

Replacement of enzyme-bound calcium with strontium alters the kinetic properties of methanol dehydrogenase

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Methanol dehydrogenase (MEDH) possesses tightly bound Ca^{2+} in addition to its pyrroloquinoline quinone (PQQ) prosthetic group. Ca^{2+} was replaced with Sr^{2+} by growing the host bacterium, *Paracoccus denitrificans*, in media in which Ca^{2+} was replaced with Sr^{2+} . MEDH, which was purified from these cells (Sr-MEDH), exhibited an increased absorption coefficient for the PQQ chromophore, and displayed certain kinetic properties which were different from those of native MEDH. Native MEDH exhibits an endogenous activity which is not stimulated by substrate and which is inhibited by cyanide. Sr-MEDH exhibited lower endogenous activity which was stimulated by substrate, and was much less sensitive to inhibition by cyanide. The V_{max} for the methanol-dependent activity of Sr-MEDH was 3-fold greater than that of the native enzyme, and the K_s for methanol was altered. Cyanide also acts as an obligatory activator and

competitive inhibitor of methanol-dependent activity in native MEDH from *P. denitrificans* [Harris and Davidson (1993) *Biochemistry* 32, 4362–4368]. Sr-MEDH exhibited a similar K_i for cyanide inhibition of methanol-dependent activity, but the K_A for cyanide activation of this activity was 17-fold greater than that for the native enzyme. The activation energy of Sr-MEDH was 13.4 kJ (3.2 kcal)/mol lower than that of the native enzyme. These data confirm and significantly extend the conclusions from genetic [Richardson and Anthony (1992) *Biochem. J.* 287, 709–715] and crystallographic [White, Boyd, Mathews, Xia, Dai, Zhang and Davidson (1993) *Biochemistry* 32, 12955–12958] studies that suggest an apparently unique role for Ca^{2+} in MEDH compared with other Ca^{2+} -dependent proteins and enzymes.

INTRODUCTION

Methanol is oxidized to formaldehyde in methylotrophic and autotrophic bacteria by the periplasmic quinoprotein methanol dehydrogenase (MEDH) (Anthony, 1986, 1993). MEDH from *Paracoccus denitrificans* consists of a pair of heterodimers having α and β subunits of 67 kDa and 9.5 kDa respectively, giving a total molecular mass of 153 kDa (Nunn et al., 1989; Davidson et al., 1992). The two α subunits each contains one molecule of tightly but non-covalently bound pyrroloquinoline quinone (PQQ) (Figure 1; Salisbury et al., 1979). Originally, the enzyme was thought not to contain any metal atoms (Anthony and Zatman, 1967). More recently, atomic-absorption spectroscopy revealed the presence of tightly bound Ca^{2+} in MEDHs from *P. denitrificans*, *Methylobacterium extorquens*, *Methylophilus methylotrophus*, *Hyphomicrobium X* (Richardson and Anthony, 1992), *Methylobacterium glycogenes* (Adachi et al., 1990a) and *Methylophaga marina* (Chan and Anthony, 1992). On the basis of the belief that MEDH possessed one atom of Ca^{2+} , Richardson and Anthony (1992) proposed a model in which a single Ca^{2+} interacting with both α subunits influenced the configuration of the two active sites. Crystallographic studies have recently revealed that Ca^{2+} is directly co-ordinated to PQQ at each active site (White et al., 1993).

Two other PQQ-dependent enzymes have been shown to possess bound Ca^{2+} . Ethanol dehydrogenase from *Pseudomonas aeruginosa* contains two Ca^{2+} atoms per α_2 dimer (Mutzel and Gorisch, 1991), and glucose dehydrogenase from *Acinetobacter calcoaceticus* contains four Ca^{2+} atoms per α_2 dimer (Geiger and Gorisch, 1989). When these enzymes were prepared in the apoenzyme form, successful reconstitution with PQQ required the presence of either Ca^{2+} or Sr^{2+} for ethanol dehydrogenase, and Ca^{2+} , Mn^{2+} or Cd^{2+} for glucose dehydrogenase. The fact that

these cations were necessary for re-activation suggests a possible structural role for Ca^{2+} in enzymes containing PQQ. We have previously shown that the thermal stability of MEDH from *P. denitrificans* was enhanced by the presence of 1 mM Ca^{2+} and diminished by the presence of 5 mM EDTA, suggesting a structural role for Ca^{2+} in this quinoprotein as well (Davidson et al., 1992). Although it has not been possible to reconstitute MEDH activity after removal of PQQ, it has been shown that mutant strains of *M. extorquens* synthesized an inactive MEDH which contained no Ca^{2+} and exhibited abnormal spectral properties (Richardson and Anthony, 1992). Normal activity and spectral properties of the MEDH from these mutants could be restored by addition of Ca^{2+} .

It has been demonstrated that the biosynthesis of the quinoprotein MEDH in *M. glycogenes* was strictly controlled by Ca^{2+} in the growth medium (Adachi et al., 1990b). Similar growth-

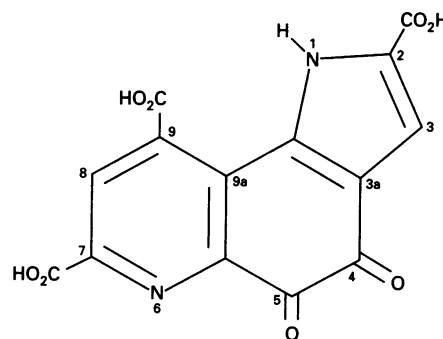


Figure 1 Structure of the PQQ prosthetic group of MEDH as deduced by Salisbury et al. (1979)

dependent effects were observed when MEDH was purified from this organism which was grown in a culture medium containing Sr^{2+} instead of Ca^{2+} . The purified MEDH from cells grown on Sr^{2+} (Sr-MEDH) contained Sr^{2+} , and no appreciable contamination by Ca^{2+} was found in the enzyme. Moreover, those authors reported that the specific activity of MEDH had increased when the organism was grown in a culture medium containing Sr^{2+} instead of Ca^{2+} , suggesting a possible kinetic role for the cation.

