Reductive half-reaction of the H172Q mutant of trimethylamine dehydrogenase: evidence against a carbanion mechanism and assignment of kinetically influential ionizations in the enzyme–substrate complex

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The reactions of wild-type trimethylamine dehydrogenase (TMADH) and of a His-172 → Gln (H172Q) mutant were studied by rapid-mixing stopped-flow spectroscopy over the pH range 6.0–10.5, to address the potential role of His-172 in abstracting a proton from the substrate in a ‘carbanion’ mechanism for C–H bond cleavage. The pH-dependence of the limiting rate for flavin reduction (k_{lim}) was studied as a function of pH for the wild-type enzyme with perdeuterated trimethylamine as substrate. The use of perdeuterated trimethylamine facilitated the unequivocal identification of two kinetically influential ionizations in the enzyme–substrate complex, with macroscopic pK_a values of 6.5 ± 0.2 and 8.4 ± 0.1 . A plot of k_{lim}/K_d revealed a bell-shaped curve and two kinetically influential ionizations with macroscopic pK_a values of 9.4 ± 0.1 and 10.5 ± 0.1 . Mutagenesis of His-172, a potential active-site base and a component of a novel Tyr-His-Asp triad in the active site of TMADH, revealed that the pK_a of 8.4 ± 0.1 for the wild-type enzyme–substrate complex represents

ionization of the imidazolium side-chain of His-172. H172Q TMADH retains catalytic competence throughout the pH range investigated. At pH 10.5, and in contrast with the wild-type enzyme, flavin reduction in H172Q TMADH is biphasic. The fast phase is dependent on the trimethylamine concentration and exhibits a kinetic isotope effect of about 3; C–H bond cleavage is thus partially rate-limiting. In contrast, the slow phase does not show hyperbolic dependence on substrate concentration, and the observed rate shows no dependence on isotope, revealing that C–H bond cleavage is not rate-limiting. The analysis of H172Q TMADH, together with data recently acquired for the Y169F mutant of TMADH, reveals that C–H bond breakage is not initiated via abstraction of a proton from the substrate by an active-site base. The transfer of reducing equivalents to flavin via a carbanion mechanism is therefore unlikely.

Key words: amine oxidation, electron transfer, flavoprotein.

INTRODUCTION

Trimethylamine dehydrogenase (TMADH; EC 1.5.99.7) is a homodimeric iron–sulphur flavoprotein from *Methylophilus methylotrophus* (sp. W3A1) [1]. The enzyme catalyses the oxidative demethylation of trimethylamine:

The crystal structure of TMADH ([2]; Brookhaven Data Bank code 2TMD) reveals that each subunit comprises a flavin-binding N-terminal β/α barrel. A peptide loop responsible for binding a ferredoxin-type 4Fe–4S centre [3] is located at the C-terminus of the barrel. This loop then leads to the C-terminal domains, which are rich in α/β structure and bind ADP [4]. These domains resemble in fold the classical dinucleotide-binding domains found, for example, in enzymes such as the flavoprotein disulphide oxidoreductases [4,5]. The enzyme contains an unusual 6-S-cysteinyl FMN cofactor, one role of which is to prevent inactivation of the enzyme during catalysis through the formation of 6-hydroxy-FMN [6,7]. The physiological electron acceptor is an electron-transferring flavoprotein (ETF), a heterodimer containing one molecule of FAD [8] and AMP [9]. ETF forms a transient complex with TMADH [10,11], the formation of which probably involves a large-scale structural reorganization in ETF [12].

The reaction of TMADH can be divided into reductive and oxidative half-reactions. Reducing equivalents are initially transferred from the substrate to the flavin centre, a process that

involves the rupture of a C–H bond in one of the methyl groups of trimethylamine. The electrons are subsequently transferred in two single-electron transfer events to two molecules of ETF via the 4Fe–4S centre [13–17]. The reductive half-reaction displays three kinetically resolvable phases [18,19]: a fast phase that represents two-electron reduction of the flavin, an intermediate phase that reflects intramolecular electron transfer from dihydroflavin to the 4Fe–4S centre to generate flavin semiquinone and reduced iron–sulphur centre, and a slow phase that involves formation of a spin-interacting state of the enzyme and product release [14–16].

The mode of cleavage of the relatively stable C–H bond in trimethylamine by TMADH remains to be elucidated. Because of the large dissociation energy of C–H bonds (approx. $420 \text{ kJ} \cdot \text{mol}^{-1}$), the mechanism of bond cleavage in many flavoproteins is unclear. In TMADH, a carbanion mechanism, involving abstraction of a proton from a substrate methyl group by an active-site base, has been proposed [18], but other possibilities include radical cleavage [19], as proposed for monoamine oxidase [20]. We have shown previously that mutation of Tyr-169, a component of a novel Tyr-His-Asp triad in the active site of TMADH also comprising residues His-172 and Asp-267, to Phe does not prevent catalysis. This demonstrates that Tyr-169 in the wild-type enzyme is not a kinetically relevant base and indicates that, if the reaction proceeds via a carbanion intermediate (formed by proton abstraction by an active-site base), some other residue must be responsible for the base-assisted catalysis [21].

