A New Kinetic Model for the Steady-State Reactions of the Quinoprotein Methanol Dehydrogenase from *Paracoccus denitrificans*†

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ABSTRACT: The reactions of methanol dehydrogenase from *Paracoccus denitrificans* with artificial electron acceptors, ammonia, cyanide, and substrates have been characterized by steady-state kinetic analysis. Phenazine ethosulfate, a commonly used electron acceptor for this enzyme, was shown to exhibit pronounced substrate inhibition with a K_i value approximately 20-fold lower than its K_m. Wurster's Blue exhibited only relatively mild substrate inhibition and was deemed a more appropriate electron acceptor. Ammonia was an obligatory activator of the enzyme at low concentrations and inhibited a high concentrations. The K_i value for this inhibition correlated closely with the K_d calculated from a titration of perturbations of the absorption spectrum of methanol dehydrogenase which were caused by the addition of ammonia. Cyanide, which suppressed the peculiar endogenous reaction of methanol dehydrogenase, was also both an activator of substrate-dependent activity and a competitive inhibitor with respect to methanol. Kinetic analysis indicated that the latter two activities corresponded to two distinct binding sites for cyanide. The K_a for cyanide activation correlated closely with the concentration required to inhibit 50% of the endogenous reaction, suggesting that a single binding event is responsible for both of these effects. A model is presented to describe the effects of ammonia and cyanide in the reaction cycle of methanol dehydrogenase, and the physiological relevance of the activation and inhibition by these compounds in vitro is discussed.

Methanol dehydrogenase is a bacterial quinoprotein which catalyzes the oxidation of methanol to formaldehyde in a variety of Gram-negative methylotrophic and autotrophic bacteria. It is a soluble protein that occurs exclusively in the periplasm of most bacteria growing on methane or methanol (Anthony, 1986, 1993). The potential importance of methylotrophic bacteria to biotechnology and the characterization of its redox cofactor as pyrroloquinoline quinone (PQQ)[1] (Figure 1; Salisbury et al., 1979) has stimulated interest in describing the reaction mechanism of methanol dehydrogenase and the role of the enzyme-bound PQQ in catalysis and electron transfer.

Paracoccus denitrificans has been the focus of several bioenergetic studies because of its versatility and the similarity of key components of its respiratory chain to those of the mitochondrial electron transport chain (John & Whatley, 1977). In addition to aerobic heterotrophic growth, it is capable of either aerobic or anaerobic autotrophic growth with carbon dioxide or single-carbon compounds, such as methanol or methylamine, as a sole source of carbon and energy. Regulation of the synthesis of a variety of electron transport proteins is mediated by growth conditions. For example, methanol and methylamine are initially oxidized, respectively, by two inducible periplasmic quinoproteins, methanol dehydrogenase (Bamford & Quayle, 1979) and methylamine dehydrogenase (Husain & Davidson, 1987). The electrons derived from these oxidations are then transferred by soluble redox proteins, which include the Type I copper protein amicyanin (Husain & Davidson, 1985) and *c*-type cytochromes (Husain & Davidson, 1986), to a membrane-bound cytochrome oxidase. Cytochrome *c*-551$_i$ appears to be the physiological electron acceptor for this methanol dehydrogenase (Long & Anthony, 1991a). These periplasmic redox enzymes and proteins of *P. denitrificans* provide a powerful system with which to study the principles of bioenergetics and electron transport.

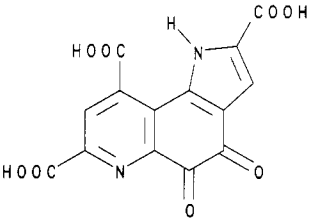

FIGURE 1: Structure of the PQQ prosthetic group of methanol dehydrogenase as deduced by Salisbury et al. (1979).

Although methanol dehydrogenase was first characterized in 1964 (Anthony & Zatman, 1964) from *Pseudomonas* sp. M27 and subsequently purified from several sources, there is still confusion in the literature regarding the steady-state kinetic properties of this enzyme and disagreement as to its reaction mechanism (Anthony, 1993). The study of this enzyme is complicated by the fact that the enzyme catalyzes the reduction of an electron acceptor in the absence of any added substrate (Anthony & Zatman, 1964; Duine & Frank, 1980; Ghosh & Quayle, 1981). This endogenous rate is not significantly stimulated by the addition of substrate. Furthermore, if allowed to react in this manner, the enzyme becomes inactivated. The commonly accepted explanation for this substrate-independent activity is that the enzyme or components of its assay are contaminated by an unidentified endogenous substrate. This endogenous activity is suppressed by cyanide, which also protects the enzyme against inactivation (Duine & Frank, 1980). Therefore, cyanide is usually included in the assay to allow the study of methanol-dependent activity. This endogenous activity is common to all methanol dehydrogenases but has never been fully characterized or explained. Studies of methanol dehydrogenase in vitro are further

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[1] Abbreviations: CHES, 2-(cyclohexylamino)ethanesulfonic acid; DCIP, 2,6-dichlorophenolindophenol; PES, phenazine ethosulfate; PMS, phenazine methosulfate; PQQ, pyrroloquinoline quinone.