All MEDHs exhibit two activities *in vitro* when assayed with dye electron acceptors. The enzyme catalyses the reduction of an electron acceptor in the absence of any added substrate. This process also results in the inactivation of the enzyme (Anthony and Zatman, 1964; Duine and Frank, 1980; Ghosh and Quayle, 1981). The commonly accepted explanation for this substrate-independent or 'endogenous' activity is that the enzyme or components of its assay are contaminated by an unidentified endogenous substrate. In our preparations of MEDH from *P. denitrificans*, the endogenous rate is not significantly stimulated by the addition of substrate (Harris and Davidson, 1993). It is suppressed by cyanide, which also protects MEDHs against inactivation which results from the endogenous activity (Duine and Frank, 1980). A second activity, which is substrate-dependent, is observed in the presence of cyanide. We have recently described a kinetic model for this enzyme which includes dual roles for ammonia and cyanide, in which each acts as an activator of methanol-dependent activity at low concentrations and as an inhibitor of this activity at higher concentrations (Harris and Davidson, 1993). MEDH can bind substrate in the presence or absence of low concentrations of cyanide, but cannot form product unless it is first activated by that cyanide. Kinetic analysis demonstrated that this activating cyanide-binding site was clearly different from the substrate-binding site. Inhibition of methanol-dependent activity by higher concentrations of cyanide was shown to be due to a separate, distinct, binding phenomenon which was competitive with respect to substrate.

In this paper, we describe the preparation of Sr-MEDH from *P. denitrificans*, examine its physical properties and present a detailed steady-state kinetic analysis of its reactions with ammonia, cyanide and substrates. The differences in the physical and kinetic properties between MEDH and Sr-MEDH are characterized and discussed in the context of the recently reported active-site structure of MEDH.

EXPERIMENTAL

P. denitrificans (A.T.C.C. 13543) was grown aerobically at 30 °C in the medium of Kornberg and Morris (1968), supplemented with 0.05 % NaHCO_3 , 0.01 % yeast extract and 0.5 % methanol. This medium contains 0.27 mM CaCl_2 . For growth of cells from which to prepare Sr-MEDH, CaCl_2 was replaced with 0.81 mM SrCl_2 . The periplasmic fraction of *P. denitrificans* was prepared as described previously (Davidson, 1990), except that the concentration of EDTA, used in the periplasmic extraction buffer, was increased 20-fold from 0.5 mM to 10 mM. Sr-MEDH was purified from that fraction as described by Davidson et al. (1992). Wurster's Blue was synthesized from *NNN'*-tetramethyl-*p*-phenylenediamine by the method of Michaelis and Grannick (1943). All other reagents were purchased from commercial sources.

The protein absorption coefficient $A_{1\text{mg/ml}}$ at 280 nm was calculated from knowledge of the amino acid sequence data (Harms et al., 1987; van Spanning et al., 1991) by the method of Gill and von Hippel (1989) and found to be 3.66 for native MEDH. A dry-weight determination of $A_{1\text{mg/ml}}$ at 280 nm yielded

a value of 3.69 under native conditions. When freeze-dried MEDH, judged pure by SDS/PAGE, was used as a standard in the colorimetric method of Bradford (1976), $A_{1\text{mg/ml}}$ at 280 nm was determined to be 3.75. These three methods yielded an average $A_{1\text{mg/ml}}$ at 280 nm of 3.70 ± 0.04 for MEDH under native conditions. When BSA was used as a standard for the Bradford (1976) assay, a value of 2.74 was determined for $A_{1\text{mg/ml}}$ at 280 nm. Thus the latter method overestimated the true concentration of MEDH by approx. 37 %.

MEDH was assayed for the presence of Ca^{2+} , and Sr-MEDH was assayed for the presence of both Sr^{2+} and Ca^{2+} , with an Inductively Coupled Plasma Emission Spectrometer. The Ca^{2+} content of native MEDH was calculated to be 1.87 atoms per molecule of holoenzyme when the $A_{1\text{mg/ml}}$ of 3.70 is used. This is consistent with the crystallographic studies, which place one Ca^{2+} per active site. The precise amount of Sr^{2+} detected in each sample varied with the preparation. However, this analysis confirmed that the bound Ca^{2+} in MEDH was replaced with Sr^{2+} in Sr-MEDH as a result of the protocol described below. In Sr-MEDH, the amounts of Ca^{2+} detected were less than 10 % of the amount of bound Sr^{2+} .

Steady-state kinetic assays were performed as described previously (Harris and Davidson, 1993). The endogenous and substrate-dependent activities of Sr-MEDH were assayed separately. Unless otherwise indicated, the standard assay mixture for the endogenous activity contained 10 $\mu\text{g/ml}$ Sr-MEDH, 6 mM $(\text{NH}_4)_2\text{SO}_4$, and 3 mM Wurster's Blue in 0.1 M potassium 2-(cyclohexylamino)ethanesulphonic acid (K-Ches) buffer, pH 9, at 30 °C. For the assay of substrate-dependent activity, the assay mixture also contained 6 mM KCN and 10 mM methanol. When endogenous activity was present together with methanol-dependent activity, the former was subtracted from the total rate to yield the true methanol-dependent activity. The reactions were initiated by the addition of the obligatory activator, $(\text{NH}_4)_2\text{SO}_4$. The rates of the reaction were determined spectrophotometrically by monitoring the decrease in absorbance of Wurster's Blue at either 560 nm ($\epsilon = 12300 \text{ M}^{-1} \cdot \text{cm}^{-1}$) or 640 nm ($\epsilon = 2776 \text{ M}^{-1} \cdot \text{cm}^{-1}$). One unit of activity is defined as the reduction of 1 μmol of electron acceptor/min under the given experimental conditions. Kinetic data were analysed by non-linear curve fitting with Enzfitter (Elsevier-Biosoft, Cambridge, U.K.) and Sigma Plot 5.0 (Jandel Scientific, San Rafael, CA, U.S.A.) computer programs. The equations used to fit the data are given in the Results section.