Abbreviations used: TMADH, trimethylamine dehydrogenase; ETF, electron-transferring flavoprotein; PMS, phenazine methosulphate; DCPIP, 2,6-dichlorophenolindophenol; KIE, kinetic isotope effect; k_{lim} , limiting rate for flavin reduction.

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Our previous studies of the pH-dependence of flavin reduction by substrate in TMADH have shown a bell-shaped dependence on the kinetic parameter k_{lim}/K_a (pK_a values of 9.3 ± 0.1 and 10.0 ± 0.1) and an apparent sigmoidal dependence on k_{lim} , the limiting rate for flavin reduction (pK_a value 7.2 ± 0.2) [19]. However, studies of the pH-dependence of wild-type TMADH have been complicated by the very high reduction rates at elevated pH (k_{lim} rises from about 100 s^{-1} to about 800 s^{-1} over the pH range 6–11 at 10°C). Here we have examined the pH-dependence of flavin reduction in wild-type TMADH using perdeuterated trimethylamine. The slower rate of C– ^2H bond cleavage has allowed more detailed analysis of the pH-dependence of flavin reduction and the identification of additional kinetically influential ionizations. We have also examined the properties of a His-172 $\rightarrow$ Gln (H172Q) mutant of TMADH, and present evidence that this mutation does not substantially compromise the rate of flavin reduction by the substrate. Kinetic analysis of H172Q TMADH has demonstrated that the ionization state of His-172 is kinetically relevant (macroscopic pK_a 8.4 ± 0.1) in the wild-type enzyme, but that this residue plays no role in abstracting a proton from a substrate methyl group. In the absence of an alternative candidate active-site base in TMADH, the oxidative demethylation of trimethylamine is therefore unlikely to proceed by a ‘carbanion’ mechanism.

EXPERIMENTAL

Chemicals

Complex bacteriological media were from Unipath, and all media were prepared as described by Sambrook et al. [22]. Trimethylamine, 2,6-dichlorophenolindophenol (DCPIP), phenazine methosulphate (PMS) and all buffer components were purchased from Sigma. Trimethyl[$^2\text{H}_9$]amine HCl (99.7% ^2H) was from CK Gas Products Ltd. All other chemicals were of analytical grade where possible.

Enzymes

Wild-type TMADH was purified from *Methylophilus methylotrophus* (sp. W3A1) as described by Steenkamp and Mallinson [1] and modified by Wilson et al. [23]. The concentration of wild-type enzyme was determined using a molar absorption coefficient of $27300 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 443 nm in 50 mM potassium phosphate buffer, pH 7.0. Recombinant H172Q TMADH was generated and isolated as described elsewhere [24], and expressed using the plasmid pSV2tmdveg [24]. Cofactor stoichiometries were determined as described elsewhere [24,25]. As with recombinantly expressed wild-type TMADH [24], the H172Q mutant was isolated with a full complement of 4Fe–4S centre and ADP, but a significant portion of the enzyme lacks the flavin. However, previous studies have indicated that the presence of deflavo TMADH in the purified enzyme sample does not affect the kinetic behaviour of TMADH. Using spectrophotometric methods reported elsewhere [24], the proportion of flavinylated enzyme in the H172Q preparations was estimated to be approx. 12%. Partial flavinylation of the enzyme is observed in other recombinant forms of TMADH [11,24,25]. Previous analyses of recombinant wild-type TMADH ($\sim 25\%$ flavinylated) and other partially flavinylated mutant TMADH enzymes ($\sim 5\text{--}60\%$ flavinylated) revealed that a significant degree of deflavinylated does not affect the kinetic behaviour of that portion of the enzyme that possesses flavin. The concentration of the H172Q mutant was determined using an effective molar absorption coefficient ($16500 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ at 443 nm) for oxidized enzyme, calculated from the extent of spectral change elicited by excess

substrate (only the flavinylated form is reducible by substrate). Enzyme solutions of the desired pH were obtained by adding microlitre volumes of a concentrated enzyme stock to buffers at the required pH values. Use of inorganic buffers is preferred in studies of TMADH, since most organic buffers contain substituted amines which either inhibit the enzyme or are slowly oxidized by it.

