Thermal stability of methanol dehydrogenase is altered by the replacement of enzyme-bound Ca^{2+} with Sr^{2+}

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Methanol dehydrogenase (MEDH) possesses tightly bound Ca^{2+} in addition to its pyrroloquinoline quinone 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+} . At temperatures in the transition region for stability, the rate constants for inactivation of MEDH purified from these cells (Sr-MEDH) were 2-fold lower than those for MEDH. However, Arrhenius plots yielded an activation energy (E_a) of 699 kJ (167 kcal)/mol for MEDH compared with 640 kJ (153 kcal)/mol for Sr-MEDH. Further analysis by transition-state theory yielded values for the activation enthalpy ($\Delta H^\ddagger$) and activation entropy ($\Delta S^\ddagger$) of 696 kJ (166 kcal)/mol and 1.73 kJ

(414 cal)/mol per K for MEDH and 637 kJ (152 kcal)/mol and 1.55 kJ (371 cal)/mol per K for Sr-MEDH. The higher rate of inactivation of MEDH than Sr-MEDH at higher temperatures is a consequence of a more favourable net gain in entropy. This positive entropy contribution increases at high temperatures, and reduces the more favourable stability obtained from the enthalpy contribution for the free energy ($\Delta G^\ddagger$) of inactivation. The differences in these thermodynamic data are discussed in relation to the recently determined crystal structure of MEDH as well as ^1H electron-nuclear double resonance studies of the influence of Sr^{2+} substitution on the structure of the pyrroloquinoline quinone-derived radical in MEDH

INTRODUCTION

Methanol is oxidized to formaldehyde in methylotrophic and autotrophic bacteria by the periplasmic quinoprotein methanol dehydrogenase (MEDH) (Anthony, 1993). MEDH from *Paracoccus denitrificans* consists of a pair of heterodimers with 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)] and tightly bound Ca^{2+} (Adachi et al., 1990; Richardson and Anthony, 1992; White et al., 1993).

The presence of Ca^{2+} appears to be a common feature of PQQ-dependent enzymes as two others 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 suggested 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 *Methylobacterium extorquens* synthesize an inactive MEDH which contains no Ca^{2+} and exhibits abnormal spectral properties (Richardson and Anthony, 1992). Normal activity

and spectral properties of the MEDH from these mutants can be restored by the addition of Ca^{2+} .

We have previously prepared an MEDH from *P. denitrificans* in which Ca^{2+} was replaced by Sr^{2+} . This was achieved by growing cells in a culture medium in which all Ca^{2+} was replaced with Sr^{2+} (Harris and Davidson, 1994). This Sr-MEDH exhibited an altered visible absorption spectrum and altered kinetic properties, suggesting that the cation is important for maintaining the correct conformation about the active site. This suggestion was confirmed by recent crystallographic studies of MEDH which identified Ca^{2+} co-ordinated to PQQ and amino acid residues at the active site (White et al., 1993). In the present paper, we characterize differences in the apparent thermodynamic parameters associated with the thermal inactivation of MEDH and Sr-MEDH, and discuss their implications with regard to the structural role of Ca^{2+} in the quinoprotein MEDH.

EXPERIMENTAL

Purification of MEDH (Davidson et al., 1992) and Sr-MEDH (Harris and Davidson, 1994) from *P. denitrificans* were as described previously. MEDH concentrations were calculated from an $\epsilon_{1\text{ mg/ml}}$ at 280 nm of 3.70 under native conditions as given by a dry-weight determination. 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.

A circulating water bath which was digitally controlled to an accuracy of $\pm 0.1^\circ\text{C}$ was used to incubate 1 mg/ml MEDH or Sr-MEDH in 1.5 ml microcentrifuge tubes. A microcentrifuge tube containing the same volume of buffer was added to the water bath at the same time as the enzyme samples. The temperature of the sample containing buffer only was monitored

Abbreviations used: E_a , activation energy for inactivation of enzyme; $\Delta G^\ddagger$, free energy of inactivation; $\Delta H^\ddagger$, enthalpy of inactivation; $\Delta S^\ddagger$, entropy of inactivation; MEDH, methanol dehydrogenase; PQQ, pyrroloquinoline quinone; Sr-MEDH, Sr^{2+} -substituted methanol dehydrogenase.