RESULTS

Purification and properties of Sr-MEDH

The production by cells of Sr-MEDH was achieved by the method of Adachi et al. (1990b). All Ca^{2+} normally present in the growth medium was replaced with Sr^{2+} . To ascertain the optimum concentration of Sr^{2+} needed to maximize production of Sr-MEDH, cell growth and MEDH activity of cell extracts were assayed at different concentrations of SrCl_2 in the growth medium. Cell growth and MEDH activity increased up to 0.81 mM SrCl_2 . When higher concentrations of SrCl_2 were present in the medium, substantial lysis of cells occurred during growth, presumably due to the toxic effect of replacing all cell Ca^{2+} with Sr^{2+} .

The procedure used previously to extract the periplasmic fraction from *P. denitrificans* (Davidson, 1990) had to be modified for the Sr-grown cells. In this procedure, the outer membrane of the cells is removed by incubation of the cells in buffer containing lysozyme, EDTA and sucrose, which prevents lysis of sphero-

plasts. Incubation of Sr-grown cells in the original buffer caused no significant degradation of the outer membrane. Successful removal of the outer membrane and release of the periplasm was achieved when the concentration of EDTA in the buffer was increased 20-fold from 0.5 mM to 10 mM. EDTA is present in this buffer to chelate Ca^{2+} , which is thought to stabilize the interactions between the outer and inner membrane. The higher concentration of EDTA was probably required because this Ca^{2+} was also replaced by Sr^{2+} , which has a much lower affinity for EDTA ($K_d = 2.3 \times 10^{-9} \text{ M}^{-1}$) than does Ca^{2+} ($K_d = 2.0 \times 10^{-11} \text{ M}^{-1}$) (Schwarzenbach, 1957).

The procedure for the purification of Sr-MEDH from the periplasm was identical with that used to purify MEDH (Davidson et al., 1992). Sr-MEDH exhibited a molecular mass and a pI identical with those of MEDH. The yield of Sr-MEDH was 174 mg/100 g wet wt. of cells.

Spectroscopic properties of Sr-MEDH

The features of the absorption spectrum of Sr-MEDH were similar to that of MEDH. However, the absorption coefficient for the PQQ chromophore differed for the two enzyme forms (Figure 2). Taking the average of three preparations, the A_{350}/A_{400} ratio was 2.42 ± 0.14 for Sr-MEDH, compared with 2.18 ± 0.08 for MEDH, the A_{280}/A_{350} ratio was 8.49 ± 0.37 for

Sr-MEDH, compared with 10.78 ± 0.27 for MEDH, and the A_{280}/A_{400} ratio was 20.50 ± 1.14 for Sr-MEDH, compared with 23.48 ± 0.82 for MEDH.

Interactions of ammonia with MEDH

As was observed for MEDH, ammonia was an essential activator for both the endogenous and methanol-dependent reactions of Sr-MEDH. No activity was observed in the absence of ammonia. The data for the interactions of ammonia with Sr-MEDH (Figure 3) were fitted reasonably well to the equation for substrate inhibition (eqn. 1):

$$v = \frac{V_{\max} [S]}{K_s + [S] (1 + [S]/K_i)} \quad (1)$$

Interestingly, the V_{\max} for the methanol-dependent activity of Sr-MEDH was 3-fold greater than that observed for MEDH, whereas the V_{\max} for the endogenous activity of Sr-MEDH was slightly lower than that of MEDH (Table 1). The V_{\max} for the endogenous activity of Sr-MEDH was 3.6-fold lower than that of the methanol-dependent activity of Sr-MEDH, whereas for MEDH the two activities were approximately equal. The methanol-dependent and endogenous activities of Sr-MEDH were somewhat more sensitive to activation by ammonia than

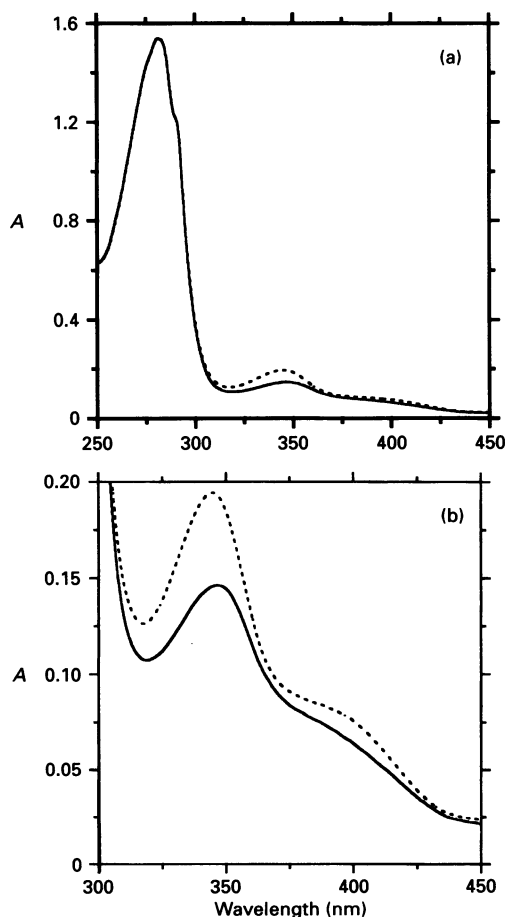


Figure 2 (a) Absorption spectra of MEDH (—) and Sr-MEDH (---); (b) expansion of the absorption spectra of MEDH (—) and Sr-MEDH (---) characteristic of the PQQ chromophore

In (a, MEDH and Sr-MEDH (0.42 mg/ml) were incubated in 0.1 M K-CHES buffer, pH 9.0, at 30°C. The absorption spectra were normalized to A_{280} for comparison.