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complicated by its high pH optimum, between pH 9.0 and 9.5, and the requirement for ammonia as an activator. For in vitro studies, most investigators have used as artificial electron acceptors the cationic dyes phenazine methosulfate (PMS) or phenazine ethosulfate (PES). Reduction of PMS or PES is measured spectrophotometrically by coupling its oxidation to the reduction of the dye 2,6-dichlorophenolindophenol (DCIP). As an alternative to PES, Wurster's Blue, the perchlorate salt of the cationic free radical of *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine, has also been used as an electron acceptor.

Despite the active interest in the periplasmic dehydrogenases and redox proteins of *P. denitrificans*, no detailed study of the kinetic properties of methanol dehydrogenase from this organism has been reported since its preliminary characterization (Bamford & Quayle, 1978). We have previously characterized reactions of the enzyme-bound PQQ with oxygen (Davidson et al., 1992a) and provided evidence for a structural role for calcium (Davidson et al., 1992b) in this enzyme. In this paper, we present a detailed steady-state kinetic analysis of the reactions of *P. denitrificans* methanol dehydrogenase with artificial electron acceptors, ammonia, cyanide, and substrates. These data reveal previously unrecognized dual roles for ammonia and cyanide, in which each acts as an activator at lower concentrations and as an inhibitor at higher concentrations. Based upon these data, a kinetic model is presented to describe the reactions of ammonia and cyanide with methanol dehydrogenase, and the physiological relevance of the activation and inhibition by these compounds in vitro is discussed.

EXPERIMENTAL PROCEDURES

P. denitrificans (ATCC 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. The periplasmic fraction of *P. denitrificans* was prepared as described previously (Davidson, 1990), and methanol dehydrogenase was purified from that fraction according to Davidson et al. (1992b). Wurster's Blue was synthesized from *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine according to the method of Michaelis and Granick (1943). All other reagents were purchased from commercial sources.

Steady-state kinetic assays were performed in 0.1 M potassium–2-(cyclohexylamino)ethanesulfonic acid [K-CHES] buffer, pH 9, at 30 °C. The endogenous and substrate-dependent activities of methanol dehydrogenase were assayed separately. Unless otherwise indicated the standard reaction mixture for the endogenous activity contained 10 μg/mL methanol dehydrogenase, 6 mM ammonium sulfate, and 3 mM Wurster's Blue. For the assay of substrate-dependent activity, the reaction 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 ammonium sulfate. The rates of the reaction were determined spectrophotometrically by monitoring the decrease in absorbance of Wurster's Blue at either 560 nm (ϵ = 12 300 M^{-1} cm^{-1}) or 640 nm (ϵ = 2776 M^{-1} cm^{-1}). For the assay of methanol dehydrogenase with PES and DCIP, the components of the assay mixtures were as described above except that Wurster's Blue was replaced with 1.8 mM PES and 0.17 mM DCIP and the concentration of ammonium sulfate was 10 mM. The reaction was initiated by the addition of ammonium sulfate and the rate of the reaction was monitored by the decrease in absorbance of DCIP at 600 nm (ϵ = 22 000 M^{-1} 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 analyzed by non-linear curve fitting with Enzfitter (Elsevier-BIOSOFT, Cambridge, England) and Sigma Plot 5.0 (Jandel Scientific, San Rafael, CA) computer programs. The equations used to fit the data are given under results.

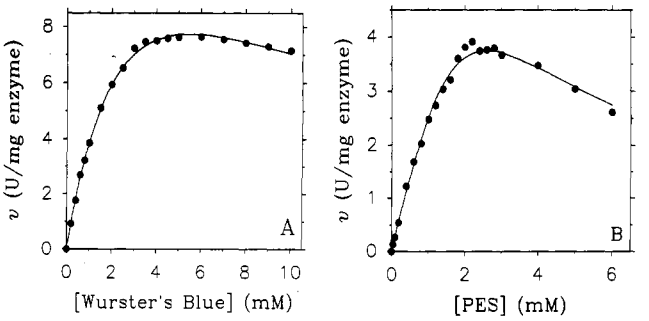


FIGURE 2: Reaction of artificial electron acceptors with methanol dehydrogenase. (A) Concentration dependence on Wurster's Blue for methanol-dependent activity. (B) Concentration dependence on PES for methanol-dependent activity. The solid line shows the fit of the data to eq 1.

RESULTS

Steady-State Kinetic Assays. Accurate and absolute determination of the kinetic parameters for methanol dehydrogenases is complicated by the high endogenous rate of reaction which the enzyme exhibits in the absence of added substrate. This activity required ammonia as an obligatory activator and was suppressed by KCN. Once the endogenous activity was inhibited by KCN, then methanol-dependent activity could be observed. A concentration of 3 mM KCN was required to give 100% inhibition of the endogenous reaction, while maximal methanol-dependent activity occurred at 6 mM KCN. The continued increase in activity observed at concentrations of KCN greater than that required to suppress the endogenous rate suggests an activation of the enzyme by KCN as well (described below). In each of the studies performed with this enzyme, kinetic parameters were determined for both the endogenous activity and the methanol-dependent activity in the presence of 6 mM KCN.