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

with the probe of a second digital thermometer with an accuracy of $\pm 0.1^\circ\text{C}$. When the temperature of the buffer in the micro-centrifuge tube equilibrated with the temperature of the water bath, the first enzyme sample was removed and assayed under standard conditions to obtain the steady-state kinetic activity at time zero.

Steady-state kinetic assays were performed in 0.1 M potassium 2-(cyclohexylamino)ethanesulphonic acid buffer, pH 9, at 30°C as described previously (Harris and Davidson, 1993). The standard assay mixture contained $10\text{ }\mu\text{g}$ MEDH/ml, 6 mM $(\text{NH}_4)_2\text{SO}_4$, 3 mM Wurster's Blue, 6 mM KCN and 10 mM methanol. Reactions were initiated by the addition of the obligatory activator, $(\text{NH}_4)_2\text{SO}_4$. Rates of 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 $1\text{ }\mu\text{mol}$ of electron acceptor/min under the given experimental conditions. The data were analysed with Enzfitter (Elsevier-BIOSOFT, Cambridge, MA, U.S.A.) 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

Thermal inactivation of MEDH

MEDH from *P. denitrificans* has been shown to be relatively stable to thermal inactivation, retaining most of its activity after a 10 min incubation at 60°C , with significant loss of activity observed at higher temperatures (Davidson et al., 1992). The kinetics of thermal inactivation of MEDH were studied by incubating samples of MEDH and Sr-MEDH in 0.1 M potassium phosphate buffer, pH 7.5, at 64, 66, 68 and 70°C . At each incubation temperature, $10\text{ }\mu\text{l}$ portions were removed at different times, diluted 100-fold into the assay mixture and assayed at 30°C under standard conditions as described in the Experimental section. The inactivation process for both MEDH and Sr-

Figure 2 Time course for thermal inactivation of (a) MEDH (1 mg/ml) and (b) Sr-MEDH (1 mg/ml) at 64 (●), 66 (▼), 68 (■) and 70°C (◆)

The solid lines represent a fit of the data to eqn. (1).

MEDH followed apparent first-order kinetics at all temperatures (Figure 2). The rate constants for this process at all temperatures were determined from fits of the data to eqn. (1) where A is the

Table 1 First-order rate constants for inactivation of MEDH

The standard error in ΔG^* is reported as the difference between the ΔG^* values calculated from the standard error maximum and minimum values of the first-order rate constants. The error in temperature measurement is negligible with temperature control being $\pm 0.1\text{ K}$ (i.e. $0.1/337.15 = 0.33\%$).

Temperature ($^\circ\text{C}$)	MEDH		Sr-MEDH	
	$10^5 \times k\text{ (s}^{-1}\text{)}$	$\Delta G^*\text{ (kJ/mol)}$	$10^5 \times k\text{ (s}^{-1}\text{)}$	$\Delta G^*\text{ (kJ/mol)}$
64	2.71 ± 0.03	112 ± 0.06	1.56 ± 0.02	114 ± 0.07
66	11.7 ± 0.1	109 ± 0.05	5.99 ± 0.07	111 ± 0.07
68	49.2 ± 0.4	105 ± 0.05	22.3 ± 0.2	108 ± 0.05
70	212 ± 2	102 ± 0.07	84.4 ± 0.07	105 ± 0.05

Figure 3 Eyring plot for thermal inactivation of MEDH (●) and Sr-MEDH (▼)

The solid lines represent a fit to eqn. (3) of the data shown in Figure 2.

percentage residual activity, t is the time incubated and b represents an offset value to account for a non-zero baseline.

$$A = \exp(-kt) + b \quad (1)$$

The first-order rate constants for MEDH and Sr-MEDH are given in Table 1. It can be seen that at each temperature the rate constant for inactivation of MEDH was approximately 2-fold greater for MEDH than for Sr-MEDH.

The temperature-dependence of the rate constant for inactivation was analysed according to the Arrhenius equation [eqn. (2)] where E_a is the activation energy, R is the gas constant, T is the absolute temperature and A is a pre-exponential factor:

$$\ln k = -\frac{E_a}{R} \left(\frac{1}{T} \right) + \ln A \quad (2)$$

Linear-regression analysis of these data indicated that the points were best fit by a straight line for both enzyme forms. The E_a for MEDH was calculated from the slope of this line to be 699 kJ (167 kcal)/mol. The E_a for Sr-MEDH was calculated to be 640 kJ (153 kcal)/mol. The standard errors in E_a for both MEDH and Sr-MEDH were less than 1%.

