Steady-state kinetic analysis of the quinoprotein methylamine dehydrogenase from *Paracoccus denitrificans*

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A steady-state kinetic analysis was performed of the reaction of methylamine and phenazine ethosulphate (PES) with the quinoprotein methylamine dehydrogenase from *Paracoccus denitrificans*. Experiments with methylamine and PES as varied-concentration substrates produced a series of parallel reciprocal plots, and when the concentrations of these substrates were varied in a constant ratio a linear reciprocal plot of initial velocity against PES concentration was obtained. Nearly identical values of V/K_m of PES were obtained with four different n-alkylamines. These data suggest that this reaction proceeds by a ping-pong type of mechanism. The enzyme reacted with a variety of n-alkylamines but not with secondary, tertiary or aromatic amines or amino acids. The substrate specificity was dictated primarily by the K_m value exhibited by the particular amine. A deuterium kinetic isotope effect was observed with deuterated methylamine as a substrate. The enzyme exhibited a pH optimum for V at pH 7.5. The absorbance spectrum of the pyrroloquinoline quinone prosthetic group of this enzyme was also effected by pH at values greater than 7.5. The enzyme was relatively insensitive to changes in ionic strength, and exhibited a linear Arrhenius plot over a range of temperatures from 10 °C to 50 °C with an energy of activation 46 kJ/mol (11 kcal/mol).

INTRODUCTION

It has been established recently that certain oxidoreductases from a variety of sources contain pyrroloquinoline quinone (PQQ) as a prosthetic group (reviewed in [1] and [2]). Some quinoproteins, such as bacterial methanol dehydrogenases [3] and glucose dehydrogenases [4], possess non-covalently associated PQQ. Other quinoproteins possess covalently bound PQQ. The latter group of enzymes includes bacterial methylamine dehydrogenases [5–7] and the copper-containing amine oxidases, such as mammalian plasma amine oxidase [8,9] and lysyl oxidase [10,11].

When grown on methylamine as a sole source of carbon and energy, *Paracoccus denitrificans* synthesizes an inducible methylamine dehydrogenase [7] that functions in the periplasmic space of this Gram-negative bacterium. It possesses covalently bound PQQ and catalyses the reaction:

where A is the electron acceptor for methylamine dehydrogenase. The physiological electron acceptor for this enzyme is a periplasmic type I blue copper protein amicyanin [12]. When assayed *in vitro* mediators such as phenazine ethosulphate (PES) are routinely used as electron acceptors. Many of the physical and redox properties of this methylamine dehydrogenase have been previously characterized in this laboratory [7,13–16]. As part of a continuing effort to elucidate the structure and function of this and other representative quinoproteins, a steady-state kinetic study of the reactions catalysed by *P. denitrificans* methylamine dehydrogenase is now presented.

EXPERIMENTAL

Methylamine dehydrogenase was purified from *P. denitrificans* (A.T.C.C. 13543) as described previously [7]. The amines used in this study were obtained from Aldrich Chemical Co. Phenazine methosulphate (PMS), PES and deuterated methylamine were obtained from Sigma Chemical Co. 2,6-Dichloroindophenol (DCIP) was obtained from General Biochemicals.

Methylamine dehydrogenase activity was assayed spectrophotometrically with a Kontron Uvicon 810 spectrophotometer as described by Eady & Large [17] with some modifications. The standard assay mixture contained 16 nM-methylamine dehydrogenase, 5 mM-PES, 100 μM methylammonium chloride and 100 μM -DCIP in 0.1 M-potassium phosphate buffer, pH 7.5. Unless otherwise indicated, assays were performed at 30 °C. Initial velocities were determined from the rate of reduction of DCIP, which was monitored at 600 nm ($\epsilon = 21\,500 \text{ M}^{-1} \cdot \text{cm}^{-1}$). To quantify the rates of reaction at different values of pH, the respective absorption coefficients of DCIP at those pH values were calculated from the data of Armstrong [18]. Absorbance spectra were recorded with a Cary 219 spectrophotometer.

