Characterization of a novel methanol dehydrogenase containing a Ba^{2+} ion at the active site

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The quinoprotein methanol dehydrogenase (MDH) contains a Ca^{2+} ion at the active site. Ca^{2+}-free enzyme (from a processing mutant) was used to obtain enzyme containing Sr^{2+} or Ba^{2+}, the Ba^{2+}-MDH being the first enzyme to be described in which a Ba^{2+} ion functions at the active site. The activation energy for oxidation of methanol by Ba^{2+}-MDH is less than half that of the reaction catalysed by Ca^{2+}-MDH (a difference of 21.4 kJ/mol), and the V_{max} value is 2-fold higher. The affinities of Ba^{2+}-MDH for substrate and activator are very much less than those of Ca^{2+}-MDH; the K_m for methanol is 3.5 mM (compared with 3 μM) and the K_A for ammonia is 52 mM (compared with 2 mM). The different activity of Ba^{2+}-MDH is probably due to a change in the conformation of the active site, leading to a decrease in the free energy of substrate binding and hence a decrease in activation energy. The kinetic model for Ba^{2+}-MDH with respect to substrate and activator is consistent with previous models for Ca^{2+}-MDH. The pronounced deuterium isotope effect (6.0–7.6) is influenced by ammonia, and is consistent with activation of the pyrroloquinoline quinone reduction step by ammonia. Because of its low affinity for substrates, it is possible to prepare the oxidized form of Ba^{2+}-MDH. No spectral intermediates could be detected during reduction by added substrate, and so it is not possible to distinguish between those mechanisms involving covalent substrate addition and those involving only hydride transfer.

INTRODUCTION

Methanol dehydrogenase (MDH) is a bacterial periplasmic quinoprotein; it has pyrroloquinoline quinone (PQQ) as its prosthetic group and it uses cytochrome c_L as electron acceptor, although it is usually assayed using an artificial electron acceptor such as phenazine ethosulphate (PES) [1–3]. When using this assay system it is necessary to add ammonia (free base) as activator, but the mechanism of this activation is not fully understood. The structure of the $\alpha_2\beta_2$ tetramer of MDH from *Methylobacterium extorquens* has recently been determined at 1.94 Å (0.194 nm) [4,5]. The active site contains a Ca^{2+} ion which is co-ordinated to residues on the protein and also to PQQ by way of the 7-carboxy group, the N-6 in the ring and the C-5 carbonyl oxygen (Figure 1).

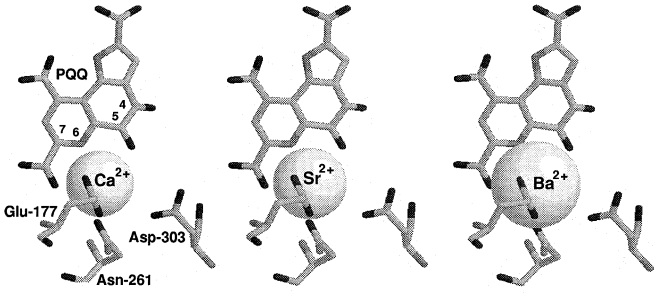

Figure 1 Co-ordination of Ca^{2+} and PQQ in the active site of MDH

The full structure is given in [5]. The structures containing Sr^{2+} and Ba^{2+} are based on the same co-ordinates as the Ca^{2+}-containing dehydrogenase by replacing the Ca^{2+} ion with appropriately sized spheres in the Rasmol program, in which the radii are presented as 1.95 for Ca^{2+} and 2.41 Å for Ba^{2+}.

It has been suggested that, besides holding PQQ in position in the active site, the Ca^{2+} ion might also play a role in the reaction mechanism by acting as a Lewis acid, facilitating attack by the substrate on the electrophilic C-5 of PQQ (Scheme 1) [4]. It is not certain whether this involves formation of a covalent hemiketal derivative of PQQ and methanol (Scheme 1, upper panel) or whether there is a relatively simple hydride transfer mechanism (Scheme 1, lower panel). The quinol form of PQQ is subsequently re-oxidized by an electron acceptor in two separate single electron transfer steps, the semiquinone free radical of PQQ being an intermediate. When MDH is isolated and purified, the prosthetic group is usually in this stable semiquinone form.

The presence of Ca^{2+} in MDH was first demonstrated in *Methylobacillus glycogenes* [6], and a function for Ca^{2+} was demonstrated by using the *mxaA* mutant of *M. extorquens*; the *mxaA* gene maps apart from the structural genes for MDH and its product is involved in processing MDH in the periplasm [7]. MDH prepared from this mutant lacks Ca^{2+}, but incubation with Ca^{2+} led to active enzyme containing irreversibly bound Ca^{2+} [8]. We have recently shown that MDH isolated from the *mxaA* mutant can be used to produce active enzyme in which Ca^{2+} is replaced by either Sr^{2+} or Ba^{2+}. The present paper provides the first description of an enzyme in which Ba^{2+} plays an active part in the reaction mechanism.

