Reconstitution of the quinoprotein methanol dehydrogenase from inactive Ca^{2+}-free enzyme with Ca^{2+}, Sr^{2+} or Ba^{2+}

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The reconstitution of active holoenzyme containing calcium from inactive calcium-free methanol dehydrogenase, isolated from a *moxA* mutant of *Methylobacterium extorquens*, has a pH optimum of about pH 10, with a well defined p*K* for the process at pH 9.3. Two Ca^{2+} ions were irreversibly incorporated per $\alpha_2\beta_2$ tetramer. Calcium could be replaced in the incorporation process by strontium or barium, the affinities for these ions being similar to that for Ca^{2+}. Arrhenius plots for measurement of the activation energy of reconstitution were biphasic; the lower activation energy was typical of most biological processes, while the higher activation energy was at least three times greater, implying the involvement of a large conformational change during incorporation of the cations. The activation energy for incorporation of Ba^{2+} was considerably higher than that for incorporation of Ca^{2+}. The novel disulphide bridge that is at the active site of the enzyme was not involved in the incorporation process. Studies of the time courses for incorporation of $^{45}Ca^{2+}$, production of active enzyme and changes in absorption spectra failed to show any intermediates in the incorporation process.

INTRODUCTION

Methanol dehydrogenase (MDH) is a bacterial periplasmic quinoprotein; it has pyrroloquinoline quinone (PQQ) as its prosthetic group, requires Ca^{2+} for activity and uses cytochrome c_L as its electron acceptor [1–3]. 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 PQQ in the active site is held in place by a co-planar tryptophan residue and by a novel disulphide ring formed between adjacent cysteines. The active site also contains a Ca^{2+} ion which is co-ordinated to residues on the protein and also to the PQQ by way of the 7-carboxy group, the N-6 in the ring and the C-5 carbonyl oxygen. It has been suggested that, besides holding the PQQ in position, the Ca^{2+} ion might play a role as a Lewis acid, facilitating attack by the substrate on the electrophilic C-5 of PQQ [4]. It is not possible to remove Ca^{2+} from active enzyme.

The requirement for Ca^{2+} in the MDH from *M. extorquens* was first shown by using the *mxaA* mutant; *mxaA* is separate from the structural genes for MDH, and its product is involved in the processing of MDH in the periplasm [6]. MDH prepared from this mutant lacks Ca^{2+} and so is inactive catalytically, but active enzyme containing Ca^{2+} could be prepared from it, after which the Ca^{2+} was very tightly bound and could not be removed, even by prolonged incubation with high concentrations of chelating agent [7].

The present paper describes the use of apoenzyme, produced from the *mxaA* mutant, to characterize the process of reconstitution of active enzyme with Ca^{2+}, and to define appropriate conditions for the production of a novel form of the enzyme in which Ba^{2+} replaces Ca^{2+} in the active site.

EXPERIMENTAL

The following methods were as described previously [8,9]: growth, harvesting and breakage of *M. extorquens* AM1 (N.C.I.M.B. 9133); purification of MDH; assay of MDH with the dye phenazine ethosulphate; and assay of MDH with cytochrome c_L using 2,6-dichlorophenol indophenol as the terminal electron acceptor. Methods for growth of the mutant *mxaA* (previously called *moxA*), and for work on its MDH, were as described previously [7]. Curve fitting for kinetic analyses was done using the Enzfitter or Sigmaplot programs.

Measurements of the prosthetic group, absorption spectra and reduction of MDH with dithiothreitol and subsequent carboxymethylation with iodoacetate were as described previously [10]. Radioactive $^{45}Ca^{2+}$ (22.3 mCi/mg $CaCl_2$ in aqueous solution) was obtained from Amersham International, and radioactivity was measured in a Beckman LS-6500 scintillation counter.