Reactions with Electron Acceptors. The first step in this investigation was to determine the kinetic parameters for Wurster's Blue and PES in both the endogenous and methanol-dependent activities of the enzyme. It should be pointed out that Wurster's Blue is a free radical so that it is reduced by one electron, whereas PES is a two-electron acceptor. The units of activities reported in this paper are defined as micromoles of electron acceptor reduced. Thus, in the case of Wurster's Blue, the observed rate must be divided by 2 to compare it with the results obtained with the two-electron acceptor PES.

Methanol dehydrogenase exhibited a maximal methanol-dependent activity of 7.6 units/mg of enzyme with approximately 6.0 mM Wurster's Blue (Figure 2A). Activity decreased slightly at concentrations greater than 6.0 mM. Because of this decrease, the data in Figure 2A were best fit to the equation for substrate inhibition:

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

Analysis of these data (Table I) yielded a K_m of 3.22 mM and

Table I: Steady-State Kinetic Parameters for the Reaction of Artificial Electron Acceptors with Methanol Dehydrogenase[a]

electron acceptor	kinetic constant	activity assayed: methanol-dependent	activity assayed: endogenous
Wurster's Blue	V_{max}	16.8 ± 0.7	17.7 ± 1.1
	K_m	3.22 ± 0.21	4.56 ± 0.38
	K_i	9.37 ± 0.86	7.19 ± 0.78
	V_{max}/K_m	5.22 ± 0.40	3.88 ± 0.40
PES/DCIP	V_{max}	36.9 ± 2.7	49.1 ± 11.8
	K_m	11.8 ± 1.0	16.1 ± 4.2
	K_i	0.59 ± 0.05	0.58 ● 0.20
	V_{max}/K_m	3.13 ± 0.35	3.05 ± 1.08

[a] The units of V_{max} are micromoles of Wurster's Blue or DCIP reduced per minute per milligram of enzyme, and those of K_m and K_i are millimolar. Activities were assayed as described under Experimental Procedures.

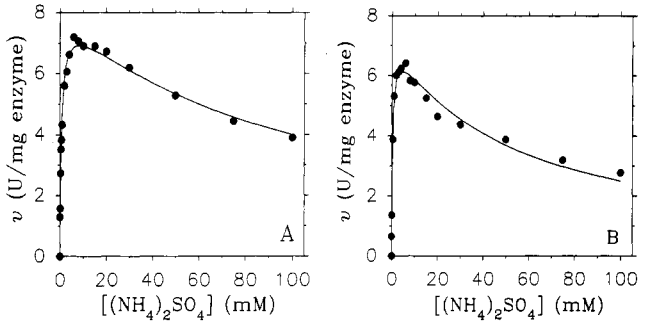


FIGURE 3: Interactions of ammonia with methanol dehydrogenase using Wurster's Blue as an electron acceptor. (A) Concentration dependence on ammonia for methanol-dependent activity. (B) Concentration dependence on ammonia for endogenous activity. The solid line shows the fit of the data to eq 1.

K_i of 9.37 mM. Because of the substrate inhibition, the calculated V_{max} of 16.8 units/mg was significantly higher than the observed maximum rate in Figure 2A. Similar results were observed for the endogenous reaction (Table I). When PES was used as an electron acceptor, a maximal methanol-dependent activity of 3.9 units/mg of enzyme was observed at approximately 2.0 mM (Figure 2B). Activity decreased dramatically at higher concentrations of PES. These data were also best fit to the equation for substrate inhibition. For PES, the apparent K_m value of 11.8 mM was considerably higher than the K_i value of 0.6 mM. Again, similar results were observed for the endogenous reaction (Table I). It should be emphasized that because of the inhibitory effects of both Wurster's Blue and PES at higher concentrations, it is not possible to saturate the enzyme with an excess of electron acceptor. Consequently, the theoretical calculated V_{max} for both electron acceptors reported in Table I is greater than the observed maximum activity. This problem is especially severe for PES given that the K_m value is nearly 20-fold greater than its K_i value. Given the extreme substrate inhibition by PES relative to the minor substrate inhibition observed with Wurster's Blue, the latter was primarily used as an electron acceptor in the subsequent studies.