EXPERIMENTAL

The following methods were as described previously [9,10]: growth, harvesting and breakage of *M. extorquens* AM1 (N.C.I.M.B. 9133); purification of MDH; assay of MDH with the dye PES; and assay of MDH with cytochrome c_L using 2,6-

Abbreviations used: MDH, methanol dehydrogenase; PES, phenazine ethosulphate; PQQ, pyrroloquinoline quinone.

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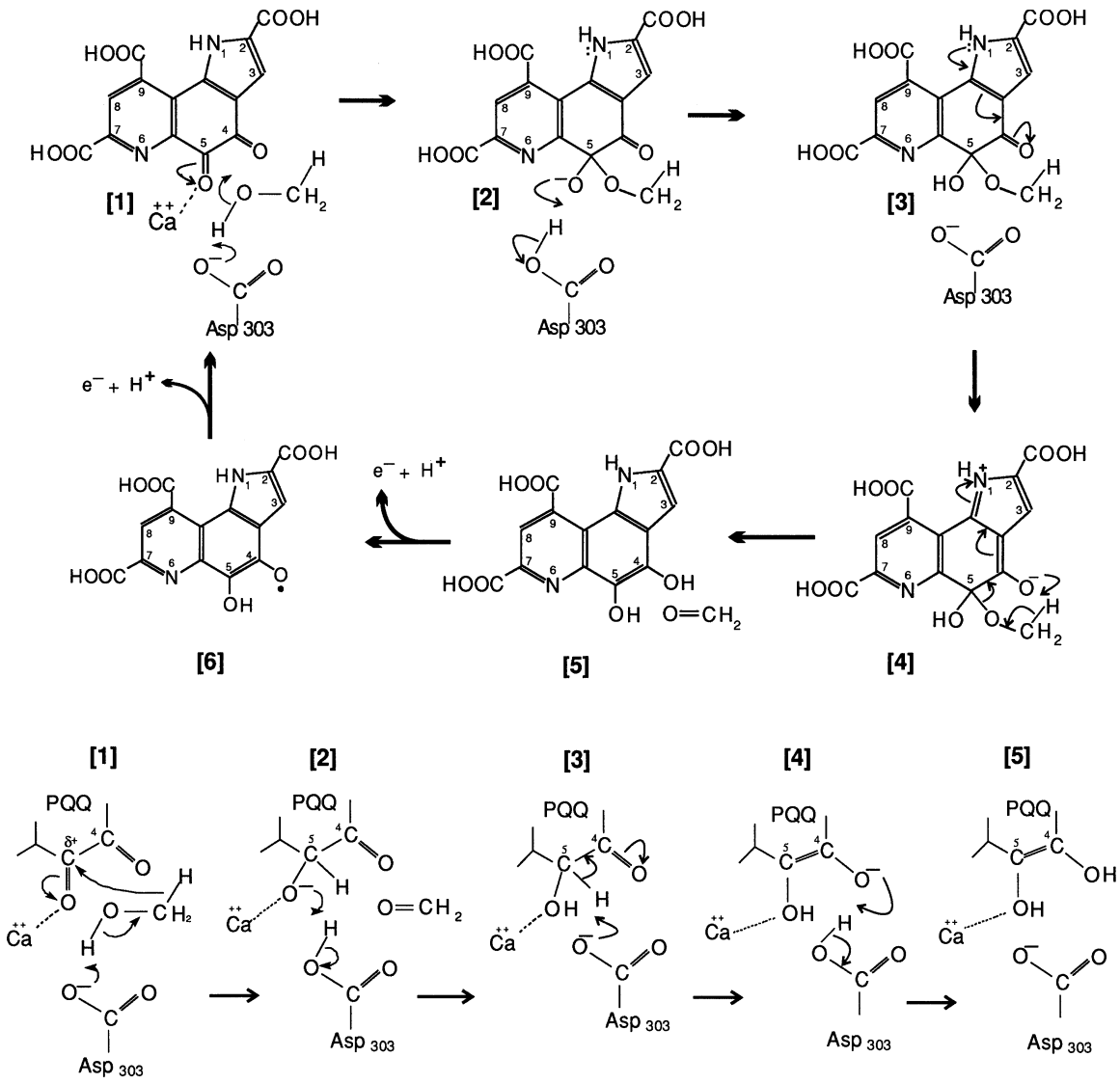


Scheme 1 Proposed mechanisms for MDH

Upper panel: mechanism involving formation of a hemiketal intermediate. It is suggested that the Ca^{2+} ion plays a catalytic role by acting as a Lewis acid, facilitating attack on the electrophilic C-5 of PQQ. The calcium ion is probably co-ordinated to PQQ throughout the reaction cycle. Proton abstraction by the base leads to an oxyanion form of the substrate which attacks the electrophilic C-5, giving the hemiketal intermediate [3] from which the methyl proton is abstracted. This mechanism is developed from that previously published [2,4] to include the possibility that this proton abstraction is facilitated by ionization of the C-5 carbonyl oxygen, which is made possible by the presence of the pyrrole nitrogen atom. The oxidative phase of the reaction cycle involves electron transfer to cytochrome c_L or PES. Lower panel: mechanism involving hydride transfer. In this case the initial proton abstraction is the same as above, but the electrophilic C-5 is involved directly in removal of the methyl hydrogen as a hydride. This mechanism is adapted from those published previously to emphasize the probable double involvement of the active-site base (Asp-303). The oxidative phase is the same as in the first mechanism.

dichlorophenol indophenol as terminal electron acceptor. Methods for growth of the mutant *mxaA* (previously called *moxA*), and for work on its MDH, were as described previously [8]. Curve fitting for kinetic analyses was done using the Enzfitter and Sigmaplot programs. Measurements of the prosthetic group, absorption spectra and CD spectra were as described in [11].