RESULTS AND DISCUSSION

Factors affecting rates of reconstitution of active holoenzyme from inactive Ca^{2+}-free MDH prepared from the *mxaA* mutant

The pH optimum for the production of active holo-MDH, by incubating apoenzyme with Ca^{2+}, was about 10.5, the best-fit curve being for a single p*K* at pH 9.3 (Figure 1). This suggests that a single arginine, lysine or tyrosine residue plays some particular role (not necessarily direct) in the incorporation process. Although the pH optimum was clearly well above pH 9, this pH was used for all further experiments because it provided a convenient time-scale and was the best pH for the subsequent assay of enzyme activity.

Figure 2 shows that the rate of incorporation of Ca^{2+} into active enzyme was approximately linear with respect to calcium concentration up to at least 20 mM when measured at pH 9 at 25 °C. Active enzyme was not produced when Ca^{2+} was replaced with the following ions (provided as their chloride salts at 20 mM): Li^+, K^+, Na^+, Cs^+, Mg^{2+} and La^{3+}. Active enzyme was produced, however, after incubation with the chloride salts (at 10 mM) of Sr^{2+} or Ba^{2+}; the relative initial rates were: Ca^{2+}, 100 %; Sr^{2+}, 94 %; Ba^{2+}, 102 %. The relative rates of methanol oxidation in the standard assay by the enzymes containing Ca^{2+}, Sr^{2+} and Ba^{2+} were 100 %, 120 % and 40 % respectively. Almost identical curves were obtained for incorporation of Sr^{2+} or Ba^{2+} to give active enzyme. Whenever reconstitution into active

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

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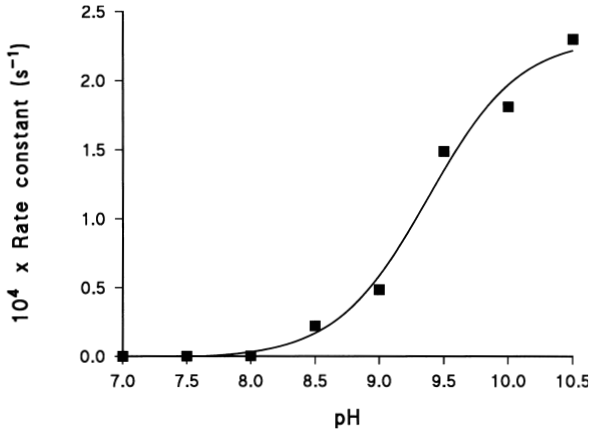

Figure 1 Effect of pH on the rate of formation of active enzyme

MDH (6.7 μM), prepared from the *mxaA* mutant, was incubated at 25 °C in 20 mM buffer containing 10 mM $CaCl_2$, and samples were removed and assayed in the dye-linked assay system. The incubation buffers were: Hepes (pH 7.0 and 7.5), Tris/HCl (pH 8.0, 8.5, 9.0 and 9.5), and 1-amino 2-methyl-1-propanol/HCl (pH 10.0 and 10.5). The apparent first-order rate constants for the formation of active holoenzyme were plotted against pH. The curve is the best-fit curve for a p*K* value of 9.3.

enzyme occurred, the process was irreversible; subsequent gel filtration or incubation with 100 mM EGTA for 3 days led to no loss of activity. The result with Sr^{2+} is consistent with previous observations that growth of some methylotrophs in the presence of this ion leads to production of active MDH containing strontium [11,12]. After attempted reconstitution with K^+, Cs^+, Mg^{2+} and La^{3+}, further incubation with Ca^{2+} led to the formation of active enzyme, indicating that these other ions had not been incorporated in the Ca^{2+} site to give inactive enzyme. Surprisingly, it was found that Na^+ ions (but not K^+ ions) were strong inhibitors of incorporation of Ca^{2+}, 50% inhibition occurring with 20 mM NaCl. Because of this, it is important that studies of the effect of pH on the incorporation process avoid the use of buffers containing sodium ions.