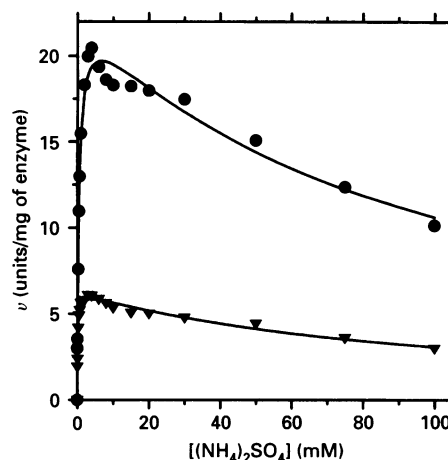


Figure 3 Interactions of ammonia with Sr-MEDH: concentration-dependence on ammonia for methanol-dependent activity (●) and endogenous activity (▼)

The continuous lines show the fit of the data to eqn. (1).

Table 1 Steady-state kinetic parameters for the interaction of $(\text{NH}_4)_2\text{SO}_4$ with MEDH

Activities were assayed with Wurster's Blue as described in the Experimental section.

Kinetic constant	Methanol-dependent activity		Endogenous activity	
	MEDH*	Sr-MEDH	MEDH*	Sr-MEDH
V_{\max} (units/mg)	8.10 ± 0.26	22.7 ± 0.6	7.18 ± 0.20	6.31 ± 0.10
K_A (mM)	0.71 ± 0.08	0.50 ± 0.05	0.38 ± 0.05	0.14 ± 0.01
K_i (mM)	98.5 ± 13.8	88.7 ± 9.7	53.2 ± 5.4	96.4 ± 8.7

* Data taken from Harris and Davidson (1993).

were those of MEDH, as shown by the lower K_a values (Table 1). Substrate inhibition by ammonia was similar for the methanol-dependent activities of both Sr-MEDH and MEDH. However, the endogenous activity of Sr-MEDH was approx. 2-fold less sensitive to inhibition by ammonia than was MEDH.

No changes in the absorption spectrum of Sr-MEDH were observed on addition of ammonia up to 600 mM. This is in contrast with the spectral changes that were observed previously for MEDH on addition of concentrations of $(\text{NH}_4)_2\text{SO}_4$ that were in the range of the K_i values obtained in kinetic studies (Harris and Davidson, 1993).

Interactions of KCN with MEDH

As was observed for MEDH, the endogenous activity of Sr-MEDH *in vitro* was suppressed in the presence of KCN. The

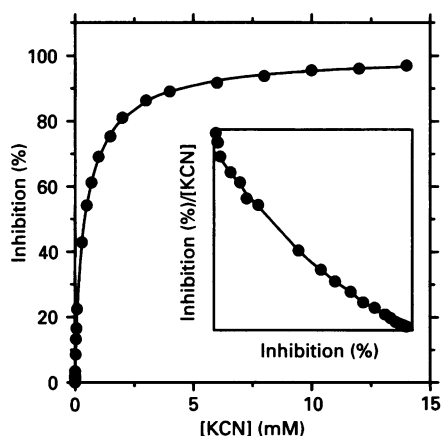


Figure 4 Inhibition by KCN of the endogenous activity of Sr-MEDH

The continuous line shows the fit of the data to eqn. (2). Inset: the same data presented in the form of a Scatchard plot.

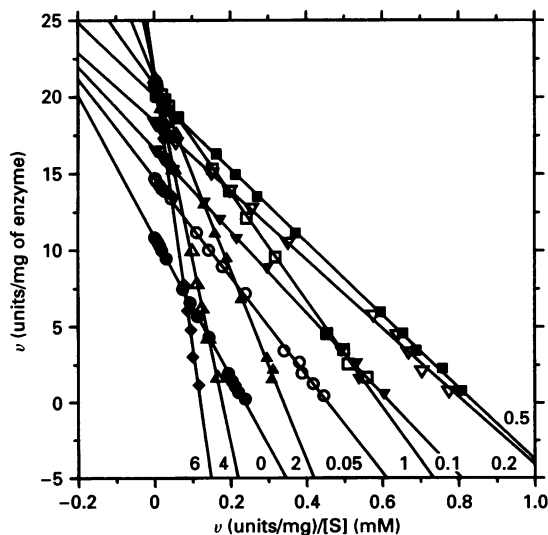


Figure 5 Effect of KCN on the steady-state reaction of Sr-MEDH with cyanide and methanol

The rates of methanol-dependent activity were assayed in the presence of 0 (●), 0.05 (○), 0.10 (▼), 0.20 (▽), 0.50 (■), 1.0 (□), 2.0 (▲), 4.0 (△) and 6.0 (◆) mM KCN. The KCN concentrations are also indicated in the Figure for clarity. Continuous lines drawn through each data set are fitted to eqn. (3). Identical lines occur when the entire data set is fitted to eqn. (5).

