PRIMARY AMINES AS UNCOUPLERS OF ELECTRON TRANSPORT FROM HYDROXYLATION IN THE SECONDARY-AMINE MONO-OXYGENASE SYSTEM OF *PSEUDOMONAS AMINOVORANS*

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SUMMARY

Methylamine, ethylamine and *n*-propylamine caused an oxidation of NADH in the presence of the partially-purified secondary-amine mono-oxygenase system of *Pseudomonas aminovorans*. The primary amine did not disappear and no hydroxylation product was formed, so it was concluded that primary amines were non-substrate effectors uncoupling NADH oxidation from hydroxylation. K_m and V_{max} values were determined for the three primary amines and compared with the values for the corresponding secondary amine substrates. Methylamine competitively inhibited the oxidation of dimethylamine to formaldehyde. It was not possible to establish whether H_2O_2 was formed since the enzyme preparation contained catalase. Substrates and non-substrate effectors were equally effective in causing conversion of a reduced-oxygenated intermediate form of the enzyme back to the original oxidized form.

Evidence with flavoprotein mono-oxygenases (equation [A]) has shown that certain substrate analogues, described as "non-substrate effectors" (1) or "pseudo-substrates" (2) are able to bring about the oxidation of the reduced nicotinamide nucleotide electron donor without the hydroxylation of substrate. Such non-substrate effectors are said to "uncouple" the hydroxylation reaction and the oxidation product in such cases is hydrogen peroxide (equation [B]).

$$RH + NAD(P)H + H^+ + O_2 \longrightarrow ROH + NAD(P)^+ + H_2O \qquad [A]$$

$$R'H + NAD(P)H + H^+ + O_2 \longrightarrow R'H + NAD(P)^+ + H_2O_2 \qquad [B]$$

This has been demonstrated for salicylate hydroxylase (2, 3), orcinol hydroxylase (4) and *p*-hydroxybenzoate hydroxylase (1, 5). Recent evidence suggests that a similar phenomenon can occur with mono-oxygenases which are independent of external electron donors such as lysine mono-oxygenase (6).

We report here evidence suggesting that primary amines can act as non-substrate effectors and produce an analogous uncoupling effect on the secondary-

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amine mono-oxygenase enzyme system of *Pseudomonas aminovorans* (equation [C]).

$$(CH_3)_2NH + NAD(P)H + H^+ + O_2 \longrightarrow CH_3NH_2 + HCHO + NAD(P)^+ + H_2O \quad [C]$$

This enzyme differs from those described above in that it is not a pure flavoprotein hydroxylase, but a carbon monoxide-sensitive haemoprotein hydroxylase system which probably also contains iron-sulphur protein and flavoprotein constituents (7). Uncoupling activity in one haemoprotein hydroxylation system has been demonstrated with liver microsomes by Ullrich and Diehl (8), who showed that perfluoro-*n*-hexane, would stimulate NADPH oxidation and oxygen uptake, but unlike its parent compound *n*-hexane, it was not hydroxylated, nor was hydrogen peroxide detected.

METHODS

Enzyme purification. The secondary-amine mono-oxygenase was purified from trimethylamine-grown *Pseudomonas aminovorans* NCIB 9039 by a modification of the procedure previously described (7). The first ammonium sulphate precipitate was applied to a DEAE-cellulose column (Whatman DE 11) under conditions in which it bound. It was eluted with a 0-0.4 M-KCl gradient in 33 mM-phosphate buffer pH 6.8 containing 5% (v/v) ethanol. The combined active fractions after dialysis were applied to a column of hydroxyapatite (Bio-Gel HTP) equilibrated with 5 mM-phosphate buffer pH 6.8. The column was eluted step-wise with increasing molarities of phosphate in 5% (v/v) ethanol. The activity was found mainly in the 125 mM eluate. The preparation had a specific activity of 2 units/mg protein; overall purification was 20-fold. The enzyme was assayed spectrophotometrically as described previously (7) at 28°C. One unit of enzyme is the amount required to catalyse the oxidation of 1 μmol of NADH/min at this temperature.

Analytical methods. Oxygen consumption was estimated with a Beckman model 777 oxygen analyser, methylamine enzymically (9), formaldehyde by the Nash (10) method, and protein by the Folin-Ciocalteu method (11). Catalase activity was measured spectrophotometrically (12). Other methods have been described previously (7).

RESULTS

Effect of methylamine on the secondary-amine mono-oxygenase

Table 1 shows that when methylamine is incubated with the partially-purified secondary-amine mono-oxygenase system, there is an oxidation of NADH and an uptake of oxygen. No methylamine disappears however and no formaldehyde (the presumed oxidation product) is formed. The cause of the enzyme-dependent oxygen uptake in the absence of substrate is not known. It is not accompanied by NADH oxidation. The ratio of methylamine-dependent NADH oxidation to oxygen uptake is 2.2:1, which is not in accordance with the stoichiometry of equation [B] above. However the enzyme preparation had catalase activity (4.5 μmol of H_2O_2 oxidized/min/mg of protein when assayed at a peroxide concentration of 12.6 mM) and so if hydrogen peroxide had been formed, it would have been decomposed, giving the observed stoichiometry.

