Kinetic isotope effects and ligand binding in PQQ-dependent methanol dehydrogenase

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The reaction of PQQ (2,7,9-tricarboxypyrroloquinoline quinone)-dependent MDH (methanol dehydrogenase) from *Methylophilus methylotrophus* has been studied under steady-state conditions in the presence of an alternative activator [GEE (glycine ethyl ester)] and compared with similar reactions performed with ammonium (used more generally as an activator in steady-state analysis of MDH). Studies of initial velocity with methanol (protiated methanol, C¹H₃O¹H) and [²H]methanol (deuteriated methanol, C²H₃O²H) as substrate, performed with different concentrations of GEE and PES (phenazine ethosulphate), indicate competitive binding effects for substrate and PES on the stimulation and inhibition of enzyme activity by GEE. GEE is more effective at stimulating activity than ammonium at low concentrations, suggesting tighter binding of GEE to the active site. Inhibition of activity at high GEE concentration is less pronounced than at high ammonium concentration. This suggests a close spatial relationship between the stimulatory (K_S) and inhibitory (K_I) binding sites in that binding of GEE to the K_S site sterically impairs the binding of GEE to the K_I site. The binding of GEE is also competitive with the binding of PES, and GEE is more effective than ammonium in competing with PES. This competitive binding of GEE and PES lowers the effective concentration of PES at the site competent for electron transfer. Accordingly, the oxidative

half-reaction, which is second-order with respect to PES concentration, is more rate-limiting in steady-state turnover with GEE than with ammonium. The smaller methanol C-¹H/C-²H kinetic isotope effects observed with GEE are consistent with a larger contribution made by the oxidative half-reaction to rate limitation. Cyanide is much less effective at suppressing 'endogenous' activity in the presence of GEE than with ammonium, which is attributed to impaired binding of cyanide to the catalytic site through steric interaction with GEE bound at the K_S site. The kinetic model developed previously for reactions of MDH with ammonium [Hothi, Basran, Sutcliffe and Scrutton (2003) *Biochemistry* **42**, 3966–3978] is consistent with data obtained with GEE, although a more detailed structural interpretation is given here. Molecular-modelling studies rationalize the kinetic observations in terms of a complex binding scenario at the molecular level involving two spatially distinct inhibitory sites (K_I and K_I'). The K_I' site caps the entrance to the active site and is interpreted as the PES binding site. The K_I site is adjacent to, and, for GEE, overlaps with, the K_S site, and is located in the active-site cavity close to the PQQ cofactor and the catalytic site for methanol oxidation.

Key words: enzyme activator, kinetic isotope effects, kinetic mechanism, methanol dehydrogenase, quinoprotein.

INTRODUCTION

MDH (methanol dehydrogenase; EC 1.1.99.8) catalyses the oxidation of methanol to formaldehyde and utilizes a specific cytochrome *c* as electron acceptor [1,2]. The enzyme contains PQQ (2,7,9-tricarboxypyrroloquinoline quinone) [3] and adopts a $\alpha_2\beta_2$ structure [4–6]. A calcium ion acts as a Lewis acid during substrate oxidation and is co-ordinated to PQQ in each α subunit. Hydride transfer and addition–elimination mechanisms have been proposed for methanol oxidation by MDH [7], but computational and high-resolution crystallographic studies favour a hydride-transfer mechanism (Scheme 1) [8]. A similar scheme was also proposed for PQQ-dependent glucose dehydrogenase [9].

MDH is assayed by reduction of PES (phenazine ethosulphate) and DCPIP (2,6-dichlorophenol-indophenol) [10]. Ammonium salts are required for activity [10], but are also inhibitory at high concentration [11]. In the absence of added methanol, dye reduction (the so-called 'endogenous' activity) is observed and is attributed to contaminating alcohols and aldehydes in laboratory

reagents and the broad specificity of MDH [12–14]. Additionally, dealkylation of PES, especially at high pH, leads to aldehyde production and contributes further to endogenous activity [15]. Cyanide is a competitive inhibitor of MDH with respect to substrate and also suppresses endogenous activity.

The main features of the reaction cycle have emerged from kinetic studies using artificial electron acceptors [14,16], and extended to studies with the physiological acceptor cytochrome *c*_L for *Hyphomicrobium X* and *Paracoccus denitrificans* MDH respectively [11,17]. On the basis of our previous studies using ammonium as an activator, a reaction cycle has been proposed for *Methylophilus methylotrophus* MDH (Scheme 2). In steady-state turnover, ammonium salts can be replaced by methylamine, but not by diamines, triamines or long-chain alkylamines [18]. MDH enzymes isolated from *Hyphomicrobium X* and *M. methylotrophus* are active in the presence of glycine esters and β -alanine esters, but not with aliphatic amines or amino acids ([19]; M. Beardmore-Gray and C. Anthony, unpublished work cited in [18]). MDH isolated from *Rhodospseudomonas acidophila* is

Abbreviations used: DCPIP, 2,6-dichlorophenol-indophenol; GEE, glycine ethyl ester; K_I, kinetic constant describing inhibitory effects of activator on enzyme activity; K_I', structurally defined inhibitory binding site in the active site of methanol dehydrogenase; K_I'', structurally defined inhibitory binding site for phenazine ethosulphate binding in the entrance to the active site cavity of methanol dehydrogenase; K_IE, kinetic isotope effect; K_S, kinetic constant describing stimulatory effects of activator on enzyme activity; K_S', structurally defined stimulatory binding site in the active site of methanol dehydrogenase; MDH, methanol dehydrogenase; PES, phenazine ethosulphate; PQQ, 2,7,9-tricarboxypyrroloquinoline quinone.