Interactions of Ammonia with Methanol Dehydrogenase. Ammonia was an essential activator for both the endogenous and methanol-dependent reactions of methanol dehydrogenase. No activity was observed in the absence of ammonia. This was true over a pH range from 6 to 10 (data not shown). At pH 9, with Wurster's Blue as the electron acceptor, a maximum methanol-dependent activity of 7.2 units/mg of enzyme was observed in the presence of 6.0 mM ammonia (Figure 3A). The endogenous activity exhibited by methanol dehydrogenase was also maximal in the presence of 6.0 mM ammonia (Figure 3B). A decrease of activity was observed in the presence of

Table II: Steady-State Kinetic Parameters for the Interaction of $(NH_4)_2SO_4$ with Methanol Dehydrogenase[a]

activity	V_{max} (units/mg)	K_a (mM)	K_i (mM)
methanol-dependent	8.10 ± 0.26	0.71 ± 0.08	98.5 ± 13.8
endogenous	7.18 ± 0.20	0.38 ± 0.05	53.2 ± 5.4

[a] Activities were assayed with Wurster's Blue as described under Experimental Procedures.

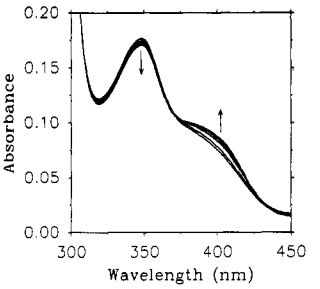


FIGURE 4: Effect of ammonia on the absorption spectra of methanol dehydrogenase. Methanol dehydrogenase (0.5 mg/mL) was incubated in 0.1 M K-CHES buffer, pH 9.0, at 30 °C. Spectra were recorded in the presence of 0, 10, 50, 100, 150, and 300 mM $(NH_4)_2SO_4$. Arrows indicate the direction of the spectral change with increasing concentration of $(NH_4)_2SO_4$.

concentrations of ammonia greater than 6.0 mM for both the endogenous and methanol-dependent activities. The data shown in Figure 3 were also fit to eq 1. This yielded K_a values for ammonia of 0.38 mM and 0.71 mM, respectively, for the endogenous and methanol-dependent reactions. K_i values for ammonia of 53.2 mM and 98.5 mM were obtained, respectively, for the endogenous and methanol-dependent reactions. The kinetic parameters for the reactions with ammonia are summarized in Table II. When PES was used as an electron acceptor, similar substrate inhibition at high concentrations of ammonia was also observed (data not shown).

It has been previously noted that no changes in the absorption spectrum of methanol dehydrogenase occur on the addition of ammonia (Anthony, 1992). We also observed no spectral change on the addition of ammonia at concentrations less than 6.0 mM. However, at higher concentrations in the range of the K_i value obtained in kinetic studies, small but reproducible ammonia-dependent changes in the absorption spectrum were observed (Figure 4). On addition of ammonia, the absorption peak at 350 nm decreased while the absorption of the shoulder region near 400 nm increased. Isosbestic points were located between the peak and shoulder region at 370 nm and also following the shoulder region at 430 nm. Data obtained from a titration of the increase in absorbance at 404 nm on addition of ammonia were fit to eq 2, which describes ligand binding to a single site where [X] is the concentration of added ammonium sulfate and C is the binding capacity in terms of the maximum ΔA. Analysis of these data yielded a K_d of 41.5 ± 4.5 mM.

$$\Delta A_{404nm} = \frac{C[X]}{K_d + [X]} \quad (2)$$

Thus, there appears to be a strong correlation between the concentration of ammonia required to perturb the absorption spectrum of methanol dehydrogenase and that which inhibits its endogenous activity.

Interactions of KCN with Methanol Dehydrogenase. The endogenous activity of methanol dehydrogenase was suppressed in the presence of KCN and exhibited 50% inhibition at 46.0 ± 4.6 μM KCN (Figure 5). To fully explore the role of KCN in the assay of methanol-dependent activity, the rates

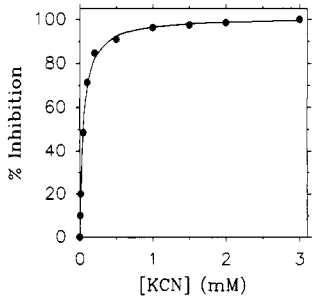


FIGURE 5: Inhibition by KCN of the endogenous activity of methanol dehydrogenase using Wurster's Blue as an electron acceptor.

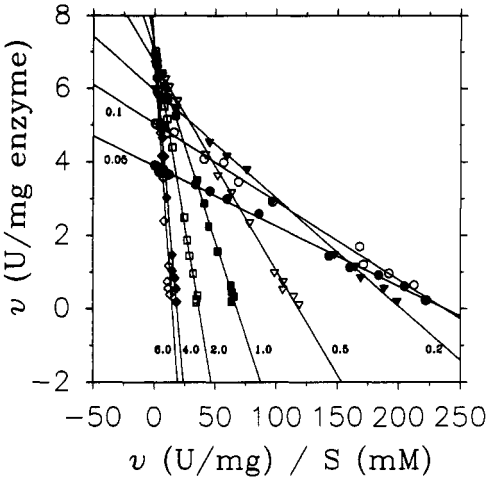


FIGURE 6: Effect of KCN on the steady-state reaction of methanol dehydrogenase with cyanide and methanol. The rates of methanol-dependent activity were assay in the presence of 0.05 (●), 0.10 (○), 0.20 (▼), 0.50 (▽), 1.0 (■), 2.0 (□), 4.0 (◆), and 6.0 mM (◇) KCN. The KCN concentrations are also indicated in the figure for clarity. Solid lines drawn through each data set are fit to eq 3.