Incubation of $^{45}Ca^{2+}$ (10 mM $CaCl_2$) with MDH purified from the *mxaA* mutant for 150 min at pH 9.0 led to the incorporation of 2.03 mol of Ca^{2+}/mol of $\alpha_2\beta_2$ tetrameric MDH. That this represented saturation of all sites on the enzyme was shown by reconstitution with non-radioactive Ca^{2+} followed by incubation for a further 2 h with $^{45}Ca^{2+}$, during which there was no incorporation of radioactivity. This is consistent with the observation that both active sites in the X-ray structure of the $\alpha_2\beta_2$ tetrameric MDH were shown to contain a Ca^{2+} ion [5,13]. The competition experiments in Figure 3 show that the affinity of MDH for Sr^{2+} and Ba^{2+} in the reconstitution system is similar to its affinity for Ca^{2+}. Subsequent incubation with 10 mM $^{45}Ca^{2+}$ had no effect on the activity of the enzymes produced with Sr^{2+} or Ba^{2+}, and there was no incorporation of $^{45}Ca^{2+}$.

Time course of Ca^{2+} incorporation concomitant with changes in activity and in absorption spectra

It was confirmed that the absorption spectrum of the *mxaA* MDH was markedly different from that of the enzyme from wild-type bacteria [7]. Incubation of *mxaA* MDH with Ca^{2+} in the presence of methanol led to the production of the active reduced form of MDH, the increase in absorbance at 345 nm providing a measure of the production of enzyme in which the PQQ is in the active configuration (Figure 4).

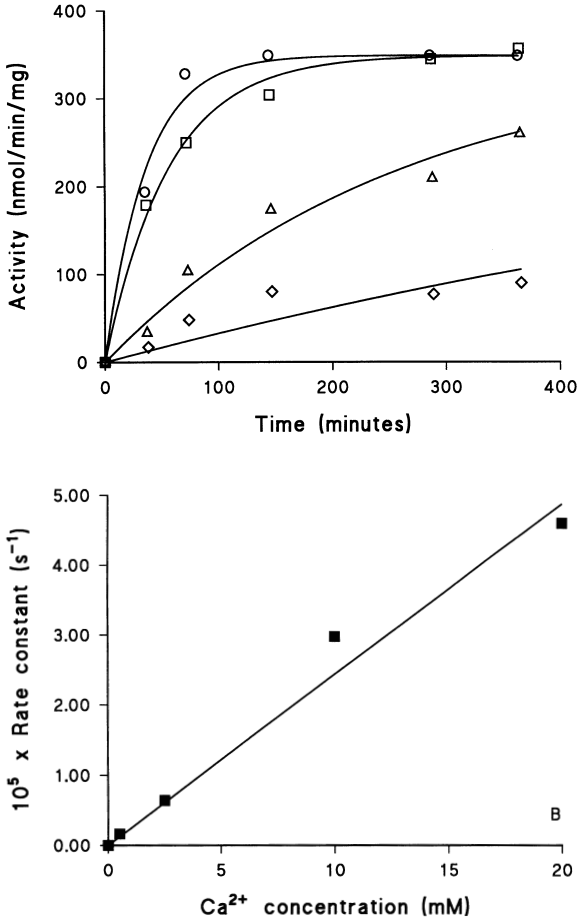

Figure 2 Effect of Ca^{2+} concentration on the rate of formation of active enzyme

MDH (6.7 μM), prepared from the *mxaA* mutant, was incubated at 25 °C in 20 mM Tris/HCl (pH 9.0) containing various concentrations of $CaCl_2$, and samples were removed and assayed in the dye-linked assay system. ◇, 2.5 mM $CaCl_2$; △, 5 mM $CaCl_2$; □, 10 mM $CaCl_2$; ○, 20 mM $CaCl_2$. The curves for the time course of the incorporation part of the reaction (top) were the best-fit curves for first-order rate reactions.

Figure 5 shows that the changes with time of many different parameters during incorporation of Ca^{2+} could be superimposed upon each other; these include the change in absorbance at 345 nm due to the change in PQQ, changes in activity and changes in the amount of bound Ca^{2+} as measured by incorporation of $^{45}Ca^{2+}$. Very similar results to those shown in Figure 5 were obtained when Ba^{2+} ions replaced Ca^{2+} in the reconstitution reaction mixtures.

These results show that it was not possible to produce active enzyme without first binding Ca^{2+}, or to produce active enzyme without a change from the original conformation of the mutant MDH. This experiment failed to determine whether Ca^{2+} binds before or after the conformational change.