Steady-state kinetic measurements

Measurements were performed with a 1-cm light path in a final volume of 1 ml. The desired concentrations of substrate, PMS and DCPIP were obtained by making microlitre additions from stock solutions to the assay mixture. Assays for the determination of kinetic parameters were performed in 100 mM sodium pyrophosphate buffer, pH 8.5. Reactions were initiated by the addition of substrate, and the decrease in absorbance at 600 nm due to the reduction of DCPIP ($\epsilon = 21900 \text{ M}^{-1} \cdot \text{cm}^{-1}$) was measured using a Hewlett-Packard 8452A single-beam diode array spectrophotometer. All data were collected at 30°C . Data were fitted to eqn. (1) [21,26] using the fitting program Grafit [27]:

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

where b is a factor by which the intrinsic maximum velocity is affected. The remaining terms have their usual meaning. The equation describes the steady-state situation where the binding of a second substrate molecule to reduced enzyme inhibits the velocity of the reaction. The mechanism underlying this inhibition has been discussed elsewhere [17,19].

Stopped-flow kinetic measurements

Stopped-flow experiments were performed using an Applied Photophysics SX.17MV or SX.18MV stopped-flow spectrophotometer. For single-wavelength studies, data collected at 443 nm were analysed using non-linear least-squares regression analysis on an Archimedes 410-1 microcomputer with Spectra-kinetics software (Applied Photophysics). Experiments were performed by mixing TMADH contained in buffer of the desired pH with an equal volume of trimethylamine at the desired concentration contained in the same buffer. The concentration of substrate was always at least 10-fold greater than that of TMADH, thereby ensuring pseudo-first-order reaction conditions. For each substrate concentration, at least four replica measurements were collected and averaged. Substrate-reduced TMADH is quite stable to re-oxidation by molecular oxygen (half-life approx. 50 min [23]), negating the need to use anaerobic conditions in the kinetic experiments.

For wild-type enzyme under pseudo-first-order conditions, the observed rate constant (k_{obs}) for the absorbance change at 443 nm (flavin reduction) is obtained from fits of the data to eqn. (2) [19,28,29]:

$$A_{443} = Ce^{-k_{\text{obs}}t} + b \quad (2)$$

where C is a constant related to the initial absorbance and b is an offset to account for a non-zero baseline. Under certain conditions, reductive transients for the wild-type enzyme deviate slightly from a true monophasic expression (see the Results section), and fits to eqn. (2) are therefore an approximation. Reductive transients for H172Q TMADH were analysed using

eqn. (2), except at pH 10.5, where transients were best fitted to the equation for a double-exponential process (eqn. 3):

$$A_{443} = C_1 e^{-k_{\text{obs}1}t} + C_2 e^{-k_{\text{obs}2}t} + b \quad (3)$$

where $k_{\text{obs}1}$ and $k_{\text{obs}2}$ are the observed rate constants for the faster and slower phases respectively, and C_1 and C_2 are related to the initial absorbance. b is an offset, again to account for a non-zero baseline. Rate constants were found to depend on substrate concentration, and the reaction sequence was modelled as shown in the general scheme:

where A is TMADH_{ox}, B is trimethylamine, C is the Michaelis complex and D is TMADH containing the two-electron-reduced (dihydroflavin) form of the 6-S-cysteinyl FMN. Data were fitted to eqn. (5) [30]:

$$k_{\text{obs}} = k_3[S]/(K_d + [S]) \quad (5)$$

where $K_d = k_2/k_1$. All curve fitting was performed using the Grafit software package [27].

Multiple-wavelength stopped-flow spectroscopy was performed using a photodiode array detector and X-SCAN software (Applied Photophysics) interfaced with the SX17.MV reaction analyser. Spectral deconvolution was performed by global analysis and numerical integration methods using PROKIN software (Applied Photophysics).

RESULTS

Kinetically influential ionizations in the reaction of wild-type enzyme with perdeuterated substrate

The pH-dependence of the wild-type enzyme using trimethylamine as substrate has been analysed in previous work [19]. Plots of limiting electron transfer rate/enzyme–substrate dissociation constant against pH (k_{lim}/K_d against pH) reveal a bell-shaped dependence governed by two $\text{p}K_a$ values of 9.3 and 10.0. The latter value is thought to reflect ionization of free substrate (trimethylamine $\text{p}K_a$ 9.8), consistent with other work indicating that substrate binding is through cation– π interactions of the trimethylammonium ion with three aromatic residues (Tyr-60, Trp-264 and Trp-355) in the active site [28,31,32]. The $\text{p}K_a$ value of 9.3 on the acid side of the bell-shaped curve has been suggested to represent the ionization of Tyr-60, since on increasing the pH beyond pH ~ 9 the major effects are on K_d for substrate [19]. Corresponding plots of k_{lim} against pH for the wild-type enzyme, which represent kinetically influential ionizations in the enzyme–substrate complex, have been difficult to analyse in detail. The data can be fitted to the equation describing a single ionization (yielding a macroscopic $\text{p}K_a$ value of 7.1), and also to the equation describing a double ionization (with poorly resolved $\text{p}K_a$ values) [19]. Whether a single or double ionization is responsible for the rate enhancement by the enzyme–substrate complex as the pH is increased is essential for a detailed understanding of the reductive half-reaction and the subsequent interpretation of mutagenesis data. A major problem in resolving this issue is the very high flavin reduction rate observed at elevated pH with trimethylamine as substrate (k_{lim} rising from about 100 s^{-1} to about 800 s^{-1} between pH 6 and pH 11 at 10°C [19]), in which a substantial portion of the spectral change takes place in the dead-time in stopped-flow experiments; these limitations compromise data analysis in the high pH and