of this activity were measured at varying methanol concentrations 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/[methanol] according to eq 3 (Figure 6), in which the y-intercept corresponds to the apparent V_{max} and the slope reflects the apparent K_m:

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

Plots of the apparent V_{max} and apparent K_m values for each concentration of KCN are given in Figure 7, panels A and B, respectively (equations for the fit of these data are given below). Activation by KCN is evident by the increase in V_{max} with increasing concentrations of KCN. However, it is clear that KCN also acts as an inhibitor as seen by the increase in K_m for methanol with increasing concentrations of KCN. A general mechanism to explain this dual role of KCN as an activator and an inhibitor is given by Scheme I, where A and I represent KCN as the activator and inhibitor, respectively. By making the rapid equilibrium assumption for substrate, activator and inhibitor binding, rate eq 4 was derived for this mechanism:

$$v = V_{max}[A][S]/\alpha K_A/\{K_s(1 + [A]/K_A + [A][I]/K_AK_I + [I]/\beta K_I) + [S](1 + [A]/\alpha K_A + [A][I]/\gamma K_I\alpha K_A + [I]/\delta K_I)\} \qquad (4)$$

This equation was fit with the 117 data points used to generate the plots in Figure 6. Although the convergence of the

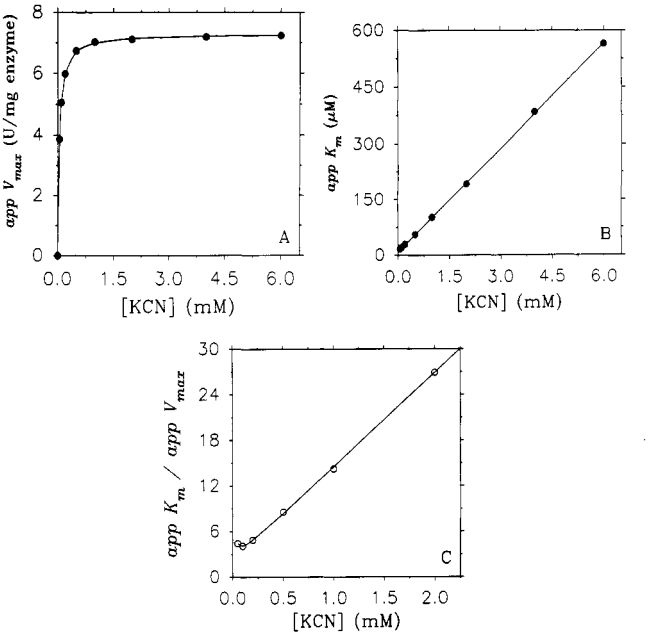
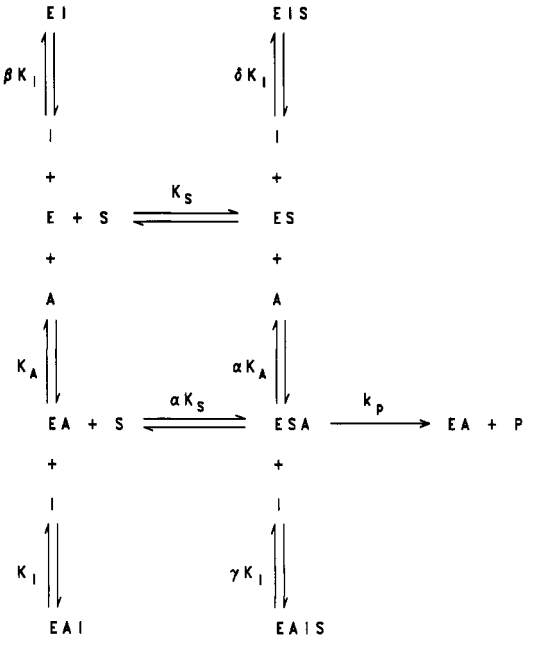


FIGURE 7: (A) Effect of KCN on the apparent V_{max} of the methanol-dependent activity of methanol dehydrogenase. (B) Effect of KCN on the apparent K_m of the methanol-dependent activity of methanol dehydrogenase. (C) Effect of KCN on the ratio of apparent K_m to apparent V_{max}. These are secondary plots in which the apparent V_{max} and K_m are deduced from the data shown in Figure 6. Solid lines drawn through the data in panels A and B are fits of the data, respectively, to eqs 6 and 7. The line in panel C is a fit of the data to an equation obtained by dividing eq 7 by eq 6.