The temperature-dependence of the rate constant of the inactivation process was also analysed according to transition-state theory by the use of eqn. (3) where ΔH^* and ΔS^* are the enthalpy and entropy of activation, h is Planck's constant and k_B is Boltzmann's constant (Figure 3).

$$\ln \frac{hk}{k_B T} = -\frac{\Delta H^*}{R} \left(\frac{1}{T} \right) + \frac{\Delta S^*}{R} \quad (3)$$

The activation enthalpy and entropy were 696 kJ (166 kcal)/mol and 1.73 kJ (414 cal)/mol per K for MEDH and 637 kJ (152 kcal)/mol and 1.55 kJ (371 cal)/mol per K for Sr-MEDH. The standard errors in ΔH^* and ΔS^* for both enzyme forms were each less than 1%.

Figure 4 Spectral changes observed on incubation of MEDH at 68 °C

(a) Absorption spectra of MEDH (0.5 mg/ml) incubated in 0.1 M potassium phosphate buffer, pH 7.5, were recorded at 5, 15, 25, 35, 45, 75, 90 and 160 min after the incubation temperature reached 68 °C. (b) Absorption spectra of Sr-MEDH (0.5 mg/ml) incubated in 0.1 M potassium phosphate buffer, pH 7.5, were recorded at 8, 23, 38, 52.5, 68, 180 and 240 min after the incubation temperature reached 68 °C. The arrow indicates the direction of the absorbance change with time.

Spectral changes associated with inactivation

To determine whether the loss of activity at high temperatures could be correlated with specific changes in the environment of the PQQ chromophore, samples of MEDH and Sr-MEDH were incubated in 0.1 M potassium phosphate buffer, pH 7.5, at 68 °C. Absorption spectra were recorded at different times during this incubation after removal of 250 μ l portions and diluting them into the same buffer at room temperature (Figure 4). The loss of absorbance at 350 nm, characteristic of the PQQ chromophore, for both MEDH and Sr-MEDH followed apparent first-order kinetics (Figure 5). The rate constant for this process was determined from a fit of these data to eqn. (1) to be $3.92 \times 10^{-4} \text{ s}^{-1}$ for MEDH and $2.39 \times 10^{-4} \text{ s}^{-1}$ for Sr-MEDH with standard errors each less than 7%. The rate constant for the loss of absorbance at 350 nm in Sr-MEDH is statistically identical with that for the loss of activity (Table 1). The rate constant for the loss of absorbance in MEDH is slightly slower than that in Table

Figure 5 Time course for the loss of absorbance in the peak at 350 nm characteristic of the PQQ prosthetic group in MEDH (●) and Sr-MEDH (▼)

Data were taken from the spectra shown in Figure 4 and additional spectra recorded at intermediate times.

1. This apparent correlation suggests that disruption of the PQQ environment at the active site is closely coupled to the loss of activity during thermal inactivation.

DISCUSSION

The thermal stability of MEDH is decreased by the replacement of enzyme-bound Ca^{2+} with Sr^{2+} as indicated by the 59 kJ (14 kcal)/mol decrease in E_a for the inactivation process. In apparent contradiction to this, the rate constant for the inactivation

of MEDH was greater than Sr-MEDH at each experimental temperature. The rate constant for the loss of absorbance of the peak at 350 nm, characteristic of the enzyme-bound PQQ prosthetic group, was also greater for MEDH than for Sr-MEDH at 68 °C. This can be explained by the values for the free energy of inactivation (ΔG^*) of the MEDHs at different temperatures (Table 1) which may be directly calculated from the first-order rate constants for the inactivation of the MEDHs by eqn. (4).

$$\Delta G^* = -RT \ln \frac{hk}{k_B T} \quad (4)$$

Over the range of temperatures studied, the 2-fold lower rate of inactivation of Sr-MEDH compared with MEDH yields values of ΔG^* for MEDH less positive than for Sr-MEDH. Because overall less energy is required to inactivate the enzyme at these temperatures, the rate of inactivation is more rapid in MEDH. These differences in ΔG^* are a consequence of the relative contribution of entropy at different temperatures as shown by eqn. (5):

$$\Delta G^* = \Delta H^* - T\Delta S^* \quad (5)$$

The more positive net gain in entropy for MEDH compared with Sr-MEDH results in the less positive ΔG^* . It is interesting to note that extrapolation of these data (Table 1) to lower temperatures indicates that values of ΔG^* for MEDH will become more positive than those for Sr-MEDH at temperatures below 55 °C (Figure 6). Therefore the overall stability of MEDH would be predicted to be greater than Sr-MEDH at physiological temperatures where the positive contribution of entropy is decreased.