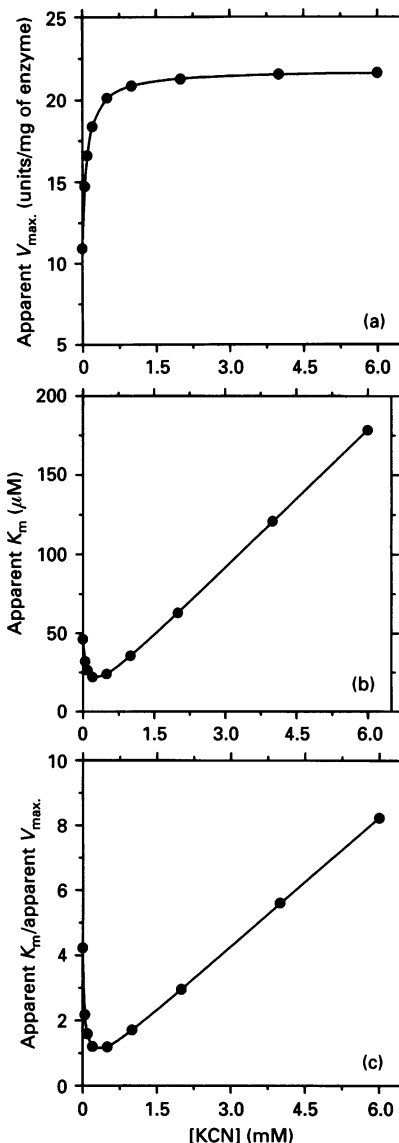


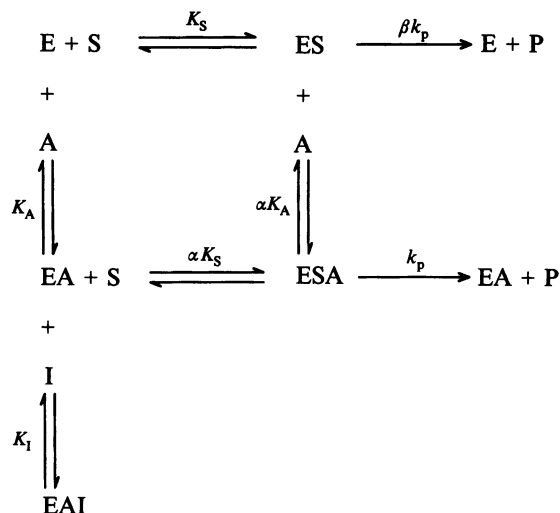
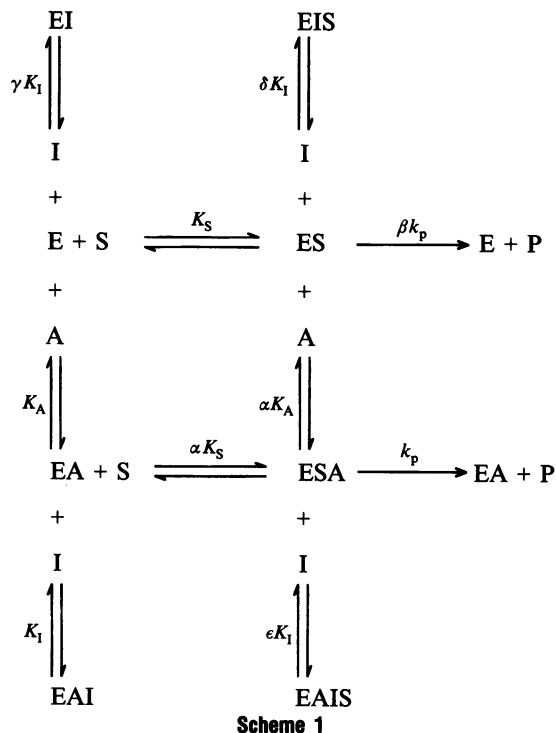
Figure 6 Effect of KCN on (a) the apparent V_{\max} of the methanol-dependent activity of Sr-MEDH, (b) the apparent K_m of the methanol-dependent activity of Sr-MEDH, and (c) the ratio of apparent K_m to apparent V_{\max} .

These are secondary plots in which the apparent V_{\max} and K_m are deduced from the data shown in Figure 5. Continuous lines drawn through the data in (a) and (b) are fits of the data to eqns. (6) and (7) respectively. The line in (c) is a fit of the data to an equation obtained by dividing eqn. (7) by eqn. (6).

previously reported data for MEDH were best fitted to the equation for ligand binding to equivalent and independent sites, which on analysis yielded a K_d value of $46.0 \mu\text{M}$ (Harris and Davidson, 1993). In contrast with this, the data obtained for percentage inhibition of endogenous activity of Sr-MEDH as a function of increasing concentrations of KCN (Figure 4) were fitted better to the equation for ligand binding to two non-equivalent sites (eqn. 2), where $[X]$ is the concentration of added KCN and C is the binding capacity in terms of the percentage inhibition of endogenous activity:

$$\text{Inhibition (\%)} = \frac{C_1[X]}{K_{d1} + [X]} + \frac{C_2[X]}{K_{d2} + [X]} \quad (2)$$

Analysis of these data yielded values of $787 \pm 61 \mu\text{M}$ for K_{d1} and $171 \pm 16 \mu\text{M}$ for K_{d2} . The slight but distinct curvature in the Scatchard-type plot of percentage inhibition/[KCN] against



Scheme 2

This equation was fitted with the 134 data points used to generate the plots in Figure 5. Although the convergence of the computer fit of the data to eqn. (4) for the general mechanism was not smooth, it yielded values of 21.8 ± 0.02 for V_{\max} , $46.1 \pm 0.3 \mu\text{M}$ for K_S , $785 \pm 29 \mu\text{M}$ for K_A , $184 \pm 6 \mu\text{M}$ for K_I , 0.117 ± 0.004 for α , and 0.500 ± 0.001 for β . The values for γ , δ and ϵ were each of the order of 10^6 , with large standard errors. This suggested that the steps defined by γK_I , δK_I and ϵK_I did not play a significant role in the reaction mechanism. The general mechanism (Scheme 1) was then modified several times so that the steps defined by K_I , γK_I , δK_I and ϵK_I could be tested individually and in all possible combinations with the other steps in this mechanism. The computer fits to the rate equations describing any of these mechanisms which included steps defined by γK_I , δK_I or ϵK_I did not converge smoothly. The final sum of the squared residuals (SSQ) for each was much larger than that for the simplified mechanism, which gave the best fit and is described in Scheme 2 and which does not contain these steps. The rate equation derived for this mechanism is described by:

$$v = \frac{V_{\max} [S] (\beta + [A]/\alpha K_A)}{K_S (1 + [A]/K_A + [A][I]/K_A K_I) + [S] (1 + [A]/\alpha K_A)} \quad (5)$$