Deuteriated methanol (C^2H_3OH) was obtained from ICN Biomedicals Inc. Wurster's Blue was prepared from *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine hydrochloride as described previously [12].

MDH containing Sr^{2+} or Ba^{2+} was prepared by a method based on that described in [8]. MDH (6.7 nmol) was incubated in a 1 ml reaction mixture at 30 °C for 150 min with 10 mM Sr^{2+} or $BaCl_2$ in 20 mM Tris buffer (pH 9.0).

RESULTS AND DISCUSSION

Effect of substituting Ca^{2+} by Sr^{2+} or Ba^{2+} ions on the kinetics of methanol oxidation

The effect of methanol concentration on MDH activity was determined at pH 9.0 wth optimal NH_4Cl in the PES-linked assay system (Figure 2; Table 1). In all measurements of MDH activity there is a transient dye-linked (endogenous) activity in

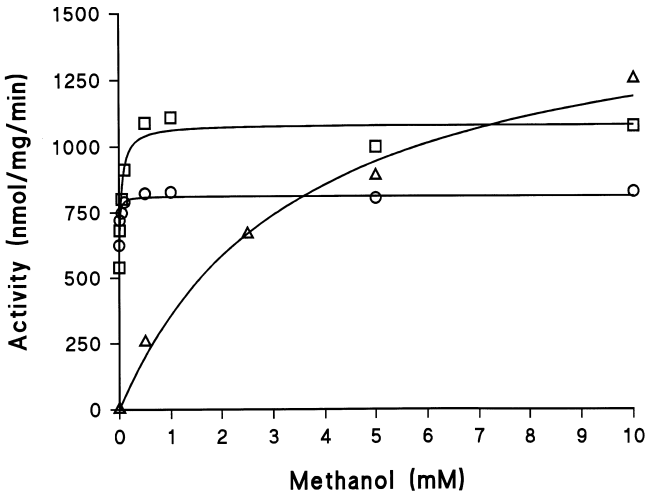


Figure 2 Effect of methanol concentration on the activity of MDHs containing Ca^{2+}, Sr^{2+} or Ba^{2+}

MDH (134 nM) containing Ca^{2+}, Sr^{2+} or Ba^{2+} was assayed using the dye-linked system at pH 9.0, with the optimum concentration of NH_4Cl (10 mM for Ca^{2+}-MDH and Sr^{2+}-MDH; 100 mM for Ba^{2+}-MDH). ○, Ca^{2+} enzyme; □, Sr^{2+} enzyme; △, Ba^{2+} enzyme. The curves are theoretical curves obtained using the equation $v = V_{max}(E+M)/(K_m+E+M)$, where E and M are the concentrations of endogenous substrate and methanol respectively.

the absence of added substrate, presumed to be due to unidentified bound substrate or to low concentrations of contaminating alcohols; this oxidation occurs by the same mechanism as is involved in methanol oxidation [1]. In Figure 2 it can be seen that the rates in the absence of added methanol catalysed by Sr^{2+}-MDH and Ca^{2+}-MDH were very high, but there was negligible endogenous activity catalysed by Ba^{2+}-MDH.

When endogenous substrate is present and there is a very high affinity for methanol, it is very difficult to obtain an accurate measure of the K_m value; previously published K_m values for the normal Ca^{2+}-MDH have all been lower than 20 μM [1]. In order to make comparisons of kinetic constants for the three enzymes it was therefore necessary to calculate approximate K_m values for Ca^{2+}-MDH and Sr^{2+}-MDH using the following equation (E and M are the concentrations of endogenous substrate and methanol respectively):

$$v = V_{max}(E+M)/(K_m+E+M)$$

This makes the assumption that the affinity for endogenous substrate and the V_{max} value are the same as measured with methanol; for the approximate calculations required here this is a reasonable assumption because many previous studies have shown that the K_m and V_{max} values for a wide range of smaller soluble alcohols are all very similar [1]. The results in Figure 2 indicate that the concentration of endogenous substrate in the reaction mixture was usually 10–20 μM. This suggests, as previously concluded [1], that the concentration of endogenous substrate is about 100 times that of the enzyme in the reaction mixture. The results in Table 1 show that the V_{max} values were higher when Sr^{2+} or Ba^{2+} replaced Ca^{2+} in MDH, the value with Ba^{2+}-MDH being twice that with Ca^{2+}-MDH. The most marked difference in the enzymes was the greatly increased K_m measured with Ba^{2+}-MDH (more than 1000-fold greater). It is presumably this greatly decreased affinity for substrate of Ba^{2+}-MDH that leads to its very low endogenous activity. It was confirmed that the altered apparent K_m value was not because of a change in affinity of Ba^{2+}-MDH for the electron acceptor (PES).