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Scheme 1 Mechanism of methanol oxidation by MDH

Scheme 2 Proposed kinetic mechanism for the reaction cycle of *M. methylotrophus* MDH with ammonium as activator (based on our recent work [21])

A represents artificial electron acceptor; S and P represent substrate and product respectively. The asterisk (*) indicates competitive binding of ammonium with respect to the binding of the artificial electron acceptor and methanol. Two binding sites for ammonium are shown, corresponding to the K_S and K_I sites. The rate of enzyme reoxidation by electron transfer to the artificial electron acceptor, A, is also affected by competitive binding of ammonium to the electron acceptor site. The species MDH_{red} and MDH_{sq} represent different forms of reduced MDH representing a distribution of ammonium-bound and ammonium-free forms. For clarity, these different forms have been represented by single species for each oxidation state (i.e. MDH_{red} and MDH_{sq}).

unusual in being activated by a wide range of primary alkylamines, as well as ammonium [18]. The enzyme from *Methylobacterium organophilum* is exceptional in displaying substantial ammonium-independent activity [20].

Reaction profiles for *M. methylotrophus* MDH as a function of ammonium and PES concentration differ between methanol (protiated methanol, $C^1H_3O^1H$) and $[^2H]$ methanol (deuterated methanol, $C^2H_3O^2H$). These differences have been attributed to force constant effects on the binding of substrate to stimulatory and inhibitory ammonium-binding sites and the competitive binding of ammonium and PES [21]. In turn, this gives rise to an unusual dependence of the observed KIEs (kinetic isotope effects) on ligand concentration [21]. Modelling studies have suggested that the stimulatory and inhibitory activator binding sites in the active site of *M. methylotrophus* MDH are in close proximity [21]. The observed KIEs at different ligand concentrations are independent of temperature, consistent with their origin in differential binding affinities of 1H - and 2H -labelled substrate at the ammonium binding sites [21]. Given the complex interplay be-

tween ammonium binding at the stimulatory and inhibitory binding sites, and the dependence of reaction rate and observed KIEs on ammonium concentration, we have extended our kinetic studies to include the use of the alternative and larger activator GEE (glycine ethyl ester). This is the first report of a detailed kinetic study with GEE. Our studies establish a general kinetic scheme for MDH regardless of activator type, and further support the notion of a close spatial relationship between the stimulatory, inhibitory activator binding sites and the catalytic substrate-binding site. Although the kinetic model is qualitatively similar for reactions performed with GEE or ammonium as activator, major quantitative differences in steady-state parameters are observed. These differences (i) impact substantially on the observed KIEs, (ii) can be rationalized in the context of the general reaction scheme for MDH and (iii) reflect competitive binding and substrate force constant effects of the C^1H-C^1H bond in methanol on the binding of GEE and methanol at the stimulatory and inhibitory activator binding sites. Additionally, we identify by modelling the binding site for PES at the entrance to the active-site cavity and provide evidence for competitive binding of activator at this site. Our molecular-modelling studies rationalize the kinetic observations in terms of a complex binding scenario involving multiple ligand sites both in the active site of MDH and in the entrance to the active site cavity.

EXPERIMENTAL

Materials

Ches [2-(*N*-cyclohexylamino)ethanesulphonic acid], Mes, DCPIP sodium salt, PES (as *N*-ethylidibenzopyrazine ethyl sulphate salt), Cyanide (KCN) and GEE hydrochloride were obtained from Sigma. $(NH_4)_2SO_4$ and methanol were from Fisher. $[^2H]$ Methanol (99.8 %) was from Aldrich. The chemical purity of the $[^2H]$ methanol was determined by the suppliers to be > 99 % by HPLC, NMR and GLC.

Purification of MDH

M. methylotrophus (strain W₃A₁) was grown aerobically at 30 °C on 0.5 % (v/v) methanol as described in [10,21]. MDH was purified as described in [21]. The enzyme was judged to be pure by SDS/PAGE and an A_{280}/A_{345} ratio of 11.3 [22]. MDH was purified in the semiquinone form (ϵ_{343} 28 300 M⁻¹ · cm⁻¹ [16]).

Steady-state kinetic analysis

Steady-state kinetic measurements were performed using a cell of 1 cm light path and 0.1 M Ches buffer, pH 9.0, at 25 °C (unless stated otherwise) in a total volume of 1 ml. MDH activity was measured using a dye-linked assay system in which the reduction of PES is monitored by coupling its oxidation to the reduction of

DCPIP at 600 nm as described in [21]. Addition of GEE to reaction mixtures resulted in a reaction pH lower than 9.0. Stock solutions of GEE were therefore titrated with KOH to $\approx$ pH 8.0 and added to 0.1 M Ches buffer ranging from pH 9 to 10.0. The required ester concentration (see the Results section) was added to the appropriate pH buffer, ensuring that reactions were maintained at pH 9.0 at all GEE concentrations. It was not feasible to use GEE in excess of 60 mM, as higher concentrations resulted in a pH lower than 9.0. Reaction mixtures typically contained KCN (6 mM; to suppress endogenous enzyme activity in the absence of added methanol), PES (1 mM), DCPIP (0.04 mM) and 20 nM MDH. $(\text{NH}_4)_2\text{SO}_4$ (0.1–200 mM), GEE (0.5–60 mM) and methanol (or ^2H methanol) were added to the reaction mix at the appropriate concentration (see the Results section). Initial velocity was expressed as μmol of product formed/s per μmol of enzyme, using an ϵ_{600} of $22\,000\text{ M}^{-1}\cdot\text{cm}^{-1}$ for DCPIP [23]. Initial-velocity data as a function of methanol concentration were analysed by fitting results to the standard Michaelis–Menten rate equation. Initial-velocity data collected as a function of activator concentration ($[\text{L}]$) were fitted to eqn (1):

$$v = \frac{\left(1 + \frac{b[\text{L}]}{K_i}\right) V_{\max}}{1 + \frac{K_s}{[\text{L}]} + \frac{K_s}{K_i} + \frac{[\text{L}]}{K_i}} \quad (1)$$

where K_s and K_i are the activation and inhibition constants for activator respectively, $V_{\max}$ is the theoretical maximum rate and b is a factor by which the $V_{\max}$ is adjusted because of inhibition. The use of eqn (1) in studies of MDH has been described [11]. In some cases, initial-velocity data collected as a function of activator concentration were also analysed by fitting to the standard Michaelis–Menten hyperbolic expression (see the Results section).