The results of the computer fit of the data shown in Figure 5 to this equation are given in Table 2. For this mechanism, a rapid and smooth convergence to a minimum SSQ in the non-linear regression analysis occurred. Rearrangement into the Michaelis-Menten form of eqn. (5) yielded eqn. (6) for the apparent V_{\max} and eqn. (7) for the apparent K_m for methanol as a function of concentration of KCN. These equations were used to fit the data in Figures 6(a) and 6(b).

$$\text{Apparent } V_{\max} = \frac{V_{\max} (\beta + [A]/\alpha K_A)}{(1 + [A]/\alpha K_A)} \quad (6)$$

$$\text{Apparent } K_m = \frac{K_S (1 + [A]/K_A + [A][I]/K_A K_I)}{1 + [A]/\alpha K_A} \quad (7)$$

That KCN is not only a simple competitive inhibitor can be seen in a Dixon-type plot of (apparent K_m /apparent V_{\max}) versus [KCN] (Figure 6c). Deviation from linearity is clearly evident in this plot, and these data are fit very well to an equation which is obtained by dividing eqn. (7) by eqn. (6).

Sr-MEDH exhibited a 3-fold greater value for the true V_{\max} .

percentage inhibition (Figure 4, inset) is consistent with either two populations of molecules or two types of sites on a single molecule for the binding of KCN responsible for this inhibition.

A kinetic mechanism which includes dual roles for cyanide as both an activator and an inhibitor of methanol-dependent activity has previously been demonstrated for *P. denitrificans* MEDH (Harris and Davidson, 1993). To describe effects of KCN on the methanol-dependent activity of Sr-MEDH, initial rates were measured at various methanol concentrations in the absence of KCN and in the presence of several different fixed concentrations of KCN. To highlight the effects of KCN on the apparent V_{\max} and K_m , the data were plotted as linear plots of v versus $v/[\text{methanol}]$ according to eqn. (3) (Figure 5), in which the ordinate intercept corresponds to the apparent V_{\max} and the slope reflects the apparent K_m :

$$v = -K_m \left(\frac{v}{[S]} \right) + V_{\max} \quad (3)$$

Secondary plots of the apparent V_{\max} and apparent K_m values for each concentration of KCN are given in Figures 6(a) and 6(b) respectively (equations for the fit of these data are given below). Sr-MEDH was similar to MEDH in that KCN acts as both an activator (increase in V_{\max} with increasing concentrations of KCN; Figure 6a) and an inhibitor (increase in K_m for methanol with increasing concentrations of KCN; Figure 6b). A general mechanism to explain this dual role of KCN as an activator and an inhibitor is given by Scheme 1, where A and I represent KCN as the activator and inhibitor, respectively. In contrast with MEDH, the endogenous activity of Sr-MEDH was stimulated by substrate, and therefore it was necessary to include a step for product formation, given by βk_p , in the absence of KCN. The endogenous activity exhibited by native MEDH was not stimulated by the addition of substrate, so that β for the native enzyme was equal to zero. By making the rapid-equilibrium assumption for substrate, activator and inhibitor binding, rate equation (4) was derived for this mechanism:

$$v = \frac{V_{\max} [S] (\beta + [A]/\alpha K_A)}{K_S (1 + [A]/K_A + [A][I]/K_A K_I + [I]/\gamma K_I) + [S] (1 + [A]/\alpha K_A + [A][I]/\epsilon K_I \alpha K_A + [I]/\delta K_I)} \quad (4)$$

Table 2 Steady-state parameters for the effect of KCN on the methanol-dependent activity of MEDH

Activities were assayed with Wurster's Blue as described in the Experimental section. The values for the parameters shown in Scheme 2 were obtained from a computer fit of the data from Figure 5 to eqn. (5). K_S , K_A and K_I are the dissociation constants for methanol, cyanide as an activator and cyanide as a competitive inhibitor, respectively. SSQ is the sum of the squared residuals from the non-linear regression of the computer analysis, and σ is the standard deviation for the velocities. The value for σ was found from the equation $[\text{SSQ}/(N-K)]^{1/2}$, where N = number of data points = 134 and K = number of estimated parameters = 6.

Parameter	MEDH*	Sr-MEDH
V_{max}	7.30 ± 0.01 units/mg	21.8 ± 0.02 units/mg
K_S	14.9 ± 1.2 μM	46.1 ± 0.3 μM
K_A	46.4 ± 5.6 μM	785 ± 29 μM
K_I	155 ± 8 μM	184 ± 6 μM
α	0.954 ± 0.116	0.117 ± 0.004
β	0	0.500 ± 0.001
SSQ	0.223 (units/mg) ²	0.347 (units/mg) ²
σ	0.045 units/mg	0.052 units/mg

* Data taken from Harris and Davidson (1993).