MDH is well known for its ability to oxidize formaldehyde, presumably using the gem-diol as substrate [1]. Ba^{2+}-MDH oxidized formaldehyde but its affinity was decreased about 1000-fold, as found for methanol and other primary alcohols (e.g. allyl alcohol). Alcohols having a low affinity for Ca^{2+}-MDH (e.g. butane 1,3-diol) were not oxidized at a measurable rate by Ba^{2+}-MDH.

Activation of MDH by NH_4Cl and glycine ethyl ester

Figure 3 and Table 1 show the effect of NH_4Cl concentration on the activity of the three enzymes, measured with saturating or near-saturating concentrations of methanol. The fitted curves in Figure 3 are for the following equation [where A is the concentration of activator (NH_4Cl)]:

$$v = V_{max}A/[K_A + A(1 + A/K_i)]$$

This suggests that there is a second ammonia-binding site on MDH, leading to inhibition at high ammonia concentrations. The concentration of ammonia required for half-maximal activity (K_A) increased when either Sr^{2+} or Ba^{2+} replaced Ca^{2+}, being 26 times greater for Ba^{2+}-MDH. Very similar results were obtained using the alternative activator, glycine ethyl ester (Table 1), although no inhibition by this activator was observed within the concentration range in which it could be used (0–100 mM).

Activation energies for methanol oxidation by Ca^{2+}-MDH, Sr^{2+}-MDH and Ba^{2+}-MDH

The activation energies for oxidation of methanol were lower for Sr^{2+}-MDH and Ba^{2+}-MDH than for Ca^{2+}-MDH, the lowest

Table 1 Kinetics and thermodynamics of methanol oxidation by MDH containing Ca^{2+}, Sr^{2+} or Ba^{2+}

The enzymes were prepared as described in the Experimental section and assayed in the PES-linked system at pH 9.0. The concentrations of NH_4Cl used for the determination of K_m values for methanol were the optimum concentrations for each enzyme, taking into account both activation and inhibition (see Figure 2). The concentration of methanol used for analysis of ammonia activation was either saturating or, with Ba^{2+}-MDH, sufficient to give more than 90% of V_{max}. These V_{max} values, and the K_A values for NH_4Cl, were calculated from the results presented in Figures 2 and 3. Activation energies were measured using the optimum concentrations of NH_4Cl and methanol (as described above). GEE, glycine ethyl ester.

			Activation/inhibition by ammonia		Activation by GEE	
Enzyme	V_{max} (nmol/min per mg)	K_m (methanol) (mM)	K_A (mM)	K_i (mM)	K_A (mM)	Activation energy (kJ/mol)
Ca^{2+}-MDH	810	~ 0.003	2	70	4.3	35.4
Sr^{2+}-MDH	1080	~ 0.022	7	72	7.6	28.2
Ba^{2+}-MDH	1610	3.5	33	> 500	37	14.0

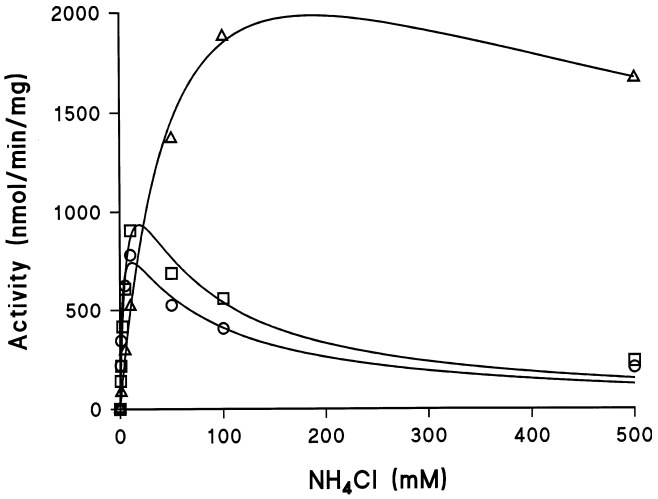


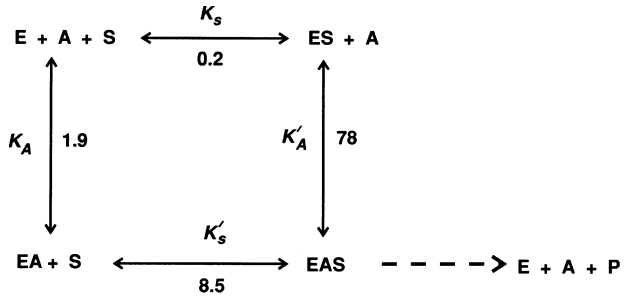
Figure 3 Effect of NH_4Cl concentration on the activity of MDHs containing Ca^{2+}, Sr^{2+} or Ba^{2+}