The differences in the values of ΔH^* and ΔS^* between MEDH and Sr-MEDH are difficult to interpret. The ΔH^* and ΔS^* values for MEDH and Sr-MEDH are quite large, but within the range typical for protein-inactivation processes (Stearn, 1949). The thermodynamic values for protein inactivation are a result of cleavage of very large numbers of weak non-covalent bonds, as this process is not expected to involve the degradation of covalent bonds. Furthermore, loss of activity most likely occurs before the complete unfolding of an enzyme so that detailed accounting of structural perturbations leading to inactivation remains elusive and cannot be directly inferred from a crystal structure. Stearn (1949) introduced a method of interpreting thermodynamic values for enzyme inactivation which assumes average values of ΔH^* and ΔS^* , associated with the cleavage of a non-covalent bond, to estimate the number of bonds broken during the inactivation process. If the average ΔH^* required for each bond broken is assumed to be 21 kJ (5.0 kcal)/mol, then this would mean that the inactivation process involves breaking about 33 bonds in MEDH and about 30 bonds in Sr-MEDH. Alternatively, if it is assumed that an average of 50 J (12 cal)/mol per K is the net gain in ΔS^* per bond broken, then $\Delta S^*/50$ yields a value of about 35 bonds broken for MEDH and 31 bonds in Sr-MEDH. Thus these two independent calculations each suggest that there are approximately three to four more bonds broken in the inactivation of MEDH than in the inactivation of Sr-MEDH. As this is a dimeric enzyme, this analysis would suggest that there are about one or two more bonds broken per active site in MEDH than in Sr-MEDH. It must be kept in mind, however, that the ΔH^* and ΔS^* differences may represent a qualitative difference in the average bond strengths rather than a quantitative difference in the actual number of bonds. Interestingly, recent ^1H electron-nuclear double resonance studies showed that any effects of Sr^{2+} substitution in MEDH from *P. denitrificans* on the participation of the PQQ-derived radical in catalysis appear to be mediated by changes in the solvation environment of the radical

Figure 6 Overall free energy of inactivation for MEDH (○) and Sr-MEDH (▼)

Lines represent a straight-line fit to the data in Table 1 and have been extrapolated to lower temperatures to illustrate the points made in the text.

(Warncke et al., 1994). Thus the differences in the inactivation parameters between MEDH and Sr-MEDH could very well be due to the same structural perturbations that caused the differences in solvation at the active site.

Correlation of the loss of absorbance with loss of activity suggests that a disruption of the PQQ-binding site is a critical component of the inactivation process. Perturbation of the active-site geometry of MEDH on replacement with Sr^{2+} is also consistent with kinetic data obtained for Sr-MEDH (Harris and Davidson, 1994). The most dramatic changes observed in that study were of the steady-state kinetic parameters that described the binding of substrate and cyanide, which serves as an activator of MEDH at low concentrations. Crystallographic studies of the active site in MEDH show that Ca^{2+} is co-ordinated to six ligands (White et al., 1993). The pyridine nitrogen (N-6), the carboxylate oxygen at C-7 and the carbonyl oxygen at C-5 of the PQQ prosthetic group (Figure 1) comprise three of the co-ordinated ligands, and the two carboxy oxygen atoms of a glutamate side chain and the side-chain oxygen of an asparagine residue complete the geometry. The Sr^{2+} ionic radius of 1.13 Å (0.113 nm) is larger than the Ca^{2+} ionic radius of 0.99 Å, 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 glutamate or asparagine side-chain ligands co-ordinated with Ca^{2+} in MEDH. Thus a steric repositioning of PQQ by replacement of

Ca^{2+} with the larger Sr^{2+} could conceivably result in changes in the number or strength of non-covalent bonds in the vicinity of PQQ which change the solvation environment of PQQ and cause differences in the thermal stability of MEDH.

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