Scheme I



computer fit of the data to eq 3 for the general mechanism was not smooth, it yielded values of 7.30 ± 0.01 units/mg for V_{max}, 14.9 ± 1.3 μM for K_s, 46.4 ± 5.7 μM for K_A, 155 ± 8 μM for K_I, and 0.953 ± 0.118 for α. The values for β, γ, and δ were each on the order of 10^6 with large standard errors. This suggested that the steps defined by βK_I, γK_I, and δK_I did not play a significant role in the reaction mechanism. The general mechanism (Scheme I) was then modified several times so that the steps defined by K_I, βK_I, γK_I, and δK_I could be tested individually and in all possible combinations with the other steps in this mechanism. The computer fits to the

Scheme II

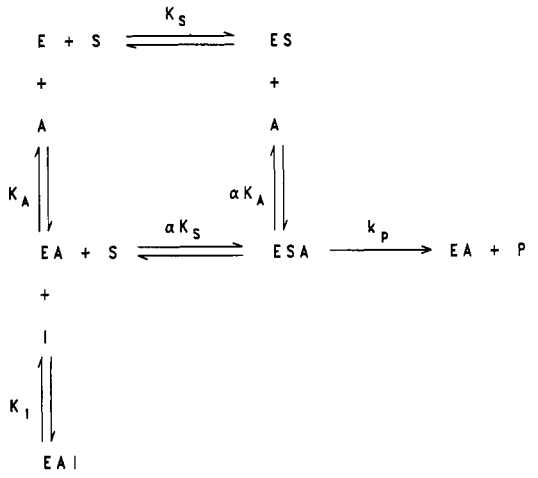


Table III: Steady-State Parameters for the Effect of KCN on the Methanol-Dependent Activity of Methanol Dehydrogenase[a]

parameter	value	parameter	value
V_{max}	7.30 ± 0.01 units/mg	α	0.954 ± 0.116
K_S	14.9 ± 1.2 μM	SSQ	0.223 (unit/mg)^2
K_A	46.4 ± 5.6 μM	σ	0.045 unit/mg
K_I	155 ± 8 μM		

[a] Activities were assayed with Wurster's Blue as described under Experimental Procedures. The values for the parameters shown in Scheme II were obtained from a computer fit of the data from Figure 6 to eq 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 nonlinear regression of the computer analysis, and σ is the standard deviation for the velocities. The value for σ was found from the equation $[SSQ/(N-K)]^{1/2}$, where N = number of data points = 117 and K = number of estimated parameters = 5.

rate equations describing any of these mechanisms which included steps defined by either βK_I, γK_I, or δK_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 II and which does not contain these steps. The rate equation derived for this mechanism is described by

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

The results of the computer fit of the data shown in Figure 6 to this equation are given in Table III. For this mechanism, a rapid and smooth convergence to a minimum sum of the squared residuals (SSQ) in the nonlinear regression analysis occurred. Rearrangement into the Michaelis–Menten form of eq 5 yielded eq 6 for the apparent V_{max} and eq 7 for the apparent K_m for methanol as a function of concentration of KCN. These equations were used to fit the data in Figure 7A,B.

$$\text{apparent } V_{max} = \frac{V_{max}[A]}{\alpha K_A + [A]} \quad (6)$$

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

Although the plot of apparent K_m versus KCN appears to indicate a simple linear relationship, the data are best fit to eq 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 7C). Deviation from linearity is evident in this plot, and these data are fit very well to an equation which is obtained by dividing eq 7 by eq 6. It should be noted that the true K_s for methanol can only be obtained by this type of analysis. Because KCN acts as a competitive inhibitor but is required as an activator, the observed K_m in the presence of the required KCN is much higher than the true calculated K_s value of 14.9 μM.

Table IV: Substrate Specificity of *P. denitrificans* Methanol Dehydrogenase[a]

substrate	V_{max} (units/mg)	K_m (mM)	V_{max}/K_m
methanol	7.33 ± 0.09	0.56 ± 0.02	13.1 ± 0.5
ethanol	7.47 ± 0.07	3.39 ± 0.14	2.20 ± 0.09
propanol	7.12 ± 0.09	10.7 ± 0.42	0.67 ± 0.03
butanol	7.61 ± 0.07	91.4 ± 0.28	0.83 ± 0.03
formaldehyde	12.6 ± 0.3	8.59 ± 0.59	1.47 ± 0.11

[a] Activities were assayed with Wurster's Blue as described under Experimental Procedures.

Substrate Specificity. To obtain information on the substrate specificity of methanol dehydrogenase, 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. In addition to methanol, other primary aliphatic alcohols were substrates for the enzyme under these conditions but with apparent K_m values significantly greater than that for methanol (Table IV). No reaction was observed with the secondary alcohols 2-propanol and isobutanol or with the tertiary alcohol *tert*-butanol. Formaldehyde was also a substrate for methanol dehydrogenase. It is interesting to note that although formaldehyde exhibited a relatively high K_m compared to the methanol, the V_{max} for formaldehyde was significantly higher than for the alcohols.