MDH (134 nM) containing Ca^{2+}, Sr^{2+} or Ba^{2+} was assayed using the dye-linked system at pH 9.0, with saturating or near-saturating methanol (10 mM for Ca^{2+}-MDH and Sr^{2+}-MDH; 50 mM for Ba^{2+}-MDH). ○, Ca^{2+} enzyme; □, Sr^{2+} enzyme; △, Ba^{2+} enzyme. The curves are theoretical curves obtained using the equation $v = (V_{max} A)/[K_A + A(1 + A/K_i)]$, where A is the concentration of activator (NH_4Cl).

value being recorded for the Ba^{2+} enzyme; this is reflected in the relative V_{max} values for the three enzymes (Table 1). The values for the Ca^{2+}- and Sr^{2+}-MDHs were in the same range as those recorded for the enzymes from *Paracoccus denitrificans* [13]; because there was little change in the K_m for methanol with that enzyme, it was concluded that the decrease in activation energy was due to an increased stability of the transition state. In the present work, the decrease in activation energy (21.4 kJ/mol) of the Ba^{2+} compared with the Ca^{2+} enzyme occurred concomitantly with more than a 1000-fold decrease in affinity for methanol. This gives a difference in the free energy for substrate binding for the two enzymes of 17.5 kJ/mol. This is sufficiently similar to the difference in activation energies to suggest that there is little difference in the transition state stability and that the differences in activation energies are probably due to an altered conformation of the active site affecting the intitial binding of substrate (see [14] for a discussion of the relationship between a change of activation energy and a change in substrate binding).

Effect of activator (ammonia) on the affinity for methanol of Ba^{2+}-MDH

Kinetic analysis of the activation of Ca^{2+}-MDH by ammonia is usually difficult because of the latter's very high affinity for methanol and its high rate of endogenous dye reduction [15,16]. The low affinity of Ba^{2+}-MDH for methanol, however, facilitated an investigation of the effect of ammonia on the binding of substrate to enzyme. This confirmed previous assumptions that the main effect of the activator is to increase the V_{max} [15], but it also demonstrated that the apparent affinity for methanol decreased with increasing concentrations of activator. The steady-state kinetic data are all consistent with Scheme 2, which shows that, although methanol and ammonia are essential for activity, each binds more strongly to the enzyme in the absence of the other. This suggests that the binding site for ammonia is likely to be independent of, but very close to, that for methanol.



Scheme 2 Kinetic scheme for the activation of Ba^{2+}-MDH by ammonia

In this scheme the combination of enzyme (E) with substrate (S) is not independent of its combination with activator (A), so E and EA have different affinities for the substrate. The equation for the reaction [23] is as follows: $v = V_{max}/(1 + K'_s/S)[1 + K_A/A(1 + S/K_s)/(1 + S/K'_s)]$. When the measured reaction rates were fitted to this equation, the values of the constants were shown to be as follows: $K_A = 1.9$ mM; $K'_A = 78$ mM; $K_s = 0.2$ mM; $K_s' = 8.5$ mM.

For formation of product it is essential to have both substrate and ammonia bound at the same time.

Deuterium isotope effect

In the dye-linked assay system, ammonia increased the V_{max} for all three enzymes with both protiated methanol (Figure 3) and deuteriated methanol (Figure 4). Because the K_A values for ammonia activation of Ca^{2+}-MDH were different with the two substrates, the isotope effect was dependent on the concentration

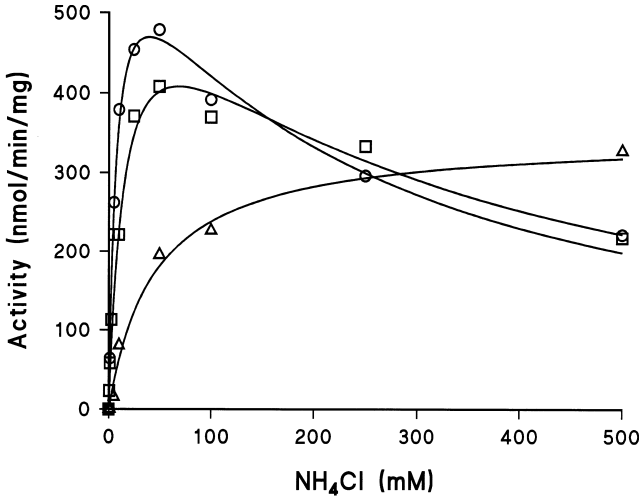


Figure 4 Effect of NH_4Cl concentration on the activity of MDHs containing Ca^{2+}, Sr^{2+} or Ba^{2+} when assayed with deuteriated methanol

MDH (134 nM) containing Ca^{2+}, Sr^{2+} or Ba^{2+} was assayed using the dye-linked system at pH 9.0, with saturating or near-saturating deuteriated methanol (10 mM for Ca^{2+}-MDH and Sr^{2+}-MDH; 50 mM for Ba^{2+}-MDH). ○, Ca^{2+} enzyme; □, Sr^{2+} enzyme; △, Ba^{2+} enzyme. The curves are theoretical curves obtained using the equation $v = V_{max} A/[K_A + A(1 + A/K_i)]$, where A is the concentration of activator (NH_4Cl).