high substrate concentration regimes. An additional complication is that kinetic transients often deviate from monophasic absorbance changes due to incomplete kinetic resolution of flavin bleaching/reduction from subsequent electron transfer to the 4Fe–4S centre; internal electron transfer makes a small, but significant, contribution to the total absorbance change at 443 nm ([18,19]; see below). The degree to which reductive transients can be analysed appropriately using a monophasic expression varies over the pH and substrate concentration ranges studied. At high pH and high substrate concentrations, the flavin reduction step is more clearly resolved from internal electron transfer, and a biphasic expression is more appropriate under these conditions. At relatively low pH and low substrate concentrations, where the rate of flavin reduction is comparatively slow and is not clearly resolved from internal electron transfer, data are adequately fitted to monophasic absorbance changes. Selection of the appropriate expression for transients recorded at the boundary between the two regimes is a matter of judgement, and frequently difficult. Clearly it would be advantageous if rates could be analysed using the same mathematical expression over the entire pH and substrate concentration ranges studied.

The absorbance changes that occur on mixing TMADH with trimethylamine are shown in Figure 1. Initially, there is a rapid and large bleaching of absorbance at 443 nm that reports on flavin reduction (conversion of spectrum A to spectrum B). This is followed by a small absorbance change at 443 nm that results from the transfer of one electron from the dihydroflavin to the 4Fe–4S centre (spectrum B $\rightarrow$ spectrum C). The final step (spectrum C $\rightarrow$ spectrum D; formation of the spin-interacting state of TMADH) does not contribute to spectral changes at 443 nm. These complex spectral changes associated with the reductive half-reaction of TMADH give rise to biphasic absorbance changes at 443 nm with trimethylamine as substrate. In order to bypass the limitations associated with analysing the plot of k_{lim} against pH for the wild-type enzyme, perdeuterated trimethylamine was used as an alternative substrate. Since C–H bond breakage is rate-limiting for flavin reduction in TMADH, the observed kinetic isotope effect (KIE) of ~ 7 for rupture of the C– ^2H bond [33] allows the full kinetic transient for flavin reduction to be observed and analysed in the stopped-flow mode at all values of pH and all substrate concentrations investigated. With perdeuterated trimethylamine all reductive transients gave rise to monophasic absorbance changes at 443 nm at all pH values and all substrate concentrations (Figure 1). The monophasic transients are the result of kinetic mixing; the rate of conversion of spectral form A to form B is decreased with perdeuterated substrate, and this phase thus becomes mixed with the small absorbance change that occurs as a result of internal electron transfer from flavin to the 4Fe–4S centre (spectrum B $\rightarrow$ spectrum C). It is important to emphasize that apparent rate constants are obtained by fitting a monophasic expression to these transients, and these apparent rate constants will underestimate the true values for flavin reduction. Nevertheless, analysis of the pH-dependence of the flavin reduction step using perdeuterated substrate is improved. This arises because (i) the lower reduction rates allow analysis of the whole kinetic transient (i.e. data are not lost in the dead-time), and (ii) the uncertainty in the measurement of apparent rate constants from monophasic transients is expected to be less than for determinations from biphasic transients. Perdeuterated substrate was therefore used to obtain values for the apparent limiting rate of flavin reduction (k_{lim}) and dissociation constant (K_d) for the enzyme–substrate complex, over the pH range 6–11 (Table 1). The errors in the determined kinetic parameters (Table 1) are now substantially smaller than those calculated for reactions with trimethylamine

Figure 1 Spectral forms of intermediates in the reductive half-reaction of wild-type TMADH, and transient observed at 443 nm with trimethylamine and perdeuterated trimethylamine

(A) Rapid-scanning photodiode array analysis of the reaction of wild-type TMADH ($6 \mu\text{M}$) mixed with $125 \mu\text{M}$ trimethylamine at 30°C , pH 7.5 (100 mM potassium phosphate buffer). (B) Deconvoluted spectra for the intermediates of the reductive half-reaction. Spectrum A, oxidized enzyme; spectrum B, reduced enzyme (dihydroflavin); spectrum C, reduced enzyme (flavin semiquinone/reduced 4Fe–4S centre); spectrum D, spin-interacting state of TMADH. (C) Reductive transient for wild-type TMADH ($3 \mu\text{M}$) with trimethylamine (2 mM) as substrate, illustrating the deviations in the fit to eqn. (2); buffer is 100 mM potassium phosphate, pH 7.5. (D) Reductive transient for wild-type TMADH ($3 \mu\text{M}$) with perdeuterated trimethylamine (2 mM) as substrate, illustrating the excellent fit to eqn. (2); buffer is 100 mM potassium phosphate, pH 7.5.