Molecular modelling

Docking studies were performed using the program GOLDv2.0 [24] with the ChemScore [25,26] fitness function. Ligands were docked into the crystal structure of MDH [8] {PDB (Protein Data Bank) [27] accession code 1g72} and the solutions ranked according to the value of the ChemScore fitness function. Inspection of the results was performed using the molecular visualization package InsightII [28]. Normal mode analysis studies were performed on the crystal structure of MDH (1g72) using the eINémo [29] server.

RESULTS

Effect of GEE on the catalytic methanol-binding site

Our previous studies indicated that PES and cyanide, but not ammonium, interfere with the binding of methanol to the catalytic methanol-binding site [21]. Prior to embarking upon a detailed study of MDH activity with the alternative activator GEE, we analysed the effect of GEE concentration on the apparent Michaelis constant for methanol.

Steady-state turnover assays were performed with methanol in the presence of various fixed concentrations of GEE (5–60 mM) at 25 °C. Cyanide and PES were held constant at 6 mM and 1 mM respectively. Plots of initial velocity versus methanol concentration were hyperbolic (Figures 1A and 1B) and kinetic constants were derived by fitting results to the Michaelis–Menten expression. The apparent K_m for methanol decreases as GEE is increased (Figure 1C). This suggests that GEE is impairing the binding of either (or both) PES or cyanide, consistent with the known

Figure 1 Plots of initial velocity (v_i) versus methanol concentration in the presence of GEE

Conditions were as follows: 0.1 M Ches, pH 9.0, various fixed concentrations of GEE, 6 mM cyanide (KCN) and 1 mM PES at 25 °C. (A) Plot of initial velocity versus methanol concentration in the presence of 5 mM GEE. (B) As (A), but in the presence of 60 mM GEE. Similar plots were also collected using 10, 12, 15, 20 and 40 mM GEE (results not shown). Apparent Michaelis constants (K_m) were determined by fitting initial-velocity data to the Michaelis–Menten expression. (C) Plot of apparent K_m for methanol versus GEE concentration.

competitive effects of PES and cyanide on methanol binding [21]. The apparent K_m obtained with 20 mM $(\text{NH}_4)_2\text{SO}_4$ ($1.06 \pm 0.05\text{ mM}$ [21]) is significantly lower with 20 mM GEE ($0.06 \pm 0.008\text{ mM}$). This implies that binding of methanol is tighter in the presence of GEE, which is consistent with apparent weaker binding of cyanide at the catalytic methanol-binding site. Assays performed with ^2H methanol produced apparent K_m values similar to those obtained with unlabelled methanol (results not shown).

Figure 2 Plots of initial velocity (v_i) for endogenous activity and methanol-dependent activity versus GEE concentration and variation in activity with cyanide (KCN)

(A) Variation in endogenous activity with GEE and cyanide concentration. Conditions were as follows: 0.1 M Ches, pH 9.0, GEE (0.5–60 mM), 1 mM PES and no added substrate at 25 °C. Closed circles, open squares, closed triangles, open circles and closed squares represent KCN concentrations of 1, 3, 6, 12 and 20 mM respectively. (B) Variation in methanol-dependent activity with GEE and cyanide concentration. Conditions were as for (A), but with 50 mM methanol. Closed circles, open squares and closed triangles represent cyanide concentrations of 1, 6 and 20 mM respectively (results were also collected in the presence of 3 and 12 mM cyanide, but are not shown for clarity). Fits shown are those to the inhibition expression (eqn 1), but data were also fitted to the general Michaelis–Menten hyperbolic expression. Parameters determined from both equations are summarized in Table 1. (C) Variation in catalytic-centre activity ('turnover number') with cyanide concentration for endogenous (closed triangles) and methanol-dependent activity (closed circles) generated from data shown in (A) and (B). (D) Variation in apparent K_s (mM) for GEE with cyanide concentration for endogenous (closed triangles) and methanol-dependent reactions (closed circles) generated from the data shown in (A) and (B).

Effect of GEE and cyanide on endogenous and methanol-dependent activity

Our previous studies established that endogenous activity is negligible when the enzyme is assayed with $(\text{NH}_4)_2\text{SO}_4$ in the presence of 6 mM cyanide [21]. With GEE, however, significant endogenous turnover was observed in the presence of 6 mM cyanide, prompting a more detailed analysis of the relationship between endogenous activity, GEE and cyanide concentration. Assays were performed using increasing concentrations of GEE (0.5–60 mM) in the presence of various fixed concentrations of cyanide (1, 3, 6, 12 and 20 mM) at 25 °C. PES was kept constant at 1 mM, and no substrate was added. Plots of endogenous activity versus GEE concentration displayed a hyperbolic dependence (Figure 2A), with little evidence of inhibition at high GEE concentration. This contrasts with initial-velocity data collected as a function of ammonium concentration, which are best fitted to an inhibition expression (eqn 1) because of the inhibitory effects observed at higher ammonium concentrations [21]. With GEE, endogenous turnover is appreciably high in the presence of 20 mM KCN (Figure 2A). The lack of significant inhibition observed to 60 mM GEE generates large errors in fitting the data shown in Figure 2A (and Figure 2B) to eqn (1), although inhibition might become more apparent at higher GEE concentrations. That said,

in using a hyperbolic fit, small inhibitory effects that might occur at high GEE concentration are not taken into account. For this reason, kinetic parameters derived from fits of the data to eqn (1) and the standard Michaelis–Menten hyperbolic expression are shown in Table 1. Qualitatively similar results were obtained by fitting to both equations.