Table 2 Effect of ammonia on the deuterium isotope effect in MDHs containing Ca^{2+}, Sr^{2+} or Ba^{2+}

The enzymes were assayed in the PES-linked system at pH 9.0. The K_m values for methanol were the same with protiated or deuteriated substrate. The concentration of methanol used for determination of the K_A for ammonia was saturating for Ca^{2+}-MDH and Sr^{2+}-MDH (10 mM), and sufficient to give 90% of V_{max} (50 mM) with Ba^{2+}-MDH. The values were calculated from the data presented in Figures 3 and 4. It should be noted that the differences in V_{max} values here compared with those in Table 1 are because in Table 1 the concentrations of ammonia were optimal (giving maximum measurable rates), whereas the values here were calculated from experiments in which the activator concentrations were varied.

	K_A (mM) for NH_4Cl		V_{max} (nmol/min per mg)		
Enzyme	CH_3OH	C^2H_3OH	CH_3OH	C^2H_3OH	Isotope effect at maximum rate
Ca^{2+}-MDH	2	7	980	636	1.54
Sr^{2+}-MDH	6	15	1591	593	2.68
Ba^{2+}-MDH	52	45	3096	347	8.92

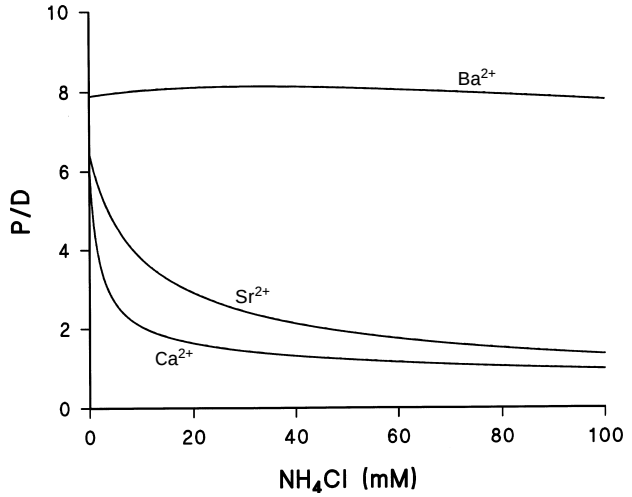


Figure 5 Effect of ammonia concentration on the deuterium isotope effect

The effect of ammonia was measured in the dye-linked assay system. The isotope effect (P/D) was calculated by subtraction of the best-fit curves given in Figures 3 and 4. The extrapolated values for zero ammonia were 6.1 for Ca^{2+}-MDH, 6.5 for Sr^{2+}-MDH and 7.9 for Ba^{2+}-MDH. In 500 mM NH_4Cl the isotope effect decreased to about 1.0 for the Ca^{2+} and Sr^{2+} enzymes, and to 5.7 for the Ba^{2+} enzyme.

of ammonia (Table 2; Figure 5). For example, at a low concentration of ammonia (1 mM) the isotope effect was 3.7, compared with 1.54 calculated from the V_{max} values with saturating substrate and activator concentrations. These results explain a previous observation, made with the enzyme from *Hyphomicrobium*, that the deuterium isotope effect was higher at lower ammonia concentrations [15]. When Sr^{2+} or Ba^{2+} replaced Ca^{2+}, the V_{max} with protiated methanol increased but that with deuteriated methanol decreased; as a result, the maximum measured isotope effect was greater with Sr^{2+}-MDH and even greater with Ba^{2+}-MDH.

With Ba^{2+}-MDH, the K_A value for ammonia was 26 times greater than with Ca^{2+}-MDH and was not markedly different when deuteriated methanol was used (Table 2). The isotope effect was, therefore, almost independent of the ammonia concentration over the range that affects the other two enzymes, although at high concentrations the isotope effect decreased (to about 5 at 500 mM NH_4Cl). Figure 5 shows that the deuterium isotope effect increases to a maximum of 6.1–7.9 when the curves are extrapolated back to zero ammonia concentration.

The steps in the mechanism that are affected by the change from protiated substrate to deuteriated substrate must be those involving the removal of the methyl hydrogen, as shown in Scheme 1. These are the conversion of **4** into **5** in the upper panel of Scheme 1, and the conversion of **1** into **2** in the lower panel. The results described above exclude the possibility that ammonia activates MDH by encouraging the initial binding of substrate, or by affecting the formation of any initial PQQ–methanol adduct, or by stimulating the re-oxidation of reduced PQQ; they confirm the previous suggestion [15] that ammonia activates the reduction of PQQ by the substrate.

A pronounced deuterium isotope effect was also measured when cytochrome c_L was used as electron acceptor; at 10 mM NH_4Cl the isotope effects in the cytochrome-linked assay were 4.3, 5.8 and 7.8 respectively for the enzymes containing Ca^{2+}, Sr^{2+} and Ba^{2+}. Because reduction of cytochrome is inhibited by high ionic strength (50% at $I = 0.02$) [17], it was not possible to do a full analysis of the effect of ammonia on this reaction.

