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

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INTRODUCTION

MDH (methanol dehydrogenase; EC 1.1.99.8) catalyses the oxidation of methanol to formaldehyde and utilizes a cytochrome c as electron acceptor [1,2]. The enzyme contains PQQ (2,7,9-tricarboxyxyproloquinoline quinone) [3] and adopts a αβ2 structure [4–6]. A calcium ion acts as a Lewis acid during substrate oxidation and is co-ordinated in 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 cL 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 methyleneamine, but not by diamines, triamines or long-chain alkyamines [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esters and β-alanine esters, but not with aliphatic amines or amino acids ([19]; M. Beardmore-Gray and C. Anthony, unpublished work cited in [18]).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 acidiphila is

Abbreviations used: DCPIP, 2,6-dichlorophenol-indophenol; GEE, glycine ethyl ester; Ks, kinetic constant describing stimulatory effects of activator on enzyme activity; Ks′, structurally defined stimulatory binding site in the active site of methanol dehydrogenase; KI, structurally defined inhibitory binding site in the active site of methanol dehydrogenase; KI′, structurally defined inhibitory binding site in the active site of methanol dehydrogenase; MDH, methanol dehydrogenase; PES, phenazine ethosulphate; PQQ, 2,7,9-tricarboxyxyproloquinoline quinone.

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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\textsuperscript{1}H\textsubscript{3}O\textsubscript{1}H) and \textsuperscript{2}H methanol (deuterated methanol, C\textsuperscript{2}H\textsubscript{3}O\textsubscript{2}H). 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]. 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].

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\textsubscript{S} and K\textsubscript{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\textsubscript{red} and MDH\textsubscript{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\textsubscript{red} and MDH\textsubscript{sq}).

EXPERIMENTAL

Materials

Ches [2-(N-cyclohexylamino)ethanesulphonic acid],Mes, DCPIP sodium salt, PES (as N-ethyldibenzopyrazine ethyl sulphate salt), Cyanide (KCN) and GEE hydrochloride were obtained from Sigma. (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} and methanol were from Fisher. \textsuperscript{2}H Methanol (99.8 %) was from Aldrich. The chemical purity of the \textsuperscript{2}H methanol was determined by the suppliers to be > 99 % by HPLC, NMR and GLC.

Purification of MDH

M. methylotrophus (strain W\textsubscript{A1}) 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\textsubscript{280}/A\textsubscript{345} ratio of 11.3 [22]. MDH was purified in the semiquinone form (\varepsilon\textsubscript{343} 28 300 M\textsuperscript{−1}·cm\textsuperscript{−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 PES.
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 ≈ 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. (NH₄)₂SO₄ (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 ε₆₀₀ of 22 000 M⁻¹·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 ([L]) were fitted to eqn (1):

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

(1)

where \( K_S \) and \( K_i \) are the activation and inhibition constants for activator respectively, \( V_{\text{max}} \) is the theoretical maximum rate and \( b \) is a factor by which the \( V_{\text{max}} \) 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 elNémo [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 competitive effects of PES and cyanide on methanol binding [21]. The apparent \( K_m \) obtained with 20 mM (NH₄)₂SO₄ (1.06 ± 0.05 mM [21]) is significantly lower with 20 mM GEE (0.06 ± 0.008 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).
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 (NH₄)₂SO₄ 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...
Inhibition expression

\[ v = \frac{v_{\text{max}}}{K_i + [GEE]} \]

where \( v \) is the initial velocity, \( v_{\text{max}} \) is the maximum velocity, \( K_i \) is the inhibition constant, and [GEE] is the concentration of glycine ethyl ester (GEE).

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

<table>
<thead>
<tr>
<th>[KCN] (mM)</th>
<th>Endogenous</th>
<th>Methanol</th>
<th>Endogenous</th>
<th>Methanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.31 ± 0.08</td>
<td>4.41 ± 0.09</td>
<td>7.18 ± 0.38</td>
<td>4.62 ± 0.49</td>
</tr>
<tr>
<td>3</td>
<td>5.22 ± 0.13</td>
<td>4.27 ± 0.08</td>
<td>15.10 ± 1.06</td>
<td>4.57 ± 0.31</td>
</tr>
<tr>
<td>6</td>
<td>4.95 ± 0.12</td>
<td>4.24 ± 0.06</td>
<td>26.38 ± 2.36</td>
<td>4.86 ± 0.27</td>
</tr>
<tr>
<td>12</td>
<td>4.75 ± 0.19</td>
<td>4.31 ± 0.05</td>
<td>35.46 ± 2.89</td>
<td>4.61 ± 0.29</td>
</tr>
<tr>
<td>20</td>
<td>4.33 ± 0.23</td>
<td>4.18 ± 0.08</td>
<td>40.56 ± 2.08</td>
<td>4.59 ± 0.37</td>
</tr>
</tbody>
</table>

(b) Inhibition expression

\[ v = \frac{v_{\text{max}}}{K_i + [GEE]} \]

where \( v \) is the initial velocity, \( v_{\text{max}} \) is the maximum velocity, \( K_i \) is the inhibition constant, and [GEE] is the concentration of glycine ethyl ester (GEE).