Table 1 Limiting rate constants for flavin reduction and enzyme–substrate dissociation constants for the reaction of wild-type TMADH with perdeuterated trimethylamine

All reactions were performed at 5°C . Experimental errors are those calculated from curve fitting.

pH	k_{lim} (s^{-1})	K_{d} (mM)	$k_{\text{lim}}/K_{\text{d}}$ ($\text{mM}^{-1} \cdot \text{s}^{-1}$)
6.0	9.7 ± 0.7	62.0 ± 9.1	0.146 ± 0.032
6.5	22.6 ± 0.8	19.9 ± 1.6	1.14 ± 0.13
7.0	34.9 ± 0.7	7.6 ± 0.5	4.59 ± 0.39
7.5	46.2 ± 0.6	2.7 ± 0.1	17.2 ± 0.86
8.0	56.2 ± 0.6	1.04 ± 0.04	54.1 ± 2.6
8.5	65.8 ± 2.0	0.39 ± 0.04	169 ± 22
9.0	77.0 ± 0.8	0.060 ± 0.002	1280 ± 56
9.5	83.7 ± 2.4	0.035 ± 0.005	2410 ± 410
10.2	83.6 ± 4.9	0.023 ± 0.008	3610 ± 1470
11.0	81.8 ± 2.0	0.11 ± 0.008	747 ± 73

[19]. A plot of k_{lim} against pH for the reaction of wild-type enzyme with perdeuterated substrate indicates clearly that the data are better fitted to the expression for a double ionization ($\text{p}K_{\text{a}}$ values 6.5 ± 0.2 and 8.4 ± 0.1) rather than the expression for a single kinetically influential ionizing species (Figure 2). The bell shape of the plot of $k_{\text{lim}}/K_{\text{d}}$ against pH is retained, and is associated with $\text{p}K_{\text{a}}$ values of 9.4 ± 0.1 and 10.5 ± 0.1 (compared

with 9.3 ± 0.1 and 10.0 ± 0.1 respectively with trimethylamine [19]).

General properties of H172Q TMADH

As for the wild-type enzyme, the steady-state kinetic behaviour of H172Q TMADH is best described by eqn. (1), implicating excess-substrate inhibition. We conclude, as for the wild type, that enzyme ‘over-reduction’ at high substrate concentrations (i.e. the population of an alternate redox cycle in which a species of TMADH can accommodate up to three reducing equivalents) is the cause of inhibition (Figure 3; [19]). A full account of the mechanism of substrate inhibition in TMADH has been published [17, 19]. The calculated kinetic parameters indicate that mutation of His-172 to Gln effects little, if any, change in the turnover number, but does give rise to an approx. 25-fold increase in the Michaelis constant for the substrate. The change in Michaelis constant is also reflected in modest increases in the enzyme–substrate–complex dissociation constant for H172Q TMADH measured in stopped-flow experiments (see below). The modest decrease in the rate of flavin reduction as a result of mutation (see stopped-flow results below) is not sufficient to affect the steady-state turnover value of H172Q TMADH, since flavin reduction is not rate-limiting for catalysis in the wild-type enzyme. Substrate inhibition in the steady state occurs at approximately the same concentration of trimethylamine ($K_{\text{i}} \sim 750 \mu\text{M}$) for both the wild-type and H172Q enzymes.

Figure 2 pH-dependence of the reductive half-reaction of wild-type TMADH with perdeuterated trimethylamine as substrate

(A) Plot of limiting flavin reduction rate constant, k_{lim} , as a function of pH. Data are shown fitted to the expression for a single (solid line; macroscopic pK_a value 7.6 ± 0.1) and double (broken line; macroscopic pK_a values 6.5 ± 0.2 and 8.4 ± 0.1) ionization. (B) Plot of k_{lim}/K_d against pH fitted to the equation describing a double ionization. Macroscopic pK_a values are 9.4 ± 0.1 and 10.5 ± 0.1 . Data at each individual pH value are taken from Table 1, and the buffer range was achieved using phosphate, pyrophosphate, borate and glycine buffers. Previous studies have indicated that the increased ionic strength due to the addition of substrate $[(C^2H_5)_3NH^+]$ does not affect the rates of flavin reduction or enzyme–substrate dissociation [19]. At very high pH values, total substrate concentration is the sum of the $(C^2H_5)_3NH^+$ and $(C^2H_5)_3N$ concentrations (pK_a); this probably reflects the modest increase in K_d above pH 10, since the total perdeuterated trimethylamine concentration is greater than the $(C^2H_5)_3NH^+$ concentration.