than did MEDH. The K_S for methanol in Sr-MEDH, in the absence of cyanide, was 3-fold higher than for MEDH. Activation of methanol-dependent activity by KCN was essential for MEDH, whereas Sr-MEDH required KCN only as a non-essential activator. On binding of cyanide as an activator, the methanol-dependent activity of Sr-MEDH increased 2-fold, since $\beta = 0.5$ (Scheme 2). The most notable differences in the parameters for MEDH and Sr-MEDH relate to the binding of cyanide as an activator. The K_A for activation of methanol-dependent activity by KCN of Sr-MEDH was 17-fold higher than for MEDH. Furthermore, in Sr-MEDH, the value of 0.117 for α indicates that the binding of either KCN, as an activator, or substrate does appreciably affect the binding of the other. This is in marked contrast with MEDH, in which the value of 0.954 for α indicated that the binding of either KCN, as an activator, or substrate did not appreciably affect the binding of the other. The K_I for inhibition by KCN is similar for both enzyme forms. The K_A for cyanide activation and K_I for cyanide inhibition correlate very closely with the K_{a1} and K_{a2} obtained for percentage inhibition of endogenous activity (Figure 4). This does not prove, but strongly suggests, that the curved Scatchard plot is due to two binding sites for cyanide. In MEDH, the K_A for cyanide, which also correlates with inhibition of endogenous activity, was much less than the K_I for cyanide, and therefore the second inhibitory binding (K_I) was not observed in the endogenous reaction. In Sr-MEDH, the unchanged K_I is now less than the elevated K_A which correlates with endogenous inhibition. Thus, inhibition of endogenous activity due to the binding which is described by K_I is also observable, and accounts for the biphasic nature of the inhibition of endogenous activity (Figure 4).

Substrate specificity

To obtain information on the substrate specificity of Sr-MEDH, steady-state kinetic experiments were performed with various alcohols and formaldehyde as substrates in the presence of optimal concentrations of Wurster's Blue, ammonia and KCN. For each substrate, the activity with Sr-MEDH was greater than that observed with MEDH (Harris and Davidson, 1993), but the substrate specificities of the two enzymes were essentially the same (results not shown).

Effects of temperature

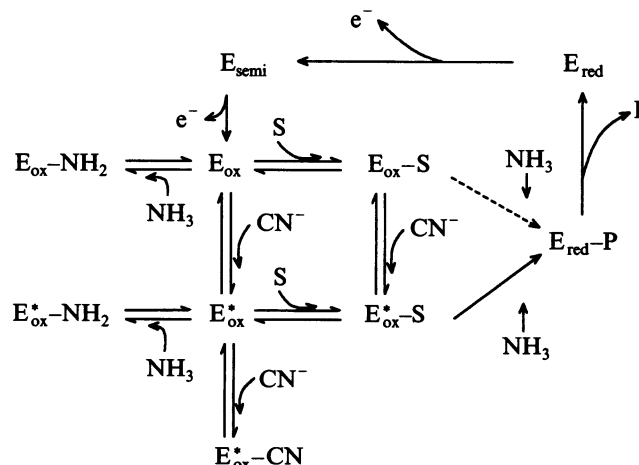
The dependence of V_{max} on temperature over the range 10–50 °C was determined for both MEDH and Sr-MEDH under optimum assay conditions. Linear-regression analysis of these data indicated that the points were best fitted by a single straight line for both enzyme forms. The energy of activation for MEDH was calculated from the slope of this line to be 53.6 kJ (12.8 kcal)/mol. The energy of activation for Sr-MEDH was calculated to be 40.2 kJ (9.6 kcal)/mol.

DISCUSSION

Replacement of the enzyme-bound Ca^{2+} of MEDH from *P. denitrificans* with Sr^{2+} affected the spectroscopic and steady-state kinetic properties of the enzyme. With the recently proposed steady-state kinetic model (Scheme 3) for the reactions of MEDH from *P. denitrificans* (Harris and Davidson, 1993), it was possible to characterize the specific effects of replacing the Ca^{2+} with Sr^{2+} on the kinetic constants which describe each step in that model.

The observed differences in the absorption coefficients for the PQQ chromophore (Figure 2) of Sr-MEDH, as compared with MEDH, are consistent with the cation-dependent spectroscopic differences reported for MEDH from mutant strains of *M. extorquens*, which contained no Ca^{2+} and exhibited a loss of the absorption maximum at 350 nm (Richardson and Anthony, 1992). These data indicate that the PQQ may bind differently in the active site in the absence of Ca^{2+} .

Low concentrations of ammonia activate MEDH by facilitating the transfer of hydrogen from methanol to PQQ (Frank et al., 1988). The K_A for this ammonia binding was not significantly altered in Sr-MEDH. At higher concentrations of ammonia, inhibition and spectral perturbations of MEDH were observed which were probably due to adduct formation with PQQ (Harris and Davidson, 1993). Similar kinetic parameters for the dual roles of ammonia were observed for Sr-MEDH. However, spectral perturbations caused by ammonia were not observed for Sr-MEDH. This does not necessarily eliminate the possibility that inhibition by ammonia is due to adduct formation. The increase in absorbance near the shoulder region of the absorption spectra of Sr-MEDH relative to MEDH (Figure 2b) is similar to

**Scheme 3** Proposed kinetic mechanism for the reaction cycle of Sr-MEDH

E* represents the cyanide-activated form of the enzyme. S and P represent substrate and product respectively. This model includes an additional step (--->) to that given by Harris and Davidson (1993) to account for product formation in the absence of cyanide. Abbreviations: ox, oxidized; red, reduced; semi, semiquinone.

the spectral perturbation caused by addition of ammonia to MEDH. Thus ammonia-dependent spectral changes in Sr-MEDH, due to adduct formation, may not be seen, owing to the overall spectral perturbations caused by the substitution of Sr^{2+} .