Activation energies for reconstitution with Ca^{2+}, Sr^{2+} and Ba^{2+}

The Arrhenius plots for the reconstitution of active MDH at pH 9.0 are shown in Figure 6. The curves all show a break at about 15 °C, indicating that there is a change in the rate-limiting step for incorporation of metal ion below this temperature. The activation energies are similar for incorporation of Ca^{2+} and

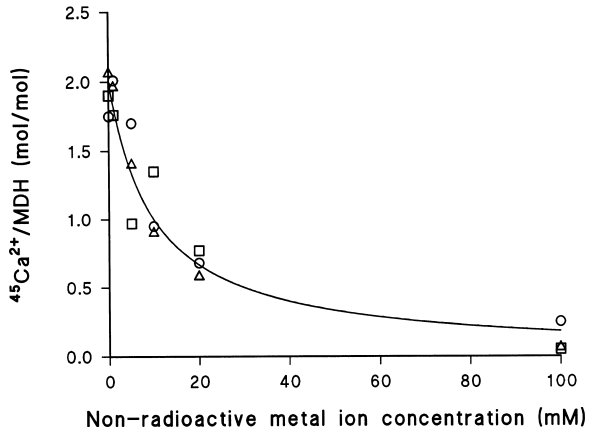

Figure 3 Incorporation of $^{45}Ca^{2+}$ into MDH in the presence of Ca^{2+}, Sr^{2+} or Ba^{2+} ions

MDH (6.7 μM), prepared from the *mxaA* mutant, was incubated at 25 °C for 150 min in 20 mM Tris/HCl (pH 9.0) containing 10 mM $^{45}CaCl_2$ and various concentrations of unlabelled $CaCl_2$ (○), $SrCl_2$ (□) or $BaCl_2$ (△). The samples were then passed down a fast-desalting column (Pharmacia G25 10/10) equilibrated in 20 mM Tris/HCl (pH 9.0) and assayed for radioactivity in a scintillation counter.

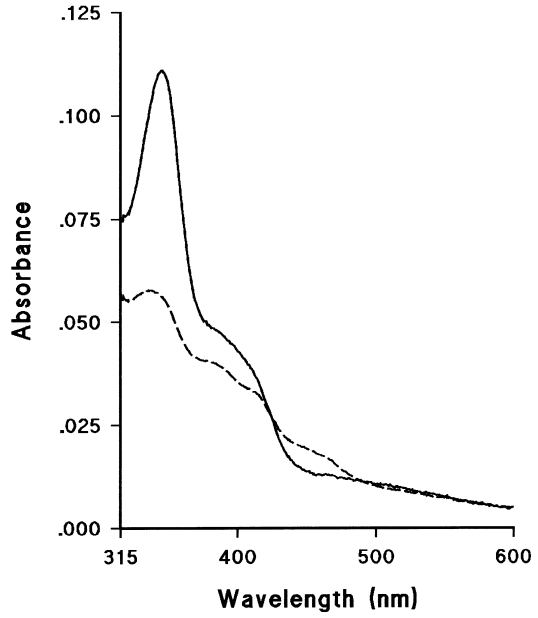

Figure 4 Absorption spectra of MDH

MDH (6.7 μM), prepared from the *mxaA* mutant, was incubated at 25 °C for 150 min in 20 mM Tris/HCl (pH 9.0) containing 10 mM $CaCl_2$ (solid line) or no metal salt (broken line).

Sr^{2+}, but are considerably greater for incorporation of the much larger Ba^{2+} ion (Table 1). The activation energies at the higher temperatures are not very different from those for most biological binding or catalytic processes (25–50 kJ·mol^{-1}). By contrast, below 15 °C the activation energies are all at least three times higher and are more similar to those characteristic of large changes in protein conformation such as occur during protein denaturation (greater than 100 kJ·mol^{-1}).