Figure 3 Plots of velocity against trimethylamine (TMA) concentration for the steady-state reactions of wild-type (O) and H172Q (●) TMADH

Reactions were conducted at pH 8.5 and 30 °C, with PMS and DCPIP as artificial electron acceptors. Data were fitted to eqn. (1). Wild-type kinetic parameters: K_m , $13.7 \pm 1.7 \mu\text{M}$; k_{cat} , $15.6 \pm 2.4 \text{ s}^{-1}$; K_i , $720 \pm 220 \mu\text{M}$; b , 0.31 ± 0.04 . H172Q kinetic parameters: K_m , $425 \pm 52 \mu\text{M}$; k_{cat} , $13.9 \pm 1.4 \text{ s}^{-1}$; K_i , $873 \pm 212 \mu\text{M}$; b , 0.41 ± 0.04 .

Figure 4 Effects of constant and increasing ionic strength on the kinetics of the reductive half-reaction of H172Q TMADH

Reductive transients were measured at 443 nm and analysed using eqn. (2). O, Transients measured employing a KCl balance (constant ionic strength) (k_{lim} , $66.1 \pm 2.7 \text{ s}^{-1}$; K_d , $37.3 \pm 3.3 \text{ mM}$); ●, transients measured without addition of KCl (increasing ionic strength) (k_{lim} , $65.5 \pm 4.2 \text{ s}^{-1}$; K_d , $33.8 \pm 5.6 \text{ mM}$). Fits to the two sets of data are essentially superimposable. Data were collected in 100 mM potassium phosphate buffer, pH 7.5, and at 5 °C. Similar controls at other values of pH likewise indicated no differences in kinetic response in the presence and absence of balancing KCl. TMA, trimethylamine.

Figure 5 Biphasic kinetic transient at 443 nm for H172Q TMADH

The transient is for H172Q TMADH ($3 \mu\text{M}$) rapidly mixed with trimethylamine ($500 \mu\text{M}$) at pH 10.5 (100 mM glycine/NaOH buffer). (A) Reductive transient showing fits to eqn. (2) (monophasic absorption change; broken line) and eqn. (3) (biphasic absorption change; solid line). (B) Plot of residuals for the fit to eqn. (3); (C) plot of residuals for the fit to eqn. (2).

Flavin reduction in H172Q TMADH

Single-turnover stopped-flow experiments performed throughout the experimental pH range used here indicated that kinetic transients were best analysed using a single-exponential process (eqn. 2) for H172Q TMADH (except at pH 10.5; see below). As for the wild-type enzyme, control experiments were performed in which the total ionic strength was kept constant by balancing with KCl the contribution made by cationic substrate. Rates of flavin reduction were found to be identical (within experimental

Figure 6 Plots of the observed high and low rates of flavin reduction against substrate concentration for H172Q TMADH at pH 10.5

(A) Observed rates for transients measured with trimethylamine (TMA). ■, Observed high rates determined by fitting to eqn. (3); □, low rates determined by fitting to eqn. (3). The observed high rates are shown fitted to eqn. (5) (k_{lim} , 78.3 ± 6.2 s⁻¹; K_d , 0.145 ± 0.033 mM). The substrate-independent low rate is 2.3 ± 0.3 s⁻¹. (B) Observed rates for transients measured with perdeuterated trimethylamine. ●, Observed high rates determined by fitting to eqn. (3); ○, low rates determined by fitting to eqn. (3). The observed high rates are shown fitted to eqn. (5) (k_{lim} , 27.3 ± 0.6 s⁻¹; K_d , 0.12 ± 0.009 mM). The low rate at 600 μM (i.e. where the electron transfer rate is not influenced by C–H bond cleavage) is 2.6 ± 0.1 s⁻¹.

error) with those measured in the absence of KCl (Figure 4).

Although kinetic transients were monophasic in the pH range 6–10 for H172Q TMADH, transients at pH 10.5 were strongly biphasic (Figure 5). The fast phase shows hyperbolic dependence on trimethylamine concentration, and has a KIE of ~ 3 , indicating that C–H bond breakage is partially rate-limiting for flavin reduction (Figure 6). [The monophasic transients measured between pH 6 and 10 all show KIEs with perdeuterated trimethylamine revealing that, as for the wild-type enzyme, C–H bond breakage is partially or fully rate-limiting for flavin reduction. A more detailed analysis of the KIEs for H172Q TMADH is to be presented elsewhere (J. Basran, M. J. Sutcliffe, R. Hille and N. S. Scrutton, unpublished work).] The slow phase, however, is essentially independent of trimethylamine concentration over the concentration range studied, and no KIE is seen with perdeuterated trimethylamine. This situation is directly analogous with that reported for the Y169F mutant of TMADH, where biphasic transients and differential effects with perdeuterated substrate on the fast and slow phases were demonstrated at pH 6.5 and below [21]. In that case, it was shown that the slow phase corresponded to the rate of electron transfer to flavin that is not rate-limited by C–H bond cleavage (hence no KIE). The fast phase was interpreted as the rate of flavin