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 \( K_a \), 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 \( k_a \)) is not affected by cyanide concentration (Table 2, Figure 2C). Furthermore, cyanide has no effect on the stimulation of activity by GEE (\( K_a \) 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 \( k_a \) values and the stimulation of activity by GEE (reflected in altered \( K_a \) 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 \(^{[2H]}\)-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 \( \mu \)M) 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 \( \mu \)M PES. In this case, the extent of inhibition by GEE was found to be comparable at 1 mM and 200 \( \mu \)M 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 \( K_i \) site for GEE and ammonium-dependent reactions. With 1 mM PES, the \( K_i \) values for GEE and ammonium are 3.4 ± 0.3 mM and 11.7 ± 2.8 mM respectively (Figures 3A and 3B). With 200 \( \mu \)M PES, the \( K_i \) 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 \( K_i \) site.

Effect of GEE concentration on KIEs

We demonstrated previously with ammonium as activator that the observed KIE with \(^1\)H- and \(^2\)H-labelled substrate decreases with increasing ammonium concentration [21]. The origin of this effect is due to (i) differential binding affinities for \(^1\)H- and \(^2\)H-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
unusual changes in KIE were not specific to ammonium-dependent reactions.

Initial-velocity data for (i) [1H]methanol, (ii) [2H]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 [1H]methanol (7.16 ± 0.25 mM) and unlabelled [1H]methanol (2.81 ± 0.14 mM). The larger $K_s$ value for [2H]methanol compared with [1H]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 [2H]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 $K_i'$; see below for a structural definition of this site) as a result of competitive binding by GEE to the $K_s$ 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 becomes 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 $K_i'$ 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 [2H]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 $K_i'$ 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 $K_i'$ 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 [2H]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 $K_i'$ site by PES might hinder passage of GEE and/or
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 \( A^{\ddagger\ddagger}/A^\ddagger \approx \text{KIE and } \Delta \Delta H^\ddagger \approx 0 \), and reflect constant effects on the binding of methanol and \([2H]\)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 \([2H]\)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 \( K_i \) site sterically hinders the binding of GEE to the \( K_s \) 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...
corresponding roughly to the position of water$^97$ and the K$_1$ site corresponding roughly to the position of water$^63$ and/or water$^65$. Our proposed role of the K$_S$ site adjacent to Glu$^{171}$ [21] was based on the positive charge on the activator withdrawing the negative charge from Glu$^{171}$, thereby enabling the calcium to act as a more efficient Lewis acid. An alternative role for Glu$^{171}$ has been proposed on the basis of molecular-dynamics studies [31], in which tail oxygen atom OE1 of Glu$^{171}$ is positioned to act as a general base (rather than Asp$^{297}$ [5,6]) to abstract the hydroxy-group hydrogen atom of methanol. The results of site-directed-mutagenesis studies in which Asp$^{297}$ had been mutated to glutamic acid [32] are consistent with, but do not unequivocally identify, Asp$^{297}$ as the active-site base. On the basis of our studies, we cannot confirm either Glu$^{171}$ or Asp$^{297}$ as the base. One possible explanation of the stimulatory role of the K$_S$ site with Glu$^{171}$ as the base is that the binding of activator, which replaces water$^9$ in hydrogen-bonding to OE1 of Glu$^{171}$, 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$_1$ site (Figure 7D). Additionally, the amino group can occupy an alternative position (Figure 7E) so that GEE is now completely within the K$_1$ site. Given that the cavity comprising the K$_S$/K$_1$ 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 Glu$^{171}$. 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 Cys$^{109}$ and Leu$^{172}$. 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$_S'$) spatially distinct from the K$_S$/K$_1$ 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$_S'$ site blocks access to the catalytic methanol site and the K$_S$/K$_1$ sites and, consistent with the kinetic results, GEE and ammonium are also predicted to bind at this K$_S'$ 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
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](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 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 $K_s'$ 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 $K_s'$ 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 $K_s'$ 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 $K_s$ 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 $K_s'$ 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 $[^2H]$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
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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Kinetic mechanism of PQQ-dependent methanol dehydrogenase with glycine ethyl ester


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