Catalytic-centre activities ('turnover numbers') for endogenous activity (Table 1) reveal that there is a mild suppression of endogenous activity with increasing cyanide concentration (Figure 2C), establishing the notion that cyanide does suppress GEE-dependent endogenous activity in MDH (albeit to a much weaker extent than with ammonium). Also, for endogenous activity, the apparent K_s value for GEE increases with cyanide concentration (Table 1; Figure 2D). This suggests that binding of GEE to the stimulatory activator binding site gradually weakens on addition of cyanide. The lack of suppression of endogenous activity with relatively high concentrations of cyanide, and the observed changes in the K_s values, support further the proposal that GEE and cyanide compete for common or overlapping sites in MDH. The data emphasize the close spatial relationship of the stimulatory activator-binding site and the catalytic methanol-binding site.

Corresponding plots for methanol-dependent activity are shown in Figure 2(B). In this case, the apparent K_m for methanol is

Table 1 Kinetic parameters determined from steady-state reactions of MDH with GEE in the presence of different cyanide (KCN) concentrations

Parameters for endogenous activity and methanol dependent activity were obtained by fitting of data to (a) the standard Michaelis–Menten hyperbolic expression or (b) the inhibition expression (eqn 1). Corresponding plots are shown in Figure 2.

(a) Hyperbolic expression

[KCN] (mM)	k_{cat} (s^{-1})		K_s (mM)	
	Endogenous	Methanol	Endogenous	Methanol
1	5.31 ± 0.08	4.41 ± 0.09	7.18 ± 0.38	4.62 ± 0.49
3	5.22 ± 0.13	4.27 ± 0.08	15.15 ± 1.06	4.57 ± 0.31
6	4.95 ± 0.12	4.24 ± 0.06	26.38 ± 2.36	4.46 ± 0.27
12	4.75 ± 0.19	4.31 ± 0.05	35.46 ± 2.89	4.61 ± 0.29
20	4.33 ± 0.23	4.18 ± 0.08	40.56 ± 2.08	4.59 ± 0.37

(b) Inhibition expression

[KCN] (mM)	k_{cat} (s^{-1})		K_s (mM)	
	Endogenous	Methanol	Endogenous	Methanol
1	6.11 ± 0.55	3.75 ± 0.16	9.01 ± 2.92	3.59 ± 0.49
3	5.72 ± 0.93	3.67 ± 0.14	13.67 ± 1.98	3.25 ± 0.47
6	5.35 ± 1.01	3.92 ± 0.18	24.23 ± 3.81	3.75 ± 0.44
12	5.03 ± 0.69	3.71 ± 0.15	30.23 ± 3.29	3.31 ± 0.36
20	4.55 ± 0.41	3.54 ± 0.19	36.99 ± 3.65	3.14 ± 0.44

0.45 ± 0.04 mM in the presence of 5 mM GEE, and this value decreases with increasing GEE concentration (Figure 1C). At low GEE concentrations (< 5 mM), the apparent K_m for methanol is > 0.45 mM. With this in mind, a methanol concentration of 50 mM was used in subsequent experiments to ensure saturation of the catalytic site with substrate. As with endogenous activity, plots of methanol-dependent activity versus GEE concentration are hyperbolic (Figure 2B). Kinetic parameters derived from eqn (1) and the standard hyperbolic expression are shown in Table 1. In contrast with endogenous turnover, methanol-dependent turnover (i.e. apparent k_{cat}) is not affected by cyanide concentration (Table 1; Figure 2C). Furthermore, cyanide has no effect on the stimulation of activity by GEE (K_s remains constant ≈ 4 mM) in the presence of methanol (Figure 2D; Table 1). These observations are consistent with our proposed kinetic mechanism for MDH (Scheme 2) in which cyanide is displaced from the catalytic methanol-binding site on the addition of methanol. Thus, cyanide concentration affects the apparent k_{cat} values and the stimulation of activity by GEE (reflected in altered K_s values) only for reactions of MDH with endogenous substrates (Figures 2A, 2C and 2D).

The relationship between endogenous substrates, methanol, GEE and cyanide is demonstrated further in supplementary Figure S1 (<http://www.BiochemJ.org/bj/388/bj3880123add.htm>). At saturating substrate concentration, cyanide has little effect on initial velocities measured in the presence of methanol and [^3H]-methanol. This is similar to observations made with ammonium as activator [21]. Unlike ammonium-dependent endogenous activity (supplementary Figure S1C; <http://www.BiochemJ.org/bj/388/bj3880123add.htm>), the effective concentration of cyanide at the active site of MDH is reduced through competitive binding with GEE, and consequently suppression of GEE-dependent endogenous activity is reduced (supplementary Figures S1A and S1B; <http://www.BiochemJ.org/bj/388/bj3880123add.htm>). Clearly, inhibition of endogenous activity by GEE requires much higher concentrations of cyanide, and these observations are consistent with the trends displayed in Figure 2.

GEE as an activator of methanol-dependent activity

Having established that the effects of cyanide are restricted to endogenous substrates only, a more detailed analysis of the effects of GEE concentration on methanol-dependent activity was possible. With ammonium, reactions are stimulated at low concentrations, but higher concentrations partially inhibit enzyme activity [21]. Also, the extent of inhibition decreases with increasing temperature. In our previous work we defined two structural sites for ammonium binding close to the PQQ cofactor, i.e. the stimulatory site (K_s) and the inhibitory site (K_i) [21]. With GEE, an inhibitory effect was not observed at 25 °C (Figure 2B). This might be attributable to either (i) occupancy by GEE of the stimulatory site (K_s), which hinders the binding, through steric interactions, of a second GEE molecule to the nearby inhibitory site (K_i), or (ii) an inhibitory site for GEE not existing in the active site of MDH. Kinetic studies were performed to distinguish between these two possibilities.