Binding of substrates and non-substrate effectors

The apparent K_m and V_{max} values for the activity of various substrates and non-substrate effectors in the oxidation of NADH by the enzyme is shown in Table 2. It is noteworthy that while the primary amine effectors have lower V_{max} values and higher apparent K_m values than the best substrates, the values are of a similar order to the less active secondary amine substrates. With mixed alkyl secondary amines, it has been shown previously (7) that aldehyde oxidation products are formed from both alkyl groups. Only in the case of methylamine has the non-disappearance of the amine been demonstrated, but it has been shown that ethylamine and *n*-propylamine both fail to produce aldehyde after incubation with enzyme and NADH. *n*-Butylamine is neither a substrate nor an uncoupler.

The uncoupling effect is specific to short chain primary amines. Methanol, ethanol and ammonium chloride did not stimulate NADH oxidation in the presence of the enzyme.

Inhibition of substrate oxidation by the non-substrate effector methylamine

As would be expected, from the precedent afforded by salicylate hydroxylase

Table 1. Stoichiometry of the methylamine-stimulated NADH oxidation by the secondary-amine mono-oxygenase.

Omission from reaction mixture	Oxygen uptake µmol	NADH remaining µmol	Methylamine remaining µmol	Formaldehyde formed µmol
None	0.383	1.39	2.93	0
Methylamine	0.108	2.01	0	0
NADH	0.103	0	2.89	0
Enzyme	0	2.01	2.82	0

Reaction mixtures contained: 3 µmol of methylamine-HCl, 2 µmol of NADH, 200 µmol of phosphate buffer pH 6.8, enzyme preparation (0.52 mg protein) and water in a total volume of 3.4 ml in the reaction vessel of an oxygen analyser. Omissions were made from this mixture as indicated. The reaction was started by addition of methylamine and followed for 20 min at 28°C. 0.4 ml of the mixture was then removed and the NADH remaining estimated spectrophotometrically at 340 nm. Trichloracetic acid (0.4 ml of 10% (w/v)) was added to the remainder of the reaction mixture, precipitated protein centrifuged off, and the supernatant neutralised with 0.2 ml of 1 M-NaOH. Methylamine and formaldehyde were then estimated as described in the Methods section.

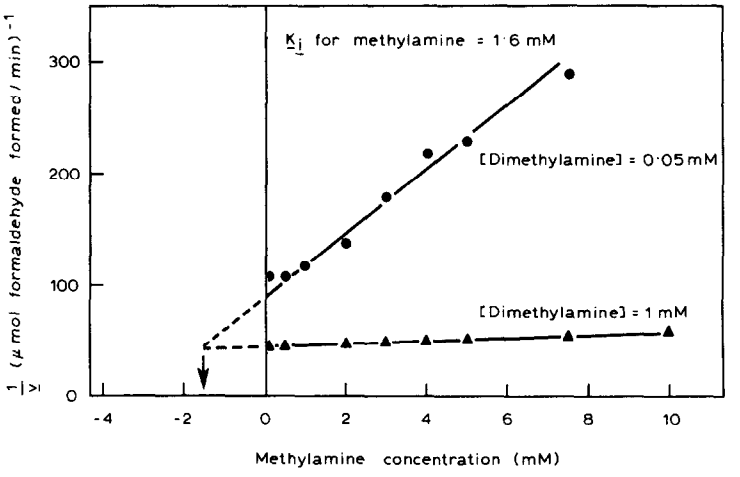

Figure 1. Dixon plot of the inhibition by methylamine of the conversion of dimethylamine to formaldehyde by the secondary-amine mono-oxygenase. Reaction mixtures contained 200 µmol of phosphate buffer, pH 6.8, 1 µmol of NADH, 3 or 0.15 µmol dimethylamine-HCl, enzyme (0.15 mg protein) various concentrations of methylamine-HCl and water to a final volume of 3 ml. After 10 min incubation at 28°C, the reaction was stopped by addition of 0.4 ml 10% (v/v)-$HClO_4$ and the formaldehyde formed estimated (10).

Table 2. Apparent K_m and V_{max} values for substrates and non-substrate effectors of the secondary-amine mono-oxygenase.

Substrate or Effector	Apparent K_m (mM)	V_{max} (μmol NADH oxidized/min/ mg protein)	Occurrence of substrate inhibition
Substrate			
Dimethylamine	0.028	0.465	Yes (>1 mM)
Ethylmethylamine	0.036	0.280	Yes (>1 mM)
Methyl-*n*-propylamine	0.595	0.412	Yes (>3 mM)
n-Butylmethylamine	9.50	0.144	-
Diethylamine	0.625	0.134	Yes (>10 mM)
Non-substrate effector			
Methylamine	1.160	0.144	Yes (>10 mM)
Ethylamine	0.233	0.146	Yes (>5 mM)
n-Propylamine	2.340	0.130	Yes (>20 mM)

Apparent K_m values and V_{max} values were determined by double reciprocal plots using the spectrophotometric assay described in (7).

