(slope) O ₂ varied	N.L.	N.L.	N.L.
$K_i^{\text{benzaldehyde}}$	(intercept) O ₂ varied	6.8 μM	N.L.	N.L.
$K_i^{\text{NH}_3}$	(slope) Benzylamine varied	63 mM	645 mM	750 mM
$K_i^{\text{NH}_3}$	(intercept) Benzylamine varied	205 mM	150 mM	150 mM
$K_i^{\text{NH}_3}$	(slope) O ₂ varied	275 mM	122 mM	—
$K_i^{\text{NH}_3}$	(intercept) O ₂ varied	120 mM	198 mM	—
$K_i^{\text{H}_2\text{O}_2}$		—	600 μM	—

Table 2. Kinetic parameters of un-ionized species of ammonia and benzylamine

The non-varied substrate was non-saturating (approx. $2 \times K_m$) during the determination of these inhibition constants. The assay pH was 7.2 and temperature 30°C, and pK_a values of 9.37 and 9.25 were assumed for benzylamine and NH_3 respectively.

		Membrane-bound enzyme	Purified soluble enzyme	Perchlorate-treated soluble enzyme
$K_m^{\text{benzylamine}}$		3.21 μM	3.86 μM	—
$K_i^{NH_3}$	(slope) Benzylamine varied	0.56 mM	5.69 mM	6.62 mM
$K_i^{NH_3}$	(intercept) Benzylamine varied	1.81 mM	1.32 mM	1.32 mM
$K_i^{NH_3}$	(slope) O_2 varied	2.43 mM	1.08 mM	—
$K_i^{NH_3}$	(intercept) Benzylamine varied	1.06 mM	1.75 mM	—

bind to oxidized species. Thus assuming steady-state conditions we can obtain the rate equation

$$\frac{V_{\max.}}{v} = 1 + \frac{K_m^{\text{amine}}}{[\text{amine}]} \left(1 + \frac{[H_2O]_2}{K_i^{H_2O_2}} \right) + \frac{K_m^{O_2}}{[O_2]}$$

which accounts for the kinetic behaviour of this product.

Some kinetic studies were performed on the $NaClO_4$ -treated enzyme, the results of which are summarized in Table 1. From the data so obtained it seems probable that the reaction pathway is comparable with that of the soluble untreated enzyme.

Discussion

The purification results in a relatively stable enzyme preparation, in that no change in activity towards benzylamine occurs after storage for 1 month at 4°C. It is readily assayed by using either the direct spectrophotometric assay of Tabor *et al.* (1954) or the coupled spectrophotometric assay with aldehyde dehydrogenase (Houslay & Tipton, 1973a). This preparation appears to be electrophoretically homogeneous as regards monoamine oxidase activity.

The reaction mechanism obeyed appears to be of the double-displacement type, and we have proposed a pathway which seems to be the simplest that accounts for the experimental results. A list of kinetic parameters comparing the data obtained from this study on the soluble enzyme with those of the membrane-bound enzyme is shown in Table 1. The kinetic parameters calculated for the un-ionized species of NH_3 and benzylamine are shown in Table 2, as McEwen *et al.* (1968, 1969) have postulated that it is these species that bind to the enzyme.

The major differences between the mode of benzylamine oxidation by the soluble and membrane-bound forms of the enzyme appear to be in product inhibition by aldehyde, and in the occurrence of an isomerization step between the free modified form of the enzyme and the species to which O_2 (the second substrate) binds. These deductions rely on the data obtained with aldehyde product-inhibition studies,

and the data obtained with all the preparations appear to be comparable, in that similar degrees of inhibition were achieved with all three preparations. These major differences in aldehyde inhibition took the form of an increase in the value of K_i (intercept) on solubilization when benzylamine was the varied substrate, and the appearance on solubilization of non-linearity in the intercept replot when O_2 was the varied substrate.

It appears that the 'E' configuration of the enzyme is held throughout the reaction pathway of the membrane-bound enzyme, whereas the free form of the soluble enzyme is the 'F' configuration, which must, however, isomerize to the 'E' form for O_2 to bind during the reaction sequence. This would imply that the configuration of the protein changes on solubilization, and thus the membrane may impose physical constraint on the tertiary structure of the protein, presumably so that the 'E' configuration is adopted; alternatively a specific lipid moiety may achieve control of the protein's tertiary structure.

Thus it seems important that studies on the enzyme, which attempt to assess its role *in vivo* and the potential effects of drugs on the enzyme, would best be carried out on a membrane-bound preparation of the enzyme and not a soluble one (see also Houslay & Tipton, 1973a,b, 1974a).

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