Methanol-dependent activity was studied as a function of GEE concentration at 5 °C, where, by analogy with reactions performed with ammonium, any inhibitory effects are expected to be more prominent [21]. Assays were performed using GEE (range 0.5–60 mM), 1 mM PES and 50 mM methanol at 5 °C. Cyanide (KCN, 6 mM) was included to allow comparison with ammonium-dependent activity, which was measured in the presence of 6 mM cyanide [21]. Initial-velocity data at 5 °C indicated inhibition of activity at high GEE concentration (Figure 3B), consistent with the binding of GEE to an inhibitory site. The large errors, however, associated with fitting to eqn (1) prevent precise determination of K_i values for comparison with ammonium-dependent data.

Changes in PES concentration affect the K_s and K_i values for ammonium binding. Low PES concentrations (200 μM) lead to smaller K_s and K_i values for ammonium binding, and larger inhibitory effects with ammonium are observed compared with assays performed at 1 mM PES [21]. Further evidence for the existence of a K_i site for GEE was obtained by investigating methanol-dependent activity at 25 °C (where inhibition is minor with 1 mM PES) as a function of GEE concentration, but with 200 μM PES. In this case, the extent of inhibition by GEE was found to be comparable at 1 mM and 200 μM PES (Figure 3C and 3D), which contrasts with observations made with ammonium. The data suggest that occupation of the K_s site by GEE sterically impairs GEE binding at the K_i site. This is consistent with (i) the larger volume occupied by GEE compared with ammonium and (ii) the close spatial relationship between the K_s and K_i sites.

Fitting of data from plots of initial velocity versus GEE concentration using eqn (1) has allowed quantitative analysis of binding at the K_s site for GEE- and ammonium-dependent reactions. With 1 mM PES, the K_s values for GEE and ammonium are 3.4 ± 0.3 mM and 11.7 ± 2.8 mM respectively (Figures 3A and 3B). With 200 μM PES, the K_s values for GEE and ammonium are 0.28 ± 0.07 mM and 1.0 ± 0.1 mM, respectively (Figures 3C and 3D). The data indicate that, under identical assay conditions, GEE binds more tightly than ammonium to the K_s site.

Effect of GEE concentration on KIEs

We demonstrated previously with ammonium as activator that the observed KIE with ^1H - and ^2H -labelled substrate decreases with increasing ammonium concentration [21]. The origin of this effect is due to (i) differential binding affinities for ^1H - and ^2H -labelled substrate, which is attributed to force constant effects on binding and (ii) competitive binding of substrate and ammonium at the activator binding sites, and not rate limitation through C– ^1H –C– ^2H bond breakage [21]. The relationship between GEE concentration and the observed KIE was studied to confirm that the

Figure 3 Plots of initial velocity (v_i) versus ammonium and GEE concentration

(A) Plot of initial velocity versus ammonium concentration at 5 °C. Conditions were as follows: 0.1 M Ches, pH 9.0, 0.5–60 mM $(\text{NH}_4)_2\text{SO}_4$, 6 mM cyanide (KCN), 1 mM PES and 10 mM methanol. Data were fitted to the inhibition expression (eqn 1). (B) Plot of initial velocity versus GEE concentration at 5 °C. Conditions were as for (A), but with 0.5–60 mM GEE and 50 mM methanol. (C) As for (A), but with $(\text{NH}_4)_2\text{SO}_4$ and 200 μM PES at 25 °C. (D) As for (B), but with GEE and 200 μM PES at 25 °C. K_s values were 11.7 ± 2.8 , 3.4 ± 0.3 , 1.0 ± 0.1 and 0.28 ± 0.07 mM for (A)–(D) respectively.

unusual changes in KIE were not specific to ammonium-dependent reactions.

Initial-velocity data for (i) $[^1\text{H}]$ methanol, (ii) $[^2\text{H}]$ methanol and (iii) endogenous substrates as a function of GEE concentration are shown in Figure 4(A). The K_s value with endogenous substrates (22.12 ± 0.17 mM) is greater than that of $[^2\text{H}]$ methanol (7.16 ± 0.25 mM) and unlabelled $[^1\text{H}]$ methanol (2.81 ± 0.14 mM). The larger K_s value for $[^2\text{H}]$ methanol compared with $[^1\text{H}]$ methanol is consistent with observations made with ammonium as activator [21], indicating weaker binding of activator (ammonium or GEE) to the K_s site with $[^2\text{H}]$ substrate. The differential binding of ^1H - and ^2H -labelled substrates at the K_s site is partially responsible for the decrease in KIE with increasing GEE concentration (Figure 4B). The KIE (3.7) with 0.5 mM GEE is significantly less compared with the KIE [15] obtained with 1 mM ammonium [21]. This possibly reflects a lower effective concentration of PES in the electron-transfer-competent site (site K_1' ; see below for a structural definition of this site) as a result of competitive binding by GEE to the K_1' site. The lower effective concentration of PES slows the rate of the oxidative half-reaction, which is second-order with respect to PES concentration and, thus, this half-reaction becomes more rate-limiting. In the presence of more than 10 mM GEE, the observed KIE is close to unity, indicating that the oxidative half-reaction is essentially fully rate-limiting (supplementary Table S1; <http://www.BiochemJ.org/bj/388/bj3880123add.htm>). This contrasts with studies with

ammonium, where the KIE does not approach unity, reflecting only partial rate-limitation by the oxidative half-reaction, even at high ammonium concentrations [21].