RESULTS

Substrate specificity

Several amines were tested as substrates for this enzyme (Table 1). The kinetic parameters for the oxidation of amines that served as substrates were derived from reciprocal plots of the initial rate of amine oxidation at different amine concentrations in the presence of a saturating concentration (5 mM) of the reoxidant PES.

Abbreviations used: PQQ, pyrroloquinoline quinone; PMS, phenazine methosulphate; PES, phenazine ethosulphate; DCIP, 2,6-dichloroindophenol.

The enzyme reacted with a variety of primary aliphatic amines. The specificity of methylamine dehydrogenase for a particular amine was determined primarily by the K_m value for that amine (Table 1). No reaction was observed with dimethylamine, trimethylamine, benzylamine, glycine or alanine.

Once the K_m value for each amine had been determined, the kinetic parameters for the interaction of methylamine dehydrogenase with PES were derived from reciprocal plots of the initial rate of amine oxidation at different PES concentrations in the presence of a saturating concentration of each amine substrate. The specificity of this enzyme for PES was relatively unaffected by the amine substrate, as indicated by the values obtained for V/K_m for PES (Table 2).

The kinetic parameters for the oxidation of methylamine were also determined with PMS, a reoxidant that has often been used in the assay of quinoproteins. Nearly identical values of V/K_m were obtained with PMS and PES. PMS was a more efficient substrate for methylamine dehydrogenase than was PES, exhibiting a value of V/K_m 3-fold greater than that for PES. When PMS was used, however, a significant blank rate was observed for the assay. Also, significant substrate inhibition was observed at PMS concentrations greater than 1 mM (results not shown). Conversely, no appreciable blank rate or substrate inhibition was observed with PES at

concentrations as high as 5 mM. For these reasons PES rather than PMS was routinely used in these studies.

Mechanistic studies

Reciprocal plots, obtained when the initial rates of methylamine oxidation were determined at different concentrations of methylamine in the presence of a series of fixed concentrations of PES, exhibited a series of parallel lines (Fig. 1a). Similarly, a series of parallel lines was obtained when the initial rates of methylamine oxidation were determined at different concentrations of

Table 1. Substrate specificity of *P. denitrificans* methylamine dehydrogenase

The units of V are μmol of DCIP reduced/min per mg of enzyme, and those of K_m are μM .

Substrate	K_m	V/K_m
Methylamine	20	0.77
Ethylamine	18	0.49
Propylamine	170	0.085
Butylamine	4500	0.004
1,3-Diaminopropane	3500	0.004
Ethanolamine	630	0.017
Histamine	1500	0.11
Spermidine	45000	0.0003

Table 2. Effect of the amine substrate on the specificity of *P. denitrificans* methylamine dehydrogenase for PES

The fixed concentrations of amine present were: methylamine, 166 μM ; ethylamine, 200 μM ; propylamine, 1.3 mM; butylamine, 16 mM; 1,3-diaminopropane, 30.1 mM; ethanolamine, 6.3 mM; histamine, 14.5 mM. The units of V are μmol of DCIP reduced/min per mg of enzyme, and those of K_m are μM .

Substrate	$10^3 \times V/K_m^{\text{PES}}$
Methylamine	4.8 ± 0.7
Ethylamine	4.8 ± 0.2
Propylamine	5.3 ± 0.2
Butylamine	5.3 ± 0.2
1,3-Diaminopropane	3.7 ± 0.6
Ethanolamine	1.3 ± 0.2
Histamine	2.1 ± 0.1

Fig. 1. Double-reciprocal plots of the reaction of methylamine and PES with methylamine dehydrogenase

(a) Initial rates of methylamine oxidation were measured at different concentrations of methylamine in the presence of 600 μM - (○), 300 μM - (●) 200 μM - (△) and 100 μM -PES (▲). (b) Initial rates of methylamine oxidation were measured at different concentrations of PES in the presence of 30 μM - (○), 15 μM - (●), 10 μM - (△) and 7.5 μM -methylamine (▲). The units of v are μmol of DCIP reduced/min per mg of enzyme.