Figure 7 pH-dependence of the reductive half-reaction of H172Q TMADH with trimethylamine as substrate

(A) Plot of limiting flavin reduction rate constant, k_{lim} , as a function of pH. Data are shown fitted to the expression for a single ionization (pK_a 6.8 ± 0.1). (B) Plot of k_{lim}/K_d against pH fitted to the equation describing a double ionization. Macroscopic pK_a values are 10.0 ± 0.2 and 10.1 ± 0.3 . Data at each individual pH are taken from Table 2, and the buffer range was achieved using phosphate, pyrophosphate, borate and glycine buffers.

reduction that is rate-limited by C–H bond cleavage. The relative amplitudes of the two phases were shown to be controlled by a macroscopic pK_a of about 6.2. Two different protonic states of Y169F TMADH are therefore responsible for the observed biphasic transients at pH 6.5. Clearly, mutation of His-172 to Gln has brought about a change in the rate-determining step for flavin reduction in much the same way as for the Y169F mutation. However, the transition in the present case occurs at a pH of approx. 10.5. As for Y169F TMADH at pH 6.5, the nature of the slow step at pH 10.5 for H172Q TMADH remains to be elucidated.

Kinetically influential ionizations in H172Q TMADH

The pH-dependence of the reductive half-reaction of H172Q TMADH was investigated at 5 °C with trimethylamine as substrate (Figure 7; Table 2). In contrast with the case with wild-type TMADH, a plot of k_{lim} against pH revealed the presence of only one kinetically influential ionization in the enzyme–substrate complex, with a pK_a value of 6.8 ± 0.1 . The data suggest, therefore, that His-172 is the residue responsible for the pK_a of 8.4 ± 0.1 in the corresponding plot for the wild-type enzyme. This is further supported by the fact that this same ionization in Y169F TMADH is perturbed by about 1 pH unit (pK_a 9.5 ± 0.3 [21]); the hydroxy group of Tyr-169 in wild-type TMADH is hydrogen-bonded to the imidazole side-chain of His-

Table 2 Limiting rate constants for flavin reduction and enzyme–substrate dissociation constants for the reaction of H172Q TMADH with trimethylamine

At pH 10.5, data are for the fast phase of the biphasic transient. All reactions were performed at 5 °C. Experimental errors are those calculated from curve fitting.

pH	k_{lim} (s ⁻¹)	K_{d} (mM)	$k_{\text{lim}}/K_{\text{d}}$ (mM ⁻¹ ·s ⁻¹)
6.0	5.27 ± 0.88	265 ± 66	0.0198 ± 0.0082
6.5	19.3 ± 2.0	149 ± 27	0.129 ± 0.037
7.0	40.0 ± 3.4	74.4 ± 14	0.537 ± 0.15
7.5	66.1 ± 2.7	37.3 ± 3.3	1.77 ± 0.23
8.0	69.9 ± 6.9	10.5 ± 3.0	6.66 ± 2.6
8.5	74.8 ± 2.6	2.31 ± 0.26	32.4 ± 4.7
9.0	70.7 ± 3.3	0.285 ± 0.050	248 ± 55.1
9.5	74.8 ± 4.9	0.188 ± 0.038	398 ± 106
10.0	73.9 ± 2.8	0.053 ± 0.009	1394 ± 289
10.5	78.3 ± 6.2	0.145 ± 0.033	540 ± 165

172 [2], and removal of this hydroxy group is expected to elevate the pK_{a} value for His-172. An unequivocal assignment of the second (lower) pK_{a} in the plot of k_{lim} against pH for wild-type TMADH is not possible at this stage, but it is essentially the same in the wild-type (6.5 ± 0.2), H172Q (6.8 ± 0.1) and Y169F (6.7 ± 0.2) enzymes. A possible assignment is Tyr-60, which would be likely to experience a pK_{a} shift upon substrate binding ([19]; and see below). Tyr-60, with Trp-355 and Trp-264, comprise the substrate-binding site for TMADH [28]. Tyr-60 has been tentatively assigned as the ionization on the acid limb of the bell-shaped curve in the $k_{\text{lim}}/K_{\text{d}}$ plot for the wild-type enzyme [19]. This tentative assignment has been made on the basis that the major effect on $k_{\text{lim}}/K_{\text{d}}$ is due to changes in K_{d} , consistent with the ionization of the Tyr-60 side-chain. In the enzyme–substrate complex, the decrease in K_{d} on substrate binding would deprotonate this residue and thus account for rate enhancement, as seen in the k_{lim} against pH plot.

A plot of $k_{\text{lim}}/K_{\text{d}}$ against pH for the H172Q TMADH gives a bell-shaped curve defined by two ionizations with macroscopic pK_{a} values of 10.0 ± 0.2 and 10.1 ± 0.3 . The plot is similar to that obtained for the wild-type enzyme (pK_{a} values of 9.4 ± 0.1 and 10.5 ± 0.1 with perdeuterated substrate). The retention of these pK_{a} values in the wild-type, H172Q and Y169F enzymes is consistent with the proposed ionization of residue Tyr-60 (lower of the two values) and free substrate (higher of the two values) [19]. Unfortunately, precise determination of these two pK_{a} values is not possible due to the lack of kinetic data in the high-pH regime (TMADH is not stable at pH > 11).