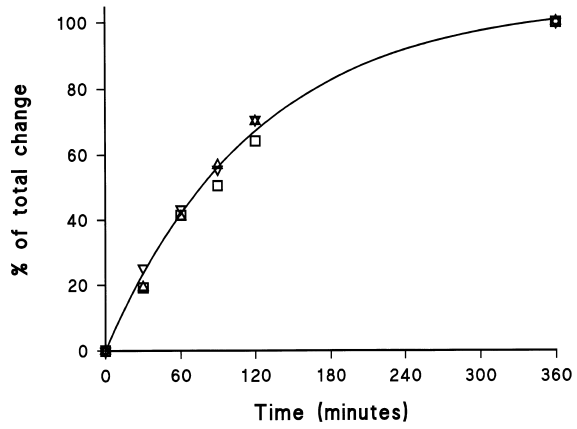

Figure 5 Relative rates of change in activity, absorption spectrum and ^{45}Ca incorporation during reconstitution of active MDH

MDH (6.7 μM), prepared from the *mxaA* mutant, was incubated at 25 °C in 20 mM Tris/HCl (pH 9.0) containing 2.5 mM $^{45}CaCl_2$. Samples were removed and assayed for changes in dye-linked activity (△), incorporation of ^{45}Ca (▽) and absorbance at 345 nm (□).

The novel disulphide bridge is not directly involved in Ca^{2+} insertion

It has previously been shown that the active site in the α subunit of MDH contains a novel eight-membered ring structure composed of two adjacent cysteine residues (positions 103 and 104) joined by a disulphide bridge, the sulphur atoms of which are within atomic distance of the PQQ [5]. When this bridge is reduced the enzyme loses activity, but when the thiol groups produced by reduction are carboxymethylated then this activity returns [10]. The possibility that this ring structure is essential for the incorporation of Ca^{2+} into the *mxaA* MDH was ruled out by incubating dithiothreitol-reduced MDH (3.5 nmol) at pH 9 in 10 mM Ca^{2+} for 16 h. This produced inactive enzyme with the same absorption spectrum as normal reduced MDH but having four free thiol groups per $\alpha_2\beta_2$ tetramer. Oxidation in air, or carboxymethylation, led to the formation of active enzyme, as shown for the enzyme from wild-type bacteria. When the inactive Ca^{2+}-free MDH from *mxaA* was carboxymethylated (after initial reduction) and incubated with Ca^{2+} in the usual way, this ion was incorporated at the usual rate to produce active carboxymethylated enzyme having the same absorption spectrum as MDH from wild-type bacteria.

Conclusion

This paper describes conditions for the production of active MDH containing Ca^{2+}, Sr^{2+} or Ba^{2+} from the inactive Ca^{2+}-free enzyme purified from the *mxaA* mutant. For the most rapid incorporation, the use of aminomethylpropanol buffer at pH 10 is recommended (at least 90% of maximum activity obtained within 15 min). When lower rates of incorporation are required, the most appropriate system involves the use of Tris/HCl at pH 9.0; in all cases the presence of sodium ions should be avoided. We also report, for the first time, the conditions required for the production of an active enzyme containing Ba^{2+} at its active site. All the evidence is consistent with the conclusion that the Ba^{2+} ion occupies the same site on MDH as does Ca^{2+} in the MDH from wild-type bacteria, and that incorporation of these ions involves a conformational change in the enzyme. In the present work the enzyme had been isolated from a *mxaA* mutant,