Fig. 2. Double-reciprocal plot for the reaction of methylamine and PES with methylamine dehydrogenase when the concentrations of substrates are varied at a constant ratio

In each assay methylamine was present at a concentration 15-fold less than that of PES.

PES in the presence of a series of fixed concentrations of methylamine (Fig. 1b). Secondary plots of the intercepts against substrate concentrations were linear and yielded K_m values of 10 μM for methylamine and 300 μM for PES, and a value of V of 15.3 $\mu\text{mol}/\text{min}$ per mg of protein. Given the molecular mass of the holoenzyme of 124 kDa [7], this corresponds to a value of k_{cat} of 1900 min^{-1} . When methylamine and PES were presented to the enzyme at different concentrations at a constant ratio, a linear relationship was observed (Fig. 2) for the reciprocal plot of the data obtained from this mixed-substrate experiment.

The kinetic parameters for the oxidation of $[\text{Me-}^2\text{H}_3]$ -methylamine were determined and compared with those obtained for methylamine (Fig. 3). A deuterium kinetic isotope effect was observed with ratios of $^2\text{H}V$ of 3.0, of $^2\text{H}K_m$ of 1.0 and of $^2\text{H}(V/K_m)$ of 3.0.

Effects of pH, ionic strength and temperature

The dependence of the kinetic parameters for the oxidation of methylamine on pH was determined from reciprocal plots obtained at different values of pH. The enzyme exhibited a pH optimum for V centred at pH 7.5 (Fig. 4). Conversely, K_m was relatively insensitive to pH (results not shown).

Given the observed dependence on pH of the activity of this enzyme, the effect of pH on the absorbance spectrum of the protein-bound PQQ was examined. Distinct perturbations of the absorbance spectrum of the oxidized enzyme were observed at pH values greater than 7.5 (Fig. 5). These perturbations occurred rapidly and were completely and rapidly reversed by shifting the pH back to pH 7.5. These changes were independent of the buffer used, as identical spectra were obtained at selected values of pH with potassium phosphate, sodium borate and sodium Bicine buffers. In order to quantify this effect

Fig. 3. Deuterium kinetic isotope effect on the oxidation of methylamine by methylamine dehydrogenase

Initial rates of the oxidation of methylamine (○) and $[\text{Me-}^2\text{H}_3]$ -methylamine (●) were measured in the presence of 5 mM-PES.

Fig. 4. Dependence of V on pH for the oxidation of methylamine by methylamine dehydrogenase

Values of V were determined from reciprocal plots obtained when the initial rates of methylamine oxidation were measured at different concentrations of methylamine in the presence of 5 mM-PES in 0.1 M-potassium phosphate buffer at the indicated values of pH.

the absorbances at 440 nm and 330 nm were measured and normalized to the absorbance at 280 nm, which was unaffected by pH. The A_{440}/A_{330} ratio was then plotted against pH (Fig. 6). By inspection, an apparent pK of 8.2 was determined for this effect.

The dependence of the kinetic parameters for the oxidation of methylamine on ionic strength was determined from reciprocal plots obtained when the initial rates of methylamine oxidation were determined at different concentrations of methylamine in the presence

Fig. 5. Effect of pH on the absorbance spectrum of methylamine dehydrogenase

Spectra were recorded of 3.2 nmol of methylamine dehydrogenase in 1 ml of 0.1 M-sodium Bicine buffer at pH 7.5 (—), pH 8.0 (---), pH 8.5 (·····) and pH 9.0 (— · —).

Fig. 6. Titration of the changes in the absorbance spectrum of methylamine dehydrogenase at different values of pH

Values of A_{440} and A_{330} were obtained from the spectra shown in Fig. 5 and from additional spectra that were recorded at other values of pH.

of a saturating concentration of PES in the presence of different concentrations of NaCl. Neither V nor K_m was significantly affected by changes in ionic strength. Even in the presence of 1.5 M-NaCl the values of V and K_m were obtained that were respectively 96% and 52% of those obtained in the absence of added NaCl.