Absorption and CD spectra of Ca^{2+}-MDH and Ba^{2+}-MDH

The absorption spectra of the enzymes from mutant *mxaA*, produced by incubation with Ca^{2+} or Ba^{2+} in the presence of added methanol, were typical of spectra of the reduced MDH. The enzyme from wild-type bacteria is usually in the semiquinone form, but that produced from the mutant is always oxidized and becomes reduced immediately by endogenous or added substrate after reconstitution of active enzyme with Ca^{2+} or Ba^{2+} [8].

The CD spectra of the Ca^{2+} and Ba^{2+} enzymes showed characteristic protein spectra between 185 and 240 nm. Calculations for secondary structure using the database matrix of Compton and Johnson [18] indicated that the enzyme is 50% β-sheet, 5–10% α-helix, 10% β-turn and 30–35% random coil; these values correlate well with those calculated from the X-ray structure of the enzyme from *Methylobacterium extorquens* (46% β-sheet, 11% α-helix, 4% β-turn and 39% random coil) [5]. The 240–300 nm region due to the aromatic residues in the protein, and the region of the prosthetic group (300–400 nm), are similar in the Ca^{2+} and Ba^{2+} enzymes, indicating that there is no major difference in the conformation of the enzyme, and in particular the configuration of PQQ in the active site appears to be similar in the two enzymes.

Production of an oxidized form of Ba^{2+}-MDH with Wurster's Blue

It is extremely difficult to investigate the mechanism of MDH by studying its absorption spectra because the enzyme is always isolated in the semiquinone form which is unable to react with substrate unless it is first oxidized [15,19,20]. The semiquinone form can be oxidized with Wurster's Blue, but this is rapidly reduced to the quinol by the endogenous substrate. When oxidized, the enzyme rapidly becomes inactivated unless it is protected by addition of the competitive inhibitor cyanide, which is assumed to form an adduct at the C-5 position of PQQ [21]. The availability of Ba^{2+}-MDH, in which the affinity for the substrate is very low, provided an opportunity to prepare the oxidized form of the enzyme. Figure 6 shows the spectrum of Ba^{2+}-MDH after oxidation at 4 °C with a 1.5-fold excess of Wurster's Blue at pH 9. The oxidation state of the enzyme was confirmed by demonstrating that its isolated prosthetic group

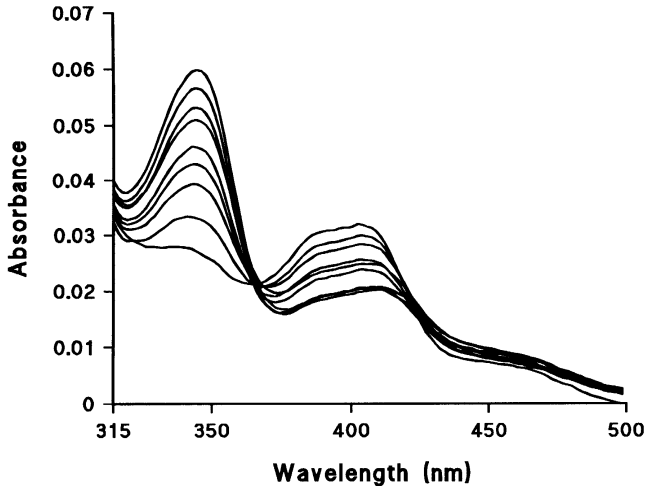


Figure 6 Absorption spectra of Ba^{2+}-MDH during reduction of the oxidized enzyme by endogenous substrate

Ba^{2+}-MDH (6.7 nM in 20 mM Tris/HCl, pH 9.0) was incubated with a slight excess of Wurster's Blue (20 nM) for 1 min and separated from the reaction mixture by rapid gel filtration in 5 mM phosphate buffer (pH 7.0) at 2 °C; spectra were then recorded at 4 °C. The time between injection and recording the first spectrum was less than 45 s, and each spectrum took 25 s to record; spectra were run at 0, 10, 20, 30, 40, 50, 70 and 90 min. The reduced enzymes have the higher absorption at 345 nm.