(2), methylamine is a competitive inhibitor of a hydroxylatable substrate like dimethylamine. Fig. 1 shows a Dixon plot demonstrating this. It may be presumed that ethylamine and *n*-propylamine would behave similarly. Some compounds such as trimethylamine, can act as competitive inhibitors without acting as non-substrate effectors of NADH oxidation.

Evidence that primary and secondary amines are interacting with the same enzyme

Since the secondary-amine mono-oxygenase is only partially purified while the uncoupling studies described in refs. 1-5 were performed with homogeneous enzyme preparations, it was important to establish that both substrates and non-substrate effectors are interacting with the same enzyme.

Table 3. Relative effectiveness of methylamine and dimethylamine as effectors of NADH oxidation under various inhibitory conditions.

Inhibitory conditions	% of Uninhibited rate	
	Dimethylamine	Methylamine
None	100*	100*
Enzyme preparation heated at 30°C for 5 min	48.5	51
Reaction mixture bubbled with CO for 10 sec	0	0
Sodium mersalyl added to 1 μM†	27	22
Cysteamine added to 10 μM. No pre-incubation	40	1.5

* Uninhibited rates were (μmol NADH oxidized/min/ml of enzyme) 1.60 for dimethylamine and 0.345 for methylamine.

† Enzyme plus NADH (0.16 mM) plus sodium mersalyl were pre-incubated for 5 min at 28°C before starting the reaction.

The spectrophotometric assay (7) was used, the concentration of dimethylamine was 1 mM and that of methylamine was 10 mM.

Enzymic evidence. Table 3 shows that both methylamine- and dimethylamine-dependent NADH oxidation are impaired to similar extents in the presence of carbon monoxide and mersalyl which are potent inhibitors of the mono-oxygenase (7), and that heat inactivation affects the activity with the two substrates to the same extent. The fact that cysteamine is a much more effective inhibitor when methylamine is the effector than when dimethylamine is the substrate would be expected, since unlike the other two inhibitors tested, cysteamine is a competitive inhibitor with K_i of 0.9 μM (7) and so would be expected to be more effective with compounds of high apparent K_m values such as methylamine.

Spectral evidence. The purified enzyme preparation in its oxidized state has

its major absorption spectral peak at 410 nm. On reduction with NADH or NADPH, this shifts to 426 nm under anaerobic conditions (7). If oxygen is admitted to a preparation in the presence of excess NADH a new intermediate is formed, with absorption maxima at 418, 542 and 578 nm (T. R. Jarman and P. J. Large, unpublished results). We think that this intermediate is analogous to the oxygenated haemoprotein found in other systems (13). Unlike the oxygenated intermediate of the camphor hydroxylase system (13) however, the formation of the derivative with absorption maximum at 418 nm does not require the presence of substrate. This intermediate reverts only slowly, if at all, to the original oxidized form of the enzyme preparation if substrate is not added, i.e. it is tightly coupled. This is evidenced by the very low NADH oxidase activity of the preparation. (A typical figure for a 20-fold purified preparation is 6 nmol/min/mg protein - about 1% of the maximal rate of NADH oxidation in the presence of excess dimethylamine.) The addition of a substrate like dimethylamine or a non-substrate effector like methylamine at concentrations comparable to those active in the NADH oxidation assay, results in the oxidation of the NADH present, and there is a partial reconversion of the intermediate with the 418 nm maximum to the original oxidized form of the enzyme after the NADH present has all been consumed in the reaction.

DISCUSSION

We originally suggested (7) that the oxidation of NAD(P)H observed when the secondary-amine mono-oxygenase was incubated with methylamine and certain other primary amines was due to the presence of secondary amine impurities, but the demonstration that aldehyde reaction products are not formed eliminates this interpretation. The possibility that the reaction products from primary amines could be compounds other than the corresponding aldehydes is made unlikely by the demonstration that no methylamine disappears when incubated with NADH and the enzyme. We can also exclude the possibility that methylamine-dependent NADH oxidation is due to a contaminating enzyme, since it is affected to exactly the

same extent as dimethylamine-dependent NADH oxidation by various treatments (Table 3).

The identical effects of dimethylamine and methylamine in allowing partial reconversion of the 418 nm intermediate of the enzyme to the original oxidized form (with Soret peak at 410 nm) suggests that both substrates and non-substrate effectors are able to combine with the reduced-oxygenated enzyme, allowing it to take up a second electron and thus turn over to the oxidized form. This mechanism shows a number of differences to the mechanisms proposed both for flavoprotein (4, 14) and haemoprotein hydroxylases (15). We are not able with the present state of purity of the enzyme to ascertain whether or not hydrogen peroxide is formed when primary amines interact with the enzyme in the presence of NADH. Further mechanistic studies must await the obtaining of a more highly purified preparation.

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