Having established that GEE affects the binding of PES at the K_1' site, the observed KIE values measured as a function of GEE concentration should also respond to changes in PES concentration. To test this assertion, reactions were performed using increasing concentrations of PES (0.02–1 mM) in the presence of fixed concentrations of GEE (1, 4 and 40 mM) at 25 °C. Cyanide and substrate (methanol or $[^2\text{H}]$ methanol) were kept constant at 6 mM and 50 mM respectively. Initial-velocity profiles were fitted using eqn (1). As seen previously with ammonium [21], the observed KIE becomes larger as the PES concentration is increased. At high GEE concentrations, the KIE remains at unity across the PES concentration range, indicating that the concentration of PES in the K_1' site is low (Figure 5A). This is attributed to competitive binding at this site with GEE. As the GEE concentration is decreased, the PES can compete more favourably for binding to the K_1' site, and the KIE becomes > 1 , reflecting only partial rate limitation by the oxidative half-reaction (Figure 5A).

As PES concentration is increased, initial velocities with methanol or $[^2\text{H}]$ methanol eventually become inhibited (Figure 5B). This inhibition is less pronounced at high GEE concentrations (Figure 5D), again reflecting the competitive binding of both GEE and PES. We suggest that, at high PES concentrations, occupation of the K_1' site by PES might hinder passage of GEE and/or

Figure 4 Plots of initial velocity versus GEE concentration and variation in the observed KIE with GEE concentration

(A) Plots of initial velocity versus GEE concentration. Closed circles, methanol; open circles, $[^2\text{H}]$ methanol; closed triangles, endogenous activity. Conditions were as follows: 0.1 M Ches, pH 9.0, GEE (0.5–60 mM), 6 mM cyanide (KCN) and 1 mM PES at 25 °C. For methanol and $[^2\text{H}]$ methanol reactions, the appropriate substrate was added at 50 mM concentration. Data were fitted to the inhibition expression (eqn 1). (B) Variation in KIE with GEE concentration generated from the data shown in (A).

substrate through the entrance cavity to the active site. This would interfere with binding at the catalytic and K_s sites, thus inhibiting the activity of the enzyme.

Thermodynamic parameters and temperature dependence of KIEs at fixed GEE and PES concentrations

The temperature-dependence of the observed KIE has previously been investigated at 1, 4 and 20 mM ammonium sulphate, with 1 mM PES and saturating concentrations of unlabelled methanol and $[^2\text{H}]$ methanol (80 mM). Eyring plots indicated that the KIE is independent of temperature, although reaction rates are strongly dependent on temperature [21]. Steady-state reactions with $[^1\text{H}]$ methanol and $[^2\text{H}]$ methanol were investigated over the temperature range 5–41 °C, at various fixed concentrations of GEE (or ammonium) in the presence of 1 mM or 200 μM PES. The parameters $\Delta H^\ddagger$ and A^H/A^D (which were defined previously [30]) were obtained by fitting to eqn (2) (supplementary Table S2; <http://www.BiochemJ.org/bj/388/bj3880123add.htm>):

$$\ln(v_i/T) = \ln k/h + \Delta S^\ddagger/R - \Delta H^\ddagger/RT \quad (2)$$

Where $\ln$ is the natural logarithm ($\log_e$), v_i is initial velocity, T is the temperature in K, k is the Boltzmann constant, h is Planck's

constant, $\Delta S^\ddagger$ is change in entropy, R is the gas constant and $\Delta H^\ddagger$ is change in enthalpy.

The temperature-independence of the KIEs at each activator and PES concentration is consistent with the value of the ratio $A^\text{H}/A^\text{D} \approx \text{KIE}$ and $\Delta\Delta H^\ddagger \approx 0$, and reflect force constant effects on the binding of methanol and $[^2\text{H}]$ methanol at the K_s and K_i sites [21]. We suggest that the different $\Delta H^\ddagger$ values obtained at different ligand/substrate concentrations reflect the different ligand occupancies at the ligand binding sites, consistent with the proposed competitive binding model (see below). The K_s term is constant over the temperature range, thus ensuring that the same fraction of enzyme remains bound to ammonium and GEE at each assay condition shown in Figure 6.

Identifying the GEE and PES binding sites

Our molecular-docking studies identified likely binding sites for GEE and PES. GEE was predicted to occupy three binding sites (Figure 7): the K_s and part of the K_i site, the K_i site, and a separate site at the entrance to the active site, which we will denote K_i' . PES docks to the K_i' site, but is too large to occupy the cavity comprising the K_s and K_i sites. Analysis of the first normal mode suggests that GEE could enter the cavity comprising the K_s and K_i sites via an opening that occurs between Cys¹⁰³ and Leu¹⁷².

DISCUSSION

Alternative activators of MDH

A number of studies have established a requirement for ammonium ions as an activator in steady-state reactions of MDH when using artificial electron acceptors [12–15]. Esters of glycine can replace ammonium ([14]; M. Beardmore-Gray and C. Anthony, unpublished work referred to in [18]), but detailed kinetic studies with alternative activators have not been reported. In the present paper we report the first detailed kinetic study of MDH with the alternative activator GEE. Our recent study of *M. methylotrophus* MDH has identified unusual kinetic effects with methanol and $[^2\text{H}]$ methanol that were attributed to force constant effects on the binding of substrate to the stimulatory (K_s) and inhibitory (K_i) activator binding sites and to the competitive binding of ammonium at the same sites [21]. On the basis of these data, we were able to elucidate the mechanistic basis for the unusual KIEs observed during steady-state reactions of MDH, and demonstrate that the value of the KIE is influenced by factors other than the bond-breakage reaction [21]. We have shown here that our previously proposed kinetic model for reactions performed in the presence of ammonium (Scheme 2) is consistent also with GEE-dependent activity, although quantitative differences exist in the derived kinetic parameters.