DISCUSSION

The most generally accepted kinetic model for the reaction cycle of methanol dehydrogenase is that proposed by Frank et al. (1988). It accounts for the role of ammonia as an activator and cyanide as an inhibitor of the enzyme. Our results support these aspects of their model. Their model, however, precludes simultaneous binding of substrate and cyanide and regards ammonia only as an activator. Given the new data concerning the previously unrecognized roles of ammonia as an inhibitor at high concentration and of cyanide as an activator at low concentration, a new model is presented (Figure 8) which extends the previous model and accounts for these dual roles of ammonia and cyanide. The rate equations derived from this model can accommodate well the steady-state kinetic data and yield reliable numbers for the rate constants which describe the interactions with ammonia, cyanide, and substrate (Tables II and III). In this model, enzyme 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. This cyanide binding site is clearly different from the substrate binding site as binding of S does not significantly affect the K_A for cyanide and binding of cyanide does not significantly affect K_s ($\alpha = 0.95$ in Scheme II). Inhibition by higher concentrations of cyanide is due to a separate, distinct binding phenomenon which is competitive with respect to substrate. Methanol dehydrogenase has an absolute requirement for ammonia when assayed with artificial electron acceptors. Stopped-flow kinetic studies with the

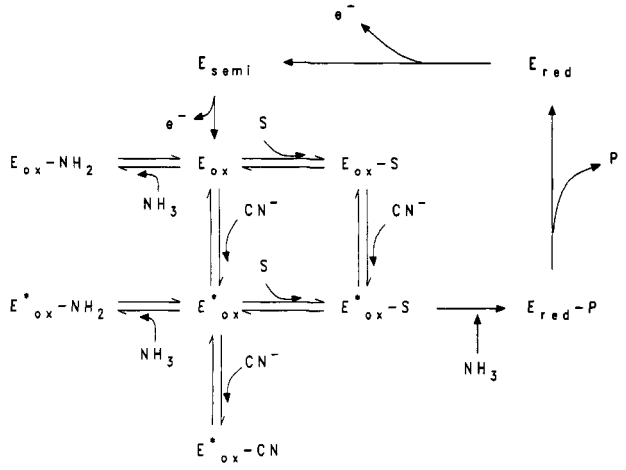


FIGURE 8: Proposed kinetic mechanism for the reaction cycle of methanol dehydrogenase. E* represents the cyanide-activated form of the enzyme. S and P represent, respectively, substrate and product.

enzyme from *Hyphomicrobium* X indicated that the magnitude of the deuterium isotope effect decreased in the presence of increased ammonia (Frank et al., 1988). This suggested that ammonia facilitated the transfer of hydrogen from methanol to PQQ. Therefore, included in this model is a role for ammonia in activating the conversion of the cyanide-activated oxidized enzyme–substrate complex to the reduced enzyme–product complex. The inhibition by ammonia is due to a completely distinct process which probably involves adduct formation with PQQ given the observed spectral perturbations caused by ammonia in the concentration range of the K_i for ammonia. Once product is released from the reduced enzyme, it is then reoxidized in two single-electron transfers to Wurster's Blue in vitro or to a *c*-type cytochrome in the physiological system.

Most of the kinetic parameters for *P. denitrificans* methanol dehydrogenase were determined in two sets of experiments using either Wurster's Blue or PES as the electron acceptor. This comparative analysis suggested that use of Wurster's Blue over PES was preferable for steady-state analysis. Particularly disturbing was the severe substrate inhibition exhibited by PES, in which the K_i value was actually 20-fold lower than the K_m for this electron acceptor. Conversely, only minor substrate inhibition is exhibited by Wurster's Blue. Also, the reduction of Wurster's Blue is monitored directly, thereby eliminating the need for an extra assay component in the mixture as in the case of PES which is further coupled to DCIP. Furthermore, it has been previously noted that oxygen competes with PES, but not Wurster's Blue, as an electron acceptor for the reduced form of PQQ in methanol dehydrogenase (Davidson et al., 1992a). The only real disadvantage of using Wurster's Blue is that it is not commercially available, however, it can be easily synthesized from a commercially available precursor. Given these data it is strongly recommended that Wurster's Blue, rather than PES, be used as an artificial electron acceptor in assays of methanol dehydrogenase activity.