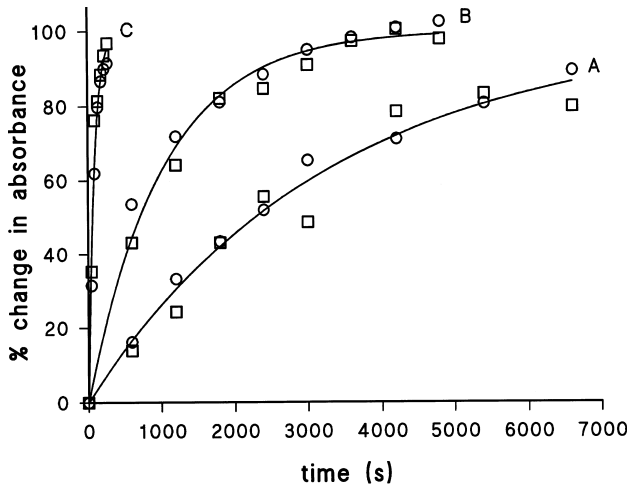


Figure 7 Reduction of oxidized Ba^{2+}-MDH

This Figure is based on experiments of the sort described in Figure 6. Points were taken from Figure 6 (and from similar experiments) and fitted to the first-order rate equation. Curve A, reduction by endogenous substrate in the absence of added activator (first-order rate constant 3.04×10^{-4} s^{-1}); curve B, reduction by added methanol in the absence of added activator (rate constant 9.77×10^{-4} s^{-1}); curve C, reduction by endogenous substrate in the presence of added activator (rate constant 113.8×10^{-4} s^{-1}). □, Change in absorbance measured at 345 nm; ○, change measured at 405 nm.

was predominantly (87%) in the oxidized (quinone) form. This is the first published spectrum of oxidized MDH in the absence of adducts. The only published spectrum of oxidized MDH was of a form produced by oxidation in the presence of ammonia and the absence of substrate; this was of transient duration only, 'changing very quickly to a structureless spectrum with concomitant loss of activity' [21].

After removal of the Wurster's Blue by rapid gel filtration, Ba^{2+}-MDH was slowly reduced (at 4 °C, pH 7.0) by endogenous substrate even in the absence of ammonia as activator (Figure 6). Addition of methanol to the oxidized enzyme (in the absence of ammonia at pH 7) increased the rate of reduction about 3.5-fold, and ammonia increased the rate of reduction by endogenous substrate about 33-fold. In the presence of methanol plus ammonia the reduction of MDH was too fast to measure. In all these experiments an isosbestic point was observed, indicating that only two spectrally observable species were present; i.e. the initial oxidized form and the final reduced form of the enzyme. If the spectra in Figure 6 represent only these two species then the rate of change at the two wavelengths (345 nm and 405 nm) should be directly proportional, and a single first-order rate equation should describe the reaction at both wavelengths. Figure 7 shows that this was observed for all three reactions.

It has previously been suggested that transient spectral changes seen during rapid reduction of MDH (after oxidation with Wurster's Blue) were due to formation of a covalent enzyme intermediate (a hemiketal adduct of oxidized PQQ and methanol); this spectrum was similar to that of the oxidized enzyme covalently modified with cyclopropanol [15,22]. Our observations of the spectral changes on reduction of Ba^{2+}-MDH do not rule out the possibility of covalent methanol or ammonia adducts. However, if such an adducts are intermediates in the mechanism, then their spectra must be similar to those of the oxidized or reduced forms, or the relative rates of their formation and conversion into product must be sufficiently similar for there to be no observable accumulation during the reaction process.

Conclusion

This paper provides the first description of an active enzyme containing Ba^{2+} as an essential component of the active site. All the results presented here are consistent with the conclusion that all features of the mechanism of the novel dehydrogenase containing Ba^{2+} are essentially the same as for that containing Ca^{2+}. It has been demonstrated previously that the spectrum of isolated PQQ is different from that of PQQ bound in the active site of MDH, and it has been concluded that this is because of specific interactions between Ca^{2+} and PQQ [5,8]. This, together with the observation that the absorption and CD spectra of Ca^{2+}-MDH and Ba^{2+}-MDH are very similar, indicates that there are no major differences between these enzymes in the interactions between PQQ and the metal ion in the active site. If, as proposed previously [4], the Ca^{2+} ion acts as a Lewis acid, contributing to the mechanism by its interaction with the C-5 carbonyl oxygen (Figure 1 and Scheme 1), then it might be expected that replacement with Ba^{2+}, a weaker Lewis acid, should decrease the activity of the enzyme; the decrease in the activation energy for Ba^{2+}-MDH was therefore unexpected. The most obvious reasons for such a decrease are that the energy of the transition state is decreased (less likely), or that the free energy change associated with substrate binding is diminished. The 1200-fold decrease in affinity for methanol of Ba^{2+}-MDH corresponds to a decrease in free energy of substrate binding (17.5 kJ/mol), and it is probably this change (predominantly) that leads to the large measured decrease in activation energy (21 kJ/mol). It is possible that the metal is involved directly in co-ordinating the oxygen of the alcohol substrate, but it seems unlikely that the change in co-ordination from Ca^{2+} to Ba^{2+} should lead to such a large change in binding energy. It is more likely that replacement of Ca^{2+} with Ba^{2+} leads to some disruption of the active-site region because of the greater size of the latter. This is indicated in Figure 1, in which the large size of the Ba^{2+} ion is clearly not consistent with retention of exactly the same conformation of all the active-site

groups in relation to each other. It is this slight change in the conformation of the active site that presumably gives rise to the decreased affinity of Ba^{2+}-MDH for its substrate and activator, which in turn manifests itself as a lower activation energy for the reaction catalysed by Ba^{2+}-MDH. Further understanding of this enzyme awaits identification of the location of the substrate and activator in the binding site, and determination of the structure of the Ba^{2+}-containing enzyme.

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