DISCUSSION

Residue His-172 in wild-type TMADH is located close to the N-1/C-2 region of the flavin isoalloxazine ring and is hydrogen-bonded to residues Tyr-169 and Asp-267 to form a triad structure in the active site. Previously, we have demonstrated that mutation of Tyr-169 (mutant Y169F [21]) or Tyr-60 (Y60Q [28]) does not severely alter the steady-state kinetics of TMADH, as is also the case on replacement of His-172 by Gln as presented here (although k_{lim} for flavin reduction is reduced about 10-fold compared with the wild-type enzyme). These three residues are the only likely candidates for an active-site base in the generation of a carbanion intermediate. The fact that mutation of the three potential active-site bases in TMADH does not substantially impair catalysis brings into question the validity of a carbanion mechanism for the transfer of reducing equivalents to the 6-S-

cysteinyl FMN. The only remaining ionizable group in the active site is Tyr-174. However, the phenolic hydroxy group of Tyr-174 is bridged by a non-active-site water to an acidic side-chain (Asp-69), and thus it is probably not available to abstract a proton from trimethylamine.

Various mechanisms have been proposed for the cleavage of C–H bonds by flavoenzymes. Early work on D-amino acid oxidase and lactate oxidase suggested that a carbanion mechanism involving an active-site base is operative; this mechanism has also been proposed for yeast flavocytochrome b_2 [34,35]. Generation of the substrate carbanion by proton abstraction followed by nucleophilic attack at the flavin N-5 atom by the carbanion is one method of transferring reducing equivalents to the flavin. A conserved histidine found throughout the α -hydroxy acid dehydrogenase/oxidase family [5], which is suitably positioned in the crystal structures of flavocytochrome b_2 [36] and glycolate oxidase [37], is consistent with this proposed mechanism. However, the crystal structure of D-amino acid oxidase reveals no likely candidate for an active-site base, thus favouring alternative modes of C–H bond breakage, such as direct hydride ion transfer or homolysis of the bond by a radical mechanism [38]. The situation with D-amino acid oxidase is further complicated by kinetic isotope studies that suggest a stepwise formation of a carbanion [39], whereas structure/linear free energy relationships with substituted phenylglycines suggest that this is not the case [40]. Additionally, studies of structure–activity relationships with L-lactate oxidase suggest that a carbanion mechanism in its classical formulation is not operative (since major charge development is not observed in the transition state), and hydride ion transfer or a concerted mechanism in which the α -C–H is abstracted as a hydride ion with the resulting charge neutralized by another event has been proposed [41].

Clearly, ambiguity remains in the mechanisms of C–H bond cleavage catalysed by the flavoproteins discussed above, but considerable controversy also surrounds the debate on the mechanism of amine oxidation by flavoproteins. A mechanism requiring proton abstraction is unlikely for TMADH, since protonation of the kinetically influential residues in the enzyme–substrate complex has only a small effect on the rate of flavin reduction. Fundamentally very different mechanisms of flavin reduction have been proposed for monoamine oxidase [20], in which substrate is oxidized initially by a single-electron transfer to the flavin to produce an amminium cation radical and anionic flavin semiquinone. The chemical similarity of the substrates for monoamine oxidase and TMADH make consideration of this type of mechanism attractive for the rupture of the substrate C–H by TMADH. Following production of the amminium cation radical in monoamine oxidase, it is proposed that the radical pair recombines to form a covalent adduct which then decays by β -elimination. An alternative mechanism, involving hydrogen atom abstraction, has been considered by Edmondson [42] to account for the lack of significant electronic influence on reaction rate using a homologous series of benzylamine derivatives. Both of these mechanisms require neutral amine. In the case of TMADH, however, protonated substrate resides in the enzyme–substrate complex. This feature is clearly inconsistent with a reaction initiated by a single-electron transfer from the substrate to the flavin. A detailed discussion of these points can be found in our recent paper [19]. For TMADH, a mechanism initiated by proton abstraction (by homolysis) thus seems more likely, but the lack of an obvious hydrogen-atom acceptor in TMADH remains problematic.

Thus, although it has not so far been possible to elucidate the precise mechanism of C–H bond cleavage in TMADH, on the basis of the present work a carbanion mechanism seems im-

probable. For the reasons discussed above, the available evidence favours a homolytic mechanism involving abstraction of hydrogen from one of the substrate methyl groups. The ionization of His-172 is influential in the reductive half-reaction of TMADH, indicating some role, albeit as yet ill-defined, for this residue in stabilizing the transition state.

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