It has been reported that the requirement for ammonia for catalysis is reduced in crude extracts which are prepared anaerobically (Duine et al., 1979) and that ammonia has little effect on the reaction of methanol dehydrogenase with its physiological cytochrome electron acceptor (Dijkstra et al., 1989). The relative decreased activation by ammonia observed when the cytochrome was used as an electron acceptor may be due to a change in the rate-limiting step of the overall reaction which was assayed or to an alteration of the ammonia binding site when the enzyme is in complex with the cytochrome (discussed below). The current study of the effects of ammonia on the endogenous and substrate-dependent reactions with the enzyme indicated that ammonia also acted as an inhibitor. It is known that ammonia reacts with free PQQ in solution to form an iminoquinone (Duine et al., 1987), and it has been proposed that this may be an intermediate in the reaction mechanism with the alcohol substrate (Forrest et al., 1980). This mechanism has been discounted, in part, because no perturbations of the absorption spectrum of methanol dehydrogenase were observed on addition of ammonia in concentrations sufficient to cause activation of the enzyme. The observation that perturbations of the absorption spectrum of the enzyme do occur on addition of ammonia (Figure 4), but only at the much higher concentrations which correlate with inhibition of activity, confirms that an iminoquinone intermediate is not necessary for catalysis but in fact may prevent catalysis. Conversely, activation by ammonia clearly must be a result of its interaction with methanol dehydrogenase at a different site than PQQ. Although the actual mechanism of inhibition by ammonia is not known, circumstantial evidence for iminoquinone formation may be inferred from model studies with PQQ derivatives. Iminoquinone formation from PQQ trimethyl ester and ammonia has been studied in organic solvents (Itoh et al., 1991). The visible spectra of the quinone and the iminoquinone are similar but the latter exhibits a slightly enhanced absorbance in the range from approximately 350 to 430 nm with an isosbestic point at approximately 430 nm. These spectral changes upon iminoquinone formation are similar to what has been observed in Figure 4. Other explanations for these spectral perturbations, however, cannot be ruled out. For example, ammonium ion may also be acting as a counterion to oxyanions in the active site and in doing so perturbs the microenvironment of the cofactor in such a way as to alter the spectrum.

It is important to note that the concentration of KCN which is present in the assay will dramatically influence the observed K_m value for methanol (Figure 7B). Cyanide, which is known to inhibit the endogenous activity of the enzyme, has also been previously reported to be a competitive inhibitor with respect to methanol (Duine & Frank, 1980). The effects that were observed here, however, are more complex than simple competitive inhibition. While the K_m of methanol did increase with increasing KCN, the V_{max} for the enzyme also increased with KCN (Figure 7A). This indicated that some activation of the enzyme by cyanide occurred concomitant with the competitive inhibition. These data suggest a mechanism which requires cyanide to react at two separate sites, one for activation and one for inhibition of the methanol-dependent reaction. The K_A for cyanide activation correlates closely with the concentration of cyanide that is required to obtain 50% inhibition of the endogenous activity. This suggests that the same cyanide interaction is responsible for both inhibiting the endogenous activity and activating the methanol-dependent activity of methanol dehydrogenase. This further suggests that the endogenous substrate is *not* methanol, which has been suggested to be present as a contaminant from commercial buffer components that have been crystallized from methanol-containing solutions. That the kinetic parameters describing the interactions of electron acceptors and ammonia with methanol dehydrogenase were very similar for the endogenous and methanol-dependent reactions suggests that the presence of 6 mM KCN did not significantly affect these interactions.

Although it is clear that ammonia and cyanide play critical roles in the reaction cycle of methanol dehydrogenase in vitro,

the physiological relevance of these effects, particularly the activation by these compounds, is unclear and requires some discussion. It is possible that ammonia and/or cyanide, as well as the high pH requirement for in vitro activity, are affecting subtle structural changes in methanol dehydrogenase which mimic the normal in vivo form of the enzyme. There is some rationale for proposing allosteric regulation of the activity of methanol dehydrogenase in vivo. A modifier protein (M-protein) has been partially purified from the periplasms of *P. denitrificans* and *Methylophilus methylotrophus*. It has been shown to act as a regulator of methanol dehydrogenase activity and proposed to play such a physiological role in vivo (Long & Anthony, 1991b). It is also quite possible that the natural electron acceptor of methanol dehydrogenase, cytochrome c-551_i in *P. denitrificans* and cytochrome c_L in many other methylotrophic bacteria, may form a complex in vivo which affects the kinetic parameters for the reductive half-reaction of the enzyme. It has been demonstrated for methylamine dehydrogenase, another periplasmic quinoprotein from *P. denitrificans*, that complex formation with its physiological electron acceptor, a Type I copper protein, causes changes in certain physical properties of its quinone cofactor (Gray et al., 1988) and alters the kinetic parameters for the reductive half-reaction of that enzyme (Davidson & Jones, 1991). Thus, it is possible that, in their roles as activators, ammonia and cyanide may be binding to methanol dehydrogenase in a manner which either induces conformational changes or modifies amino acid residues in such a way as to mimic the in vivo form of the enzyme when it is in contact with the modifier protein, cytochrome c-551_i, or some other component of the cell.

Given the model proposed in Figure 8 as a working hypothesis and the capability of extracting values for kinetic constants which describe each step in that model, we are in a position to pursue mechanistic studies with methanol dehydrogenase. It will be possible to modify the enzyme and alter reaction conditions in an attempt to selectively alter the kinetic parameters for substrates and the activation and inhibition by ammonia and cyanide. The complete amino acid sequence of *P. denitrificans* methanol dehydrogenase is known (Harms et al., 1987; von Spanning et al., 1991). Furthermore, the structures of two homologous methanol dehydrogenases have recently been determined from X-ray crystallographic studies (Xia et al., 1992). Thus, it should also be possible to correlate such mechanistic studies with structural information.

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