It has been previously demonstrated (Harris and Davidson, 1993) that MEDH possesses two cyanide-binding sites. One cyanide-binding site, described by K_A (Scheme 2), is necessary for activation of methanol-dependent activity, and is also responsible for inhibition of endogenous activity. This cyanide binding does not significantly alter the binding of substrate (Table 2, $\alpha = 0.954$). Inhibition by cyanide of substrate-dependent activity is due to a separate distinct binding phenomenon which is competitive with respect to substrate and described by K_I . In Sr-MEDH, the properties of the former site are significantly altered. The binding is 17-fold weaker, and binding of this cyanide facilitates the binding of substrate, and vice versa (Table 2, $\alpha = 0.117$). It is noteworthy that the binding of cyanide as an activator in Sr-MEDH, described by K_A , lowers the K_S for methanol from $46.1 \mu\text{M}$ (3-fold greater than the value of $14.9 \mu\text{M}$ for MEDH) to $5.4 \mu\text{M}$, thus making Sr-MEDH a much more efficient enzyme than MEDH (V_{max}/K_S is approx. 8-fold higher) in the presence of KCN. The K_I for the binding of cyanide as a competitive inhibitor of substrate-dependent activity of Sr-MEDH was not significantly altered. Although the physiological relevance of the cyanide-binding sites is not known, these results strongly suggest that substitution of Sr^{2+} for Ca^{2+} is affecting structural aspects of the active site, in particular the cyanide-binding site responsible for enzyme activation.

The decrease in the activation energy observed with Sr-MEDH further suggests some perturbation of the active site caused by the cation substitution. These data suggest that in Sr-MEDH there is an increased stability of the transition state, which could be caused by subtle differences in the geometry of the active site.

Two broad classes of Ca^{2+} -binding proteins are evident in the literature thus far. The first is the Ca^{2+} -modulated proteins, which can bind Ca^{2+} reversibly and as a result, regulate interactions with other proteins. The Ca^{2+} -binding sites in many of these proteins consist of a helix-loop-helix motif and is typified in crystal structures of troponin C (Herzberg and James, 1985), calmodulin (Babu et al., 1985), parvalbumin (Kumar et al., 1990) and α -amylase (Boel et al., 1990). The role of Ca^{2+} in the second group appears to be one of stabilization of structure. The Ca^{2+} -binding sites found in this group are more diverse, as shown by crystal structures of the serine proteinases, bovine trypsin (Bode and Schwager, 1975), elastase (Meyer et al., 1988), subtilisin (McPhalen et al., 1985), proteinase K (Bajorath et al., 1989) and thermitase (Gros et al., 1989). In the relatively constant Ca^{2+} levels of the extracellular environment, protein-bound Ca^{2+} is most often a stabilizing factor, rather than an action signal as seen for many Ca^{2+} -binding proteins for intracellular functions where Ca^{2+} levels are much lower. Given the periplasmic location of MEDH, where there is no evidence of a Ca^{2+} -signalling system, one might expect that Ca^{2+} would act as a stabilizing factor in this enzyme.

Relatively little is known about possible catalytic roles of Ca^{2+} in enzymes. The catalytic Ca^{2+} -binding site in DNAase I correctly positions the phosphodiester bond and facilitates the nucleophilic attack of the water hydroxyl group through its positive charge (Oefner and Suck, 1986). In *Staphylococcus aureus* nuclease, the role of the Ca^{2+} ion may be for the polarization of the phosphate at the scissile phosphoester bond of a substrate (Cotton et al., 1979). The Ca^{2+} ion in phospholipase A_2 is proposed to serve as part of an oxyanion hole (Dijkstra et al., 1981). In these examples, the Ca^{2+} ion directly interacts with the enzyme and substrate at the active site. Conversely, a catalytic effect has been observed in

one enzyme which binds Ca^{2+} at a distance from the active site. Although the Ca^{2+} -binding site is more than 1 nm (10 \AA) away from the active site, the crystal structure of EDTA-treated proteinase K shows small conformational changes in this region that may affect substrate binding and decrease catalytic activity (Bajorath et al., 1989).

Crystallographic studies of the active site in MEDH show that the Ca^{2+} is co-ordinated to six ligands (White et al., 1993). The pyridine nitrogen (N-6), the carboxylate oxygen at the C-7 position and the carbonyl oxygen at C-5 of the PQQ prosthetic group (Figure 1) comprise three of the co-ordinated ligands, and the two carboxyl oxygen atoms of a Glu side chain and the side-chain oxygen of an Asn residue complete the geometry. The Sr^{2+} ionic radius of 0.113 nm (1.13 \AA) is larger than the Ca^{2+} ionic radius of 0.099 nm (0.99 \AA), whereas the electronegativities of these two metal ions are approximately equal. Replacement of enzyme-bound Ca^{2+} with Sr^{2+} could result in a steric perturbation in the positioning of PQQ or the Glu or Asn side-chain ligands co-ordinated with Ca^{2+} in MEDH. We have shown that several properties of MEDH are altered on replacement of Ca^{2+} with Sr^{2+} . (1) The absorption coefficient of the PQQ chromophore is increased. (2) The endogenous activity is decreased, and may be stimulated by the addition of substrate. (3) Inhibition of endogenous activity by cyanide becomes biphasic. (4) The true V_{max} for methanol-dependent activity is increased 3-fold. (5) The K_S for methanol is increased 3-fold and is affected by the presence of cyanide. (6) The K_A for activation of methanol-dependent activity by cyanide is increased 17-fold and is affected by the presence of substrate. (7) The activation energy is decreased by 13.4 kJ (3.2 kcal)/mol. Thus, in MEDH Ca^{2+} may not only be holding the cofactor in place, but also positioning active-site amino acid residues for the binding of substrates, activators and inhibitors and maintaining the geometry of the transition state which influences the rate of the reaction. This appears to represent a new role for Ca^{2+} in the structure and activity of an oxidoreductase.

We thank Dr. Clifford J. Unkefer from the Los Alamos National Laboratory for performing the metal analysis. This work was supported by National Institutes of Health grant GM-41574.

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Received 27 September 1993/5 January 1994; accepted 14 January 1994