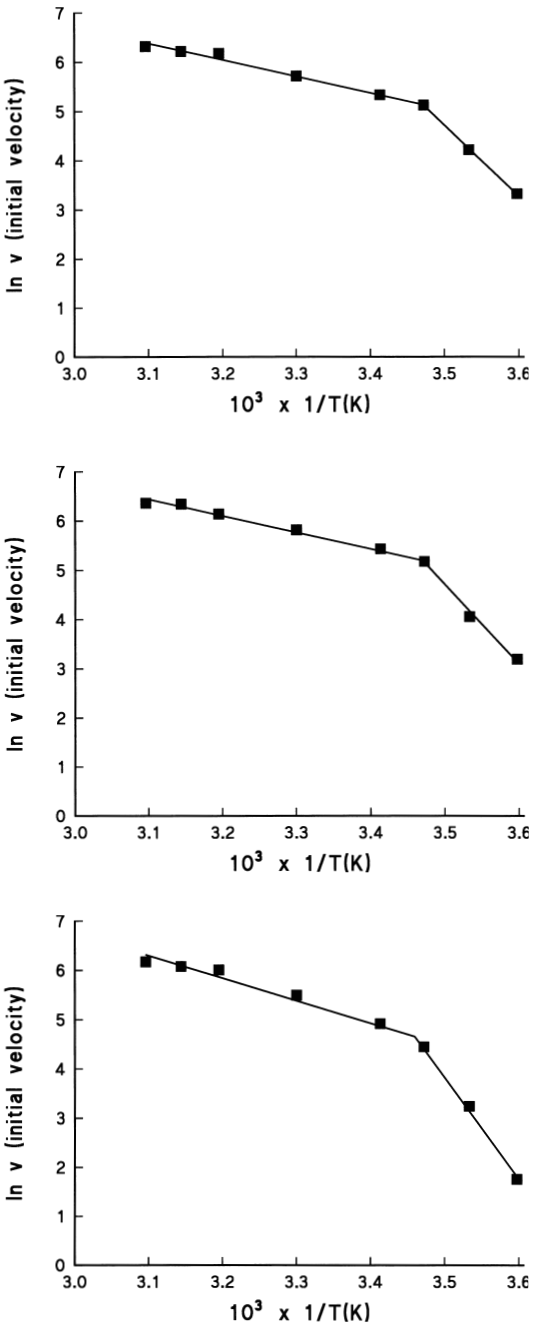

Figure 6 Arrhenius plots of the rate of incorporation of Ca^{2+}, Sr^{2+} and Ba^{2+} into MDH

MDH (6.7 μM), prepared from the *mxaA* mutant, was incubated in 20 mM Tris/HCl (pH 9.0) containing Ca^{2+}, Sr^{2+} or Ba^{2+} (10 mM); samples were removed and assayed in the dye-linked activity assay system. Initial rates were calculated and plotted against temperature: top, Ca^{2+}-MDH; middle, Sr^{2+}-MDH; bottom, Ba^{2+}-MDH.

and the formation of holoenzyme required a high pH and high concentrations of bivalent cations. It remains unclear how the process is facilitated in the periplasm, where the concentration of cations will be low and the pH will usually be about 7.0.

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Table 1 Activation energy values for the reconstitution of active MDH from the *mxaA* mutant using bivalent cations

The values shown were calculated from the data in Figure 6.

	Activation energy ($kJ \cdot mol^{-1}$)	
Metal ion	5–15 °C	15–50 °C
Ca^{2+}	120.5	27.8
Sr^{2+}	134.7	27.9
Ba^{2+}	172.0	37.9

It has been shown previously that at least three periplasmic proteins (MxaA, MxaK and MxaL) are required for the insertion of Ca^{2+} into MDH, and that mutation of their genes leads to similar phenotypes and to the production of inactive MDH lacking Ca^{2+} [7]. It has been suggested that the function of one of these proteins might be to produce a high local concentration of Ca^{2+} in the periplasm [7], and a recent analysis of the primary sequence of MxaA suggests that this periplasmic protein might have a Ca^{2+}-binding function [14]. Sequence analysis indicated that MxaK is probably a cytoplasmic protein and that MxaL might be located in the membrane. In the same study at least two other genes were shown to be closely linked to *mxaA*, *mxaK* and *mxaL*, and so were suggested to encode further proteins involved in calcium insertion; these proteins are MxaC (cytoplasmic) and MxaD (periplasmic) [14]. The present work shows that a large conformational change (with a high activation energy) is involved in the incorporation of the Ca^{2+} ion into apo-MDH, and this suggests that at least one of the MxaACKLD proteins might have a processing role, perhaps analogous to that of chaperones, in maintaining the appropriate conformation of the apoprotein for insertion of Ca^{2+} when present at low concentrations and in conditions of neutral pH. A full characterization of the MDHs containing Sr^{2+} and Ba^{2+} is described in [15].

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