The dependence of the kinetic parameters for the oxidation of methylamine on temperature was determined from reciprocal plots obtained when the initial rates of methylamine oxidation were determined at

Fig. 7. Arrhenius plot for the oxidation of methylamine by methylamine dehydrogenase

V was determined from reciprocal plots obtained when the initial rates of methylamine oxidation were measured at different concentrations of methylamine in the presence of 5 mM-PES in 0.1 M-potassium phosphate buffer, pH 7.5, at the indicated temperatures.

different concentrations of methylamine in the presence of a saturating concentration of PES at temperatures ranging from 10 °C to 50 °C. Linear-regression analysis of these data indicated that the points were best fitted by a single straight line (Fig. 7). The energy of activation was calculated from the slope of this line to be 46 kJ/mol (11 kcal/mol). A plot of pK_m against $1/T$ was linear as well (results not shown).

DISCUSSION

The data obtained from steady-state kinetic experiments with methylamine and PES as substrates (Figs. 1 and 2) are suggestive of a ping-pong type of mechanism [19] in which the aldehyde is released from the enzyme before the interaction with the reoxidant. Also consistent with these data is a sequential mechanism in which $K_{ia} < K_a$. A ping-pong mechanism, however, is most likely, given the constancy of the values of V/K_m for PES that were obtained with alternative amine substrates (Table 1). These values were nearly identical for the four n-alkylamines. The substituted alkylamines did exhibit some variation in the values of V/K_m for PES. This variation was not great, but it may suggest a subtle change in mechanism for these substrates. A deuterium kinetic isotope effect of $^3H(V/K_m)$ of 3.0 was observed for methylamine oxidation, which suggests that removal of a methyl proton contributes significantly to the overall rate of this reaction. Similar kinetic data indicative of a ping-pong mechanism have been obtained for the methylamine dehydrogenase of the facultative methylotroph *Pseudomonas* AM1 with n-butylamine and PMS as substrates [20]. Ping-pong mechanisms are also exhibited by the PQQ-containing lysyl oxidases [21], diamine oxidases [22] and plasma amine oxidases [23],

in which aldehyde release precedes the binding of the second substrate, O_2 . Large deuterium kinetic isotope effects have also been reported for the reduction of plasma amine oxidase [24]. Thus, although these eukaryotic amine oxidases differ from the prokaryotic methylamine dehydrogenases in substrate specificity and in their reaction with O_2 , a common mechanism may exist for the oxidation of primary amines by PQQ-containing enzymes.

Previous studies indicated that *P. denitrificans* methylamine dehydrogenase was very stable against denaturation at extremes of pH and temperature [7]. This has allowed the study of the steady-state kinetic parameters of the reactions that are catalysed by this enzyme over a wide range of experimental conditions. The relatively narrow pH optimum for activity exhibited by this enzyme (Fig. 4) is of interest because the loss of activity is not simply due to denaturation of the enzyme but must be due to the ionization of residues that are critical for activity. Methylamine dehydrogenase, which exhibits a pI value of 4.3 [7], and methylamine, which exhibits a pK value of 10.6, will not experience an overall change in charge over the range of pH values in which large changes in activity are observed. The PQQ prosthetic group of methylamine dehydrogenase also possesses ionizable groups [5]. Nothing is known about the pK values for these groups in free PQQ or protein-bound PQQ. The perturbations in the absorbance spectrum of methylamine dehydrogenase observed at high pH may be due to the ionization of group(s) on PQQ. Further studies are necessary to determine the cause of these spectral changes and whether this effect is relevant to catalysis.

This enzyme is well suited for the study of the mechanism of catalysis by the novel PQQ prosthetic group. The PQQ chromophore exhibits large changes in absorbance on change in redox state [7]. These changes are rapid and could be further studied by stopped-flow techniques. Furthermore, complex-formation has recently been demonstrated between *P. denitrificans* methylamine dehydrogenase and its physiological electron acceptor, amicyanin [15,16]. As a prelude to pre-steady-state kinetic studies and mechanistic studies with amicyanin, this description of the steady-state kinetics of methylamine oxidation by methylamine dehydrogenase is presented.

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