We have shown that GEE binds to stimulatory (K_s) and inhibitory (K_i) binding sites (Figures 3A and 3B), which provides two kinetic pathways to the two-electron reduced form of MDH (Scheme 2). Although methanol-dependent turnover is faster with ammonium, MDH has a higher affinity for GEE, indicated by the smaller K_s value for GEE compared with ammonium under similar reactions conditions. The binding of GEE to the inhibitory K_i site is weak (Figures 3C and 3D), and it is suggested that the binding of GEE to the K_s site sterically hinders the binding of GEE to the K_i site, consistent with a close spatial separation of the two sites (as suggested in our previous study with ammonium [21]) and the larger volume occupied by GEE compared with ammonium.

Stimulatory and inhibitory sites in MDH

Our previous studies identified K_s and K_i sites in the same water-filled cavity in the crystal structure of MDH, with the K_s site

Figure 5 Dependence of the observed KIE value on GEE and PES concentration

Conditions were as follows: 0.1 M Ches, pH 9.0, PES (0.02–1 mM), different fixed concentrations of GEE, 6 mM cyanide (KCN) and 50 mM substrate at 25 °C. **(A)** Variation in KIE with GEE and PES concentration. Closed circles, open squares and closed triangles represent GEE concentrations of 1, 4 and 40 mM respectively. **(B)–(D)** Initial velocities (v_i) for methanol and $[^2\text{H}]$ methanol (in the presence of 1, 4 and 40 mM GEE respectively) as a function of PES concentration. Plots indicate reaction rates used to calculate the KIE values shown in **(A)**. Closed circles, methanol; open circles, $[^2\text{H}]$ methanol.

corresponding roughly to the position of water⁹⁷ and the K_i site corresponding roughly to the position of water⁶³ and/or water⁶⁵. Our proposed role of the K_s site adjacent to Glu¹⁷¹ [21] was based on the positive charge on the activator withdrawing the negative charge from Glu¹⁷¹, thereby enabling the calcium to act as a more efficient Lewis acid. An alternative role for Glu¹⁷¹ has been proposed on the basis of molecular-dynamics studies [31], in which tail oxygen atom OE1 of Glu¹⁷¹ is positioned to act as a general base (rather than Asp²⁹⁷ [5,6]) to abstract the hydroxy-group hydrogen atom of methanol. The results of site-directed-mutagenesis studies in which Asp²⁹⁷ had been mutated to glutamic acid [32] are consistent with, but do not unequivocally identify, Asp²⁹⁷ as the active-site base. On the basis of our studies, we cannot confirm either Glu¹⁷¹ or Asp²⁹⁷ as the base. One possible explanation of the stimulatory role of the K_s site with Glu¹⁷¹ as the base is that the binding of activator, which replaces water⁹⁷ in hydrogen-bonding to OE1 of Glu¹⁷¹, produces a subtle structural change that enhances hydrogen abstraction to a greater extent than the electron-withdrawing effect of the ammonium/amino group in the activator. Irrespective of which residue acts as the active-site base, another possible explanation for the stimulatory role of the ammonium/amino group in the K_s site, which lies over the PQQ ring, could be the enhancement of hydride transfer by withdrawing electron density from C-5.

GEE (Figure 7C) is larger than the ammonium used in our previous studies – on binding to the K_s site it also occupies part of the K_i site (Figure 7D). Additionally, the amino group can occupy

an alternative position (Figure 7E) so that GEE is now completely within the K_i site. Given that the cavity comprising the K_s/K_i sites is buried, the question arises as to how activator/inhibitor molecules access this site. It has been suggested [31], on the basis of molecular-dynamics studies, that small molecules can gain access to this cavity from the catalytic methanol site via interaction with the OE1 atom of Glu¹⁷¹. An alternative route is suggested by our normal-mode-analysis studies, which runs directly from the substrate access channel via an opening that appears between Cys¹⁰³ and Leu¹⁷². Although we suggested [21], on the basis of the kinetic model, that PES could occupy the K_s site, we were concerned that PES (Figure 7C) is too large to fit in this site in the structure. Our docking studies of PES – on both the crystal structure and a series of structures generated by the normal mode studies – indicated that PES is indeed too large to occupy this site. However, our docking studies of PES suggest that it binds at a site (K_i') spatially distinct from the K_s/K_i sites. Importantly, this site is within the 14 Å (1.4 nm) upper limit [33] required for electron transfer from PQQ to PES. PES binding at this K_i' site blocks access to the catalytic methanol site and the K_s/K_i sites and, consistent with the kinetic results, GEE and ammonium are also predicted to bind at this K_i' site. This, in turn, leads to an alternative interpretation of the kinetic data. The kinetic data are consistent with one or more inhibitory sites, but cannot distinguish between direct and indirect competition for a given binding site. In conjunction with our modelling studies, the kinetic data (discussed in detail below) can be interpreted in terms of a single

Figure 6 Eyring plots for steady-state reactions of MDH with methanol and $[^2\text{H}]$ methanol

(A) Plot of $\ln(v_i/T)$ versus $1/T$ at 1 mM GEE and 1 mM PES. Closed circles, $[^1\text{H}]$ methanol; open circles, $[^2\text{H}]$ methanol. The inset shows a plot of $\ln(\text{KIE})$ versus $1/T$. (B) As for (A), except with 50 mM GEE and 1 mM PES. (C) Plot of $\ln(v_i/T)$ versus $1/T$ at 20 mM ammonium and 200 μM PES. Conditions were as follows: 80 mM methanol or 80 mM $[^2\text{H}]$ methanol and enzyme concentration 40 nM. In these assays, 1 S.D. in each activity measurement ($n=5$) at a defined temperature and activator concentration is $< 6\%$ of the determined value. Parameters derived from fitting of the data to the Eyring equation are given in supplementary Table S2 (<http://www.BiochemJ.org/bj/388/bj3880123add.htm>)

stimulatory site and two spatially distinct inhibitory sites (Figures 7A and 7B).

Activator-dependent changes in complex binding equilibria and KIEs

Owing to force constant effects on the binding of substrate to the K_S and K_I sites, the K_S value with $[^2\text{H}]$ methanol is higher than

the K_S value for methanol (Figure 4A). As with ammonium [21], this differential binding is partially responsible for the decrease in KIE with increasing activator concentration (Figure 4B). As the activator concentration is increased, the oxidative half-reaction becomes more rate-limiting, which also accounts for the reduction in the observed KIE. This occurs because of the competitive binding of activator and PES at the K_I' site, which reduces the effective concentration of PES at the electron-transfer competent site. Our previous stopped-flow studies established that enzyme oxidation is not rate-limiting at low ammonium concentrations (< 4 mM) during steady-state turnover. At higher ammonium concentrations (> 20 mM), however, we demonstrated that the effective concentration of PES is sufficiently low that the oxidative half-reaction becomes more rate-limiting [21]. We suggest that a similar mechanism occurs with GEE, but that the tighter binding of GEE to the K_I' site compared with ammonium enables the oxidative half-reaction to become fully rate-limiting at lower activator concentrations, resulting in a KIE of unity (Figure 4B). The temperature-independence of the observed KIE seen at different ligand/substrate concentrations (Figure 6) is consistent with a mechanism in which competitive binding of the ligands and substrate at the K_S , K_I and K_I' sites influences the KIE.

Kinetic evidence for the close spatial relationship between the catalytic methanol binding site and the K_S and K_I sites in MDH

GEE has a pronounced effect on the catalytic methanol-binding site (Figure 1). The apparent K_m for methanol decreases with increasing concentrations of GEE (Figure 1C). This suggests that GEE reduces the binding of cyanide (previously shown to be a competitive inhibitor with respect to methanol [21]) to the catalytic methanol-binding site and the ability of PES to bind to the K_I' site. The kinetic data suggest that the K_S and K_I sites are in close proximity to the catalytic methanol-binding site, and this is consistent with our proposed model of ligand binding sites in MDH (Figure 7). Our model predicts that occupation of the K_S site by a large activator (i.e. GEE rather than ammonium) reduces the accessibility of other ligands to the catalytic methanol-binding site, and this is supported by the kinetic data. The structural model is also consistent with endogenous activity being greater in the presence of GEE than with ammonium [Figure 2A; see also supplementary Figure S1 (<http://www.BiochemJ.org/bj/388/bj3880123add.htm>)], owing to weaker binding of cyanide in the catalytic site when the larger activator GEE is bound to the spatially close K_S site. Cyanide does not compete directly for the K_S and K_I sites, as it has no effect on K_S and K_I values for the binding of ammonium in methanol and endogenous reactions (results not shown). The changes in K_S value for GEE as a function of cyanide concentration are consistent with a spatially close catalytic and K_S site. We have demonstrated that cyanide is displaced from the catalytic methanol-binding site on the addition of methanol and thus has no effect on initial velocities measured with methanol (Figure 2B) or $[^2\text{H}]$ methanol (supplementary Figure S1; <http://www.BiochemJ.org/bj/388/bj3880123add.htm>). This is consistent with our proposed kinetic mechanism (lower triangular route in Scheme 2) and confirms our previous conclusions that cyanide does not function as an activator of methanol-dependent activity [21].

The present study furthers our understanding of isotope effects by providing a mechanistic basis for the unusual KIEs observed in MDH and highlights the potential complications arising from multiple ligand binding to an enzyme active site. More importantly, this work illustrates that temperature-independent KIE values are not necessarily indicative of H-tunnelling. Given the recent interest in H-tunnelling, it is essential to appreciate the

Figure 7 Ligand binding sites in MDH

(A) The active site of MDH (PDB accession code 1g72). Residues lining the substrate access channel (broken arrow) are shown. Also shown are the stimulatory (K_S) and inhibitory (K_I) sites (consistent with the sites discussed in our earlier study [21]; see D and E), and an additional inhibitory site (K_I'), which caps the entrance to the substrate access channel and has been identified during our molecular-docking studies. (B) Schematic diagram indicating the predicted binding site(s) of the ligands studied. (C) Structures of PES and GEE. (D) The predicted binding mode of GEE when acting as a stimulator. (E) The predicted binding mode of GEE when acting as an inhibitor. In (D) and (E) the spheres labelled 'K_S' and 'K_I' denote the locations of the stimulatory and two possible inhibitory sites (which could merge, as A, to form a larger single inhibitory site) respectively, identified for ammonium in our previous study [21]; note that when GEE binds to K_S, it also occupies part of K_I.

mechanistic basis of KIE values prior to using the temperature-dependence of these values as probes for tunnelling regimes.

The results obtained in the present study also highlight the potential complications arising from multiple ligand binding, and in particular the complications arising from the use of artificial electron acceptors. This is important given the prevalence in the MDH literature of erroneously associating steady-state KIEs exclusively to the chemical step involving C-¹H-C-²H bond breakage.

Concluding remarks

We have provided a detailed kinetic analysis of the reaction catalysed by MDH in the presence of the alternative activator GEE. Our studies have established the robust nature of the general kinetic model proposed in our previous work with ammonium, but has revealed quantitative differences in the kinetic parameters obtained with GEE. Moreover, our studies with GEE have provided additional data to support the close spatial relationships for ligand binding and catalytic sites in MDH. We have described a structural model for these ligand-binding sites, in which we have identified an additional and distinct binding site for PES. The structural model is consistent with kinetic data obtained with ammonium and GEE as activators.

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