Probing the catalytic mechanism of *Escherichia coli* amine oxidase using mutational variants and a reversible inhibitor as a substrate analogue

Colin G. SAYSELL, Winston S. TAMBYRAJAH[1], Jeremy M. MURRAY[2], Carrie M. WILMOT[3], Simon E. V. PHILLIPS, Michael J. McPHERSON and Peter F. KNOWLES[4]

Astbury Centre for Structural Molecular Biology, School of Biochemistry and Molecular Biology, University of Leeds, Leeds LS2 9JT, U.K.

Copper amine oxidases are homodimeric enzymes containing one Cu^{2+} ion and one 2,4,5-trihydroxyphenylalanine quinone (TPQ) per monomer. Previous studies with the copper amine oxidase from *Escherichia coli* (ECAO) have elucidated the structure of the active site and established the importance in catalysis of an active-site base, Asp-383. To explore the early interactions of substrate with enzyme, we have used tranylcypromine (TCP), a fully reversible competitive inhibitor, with wild-type ECAO and with the active-site base variants D383E and D383N. The formation of an adduct, analogous to the substrate Schiff base, between TCP and the TPQ cofactor in the active site of wild-type ECAO and in the D383E and D383N variants has been investigated over the pH range 5.5–9.4. For the wild-type enzyme, the plot of the binding constant for adduct formation (K_b) against pH is bell-shaped, indicating two pK_as of 5.8 and ~ 8, consistent with the preferred reaction partners being the unprotonated active-site base and the protonated TCP. For the D383N variant, the reaction pathway involving unprotonated base and protonated TCP cannot occur, and binding must follow a less favoured pathway with unprotonated TCP as reactant. Surprisingly, for the D383E variant, the K_b versus pH behaviour is qualitatively similar to that of D383N, supporting a reaction pathway involving unprotonated TCP. The TCP binding data are consistent with substrate binding data for the wild type and the D383E variant using steady-state kinetics. The results provide strong support for a protonated amine being the preferred substrate for the wild-type enzyme, and emphasize the importance of the active-site base, Asp-383, in the primary binding event.

Key words: copper, hydrazines, inhibition, topa-quinone, tranylcypromine.

INTRODUCTION

The copper amine oxidases (EC 1.4.3.6) are widely distributed in Nature, mediating the oxidative deamination of a wide range of amine substrates (eqn 1):

$$RCH_2NH_2 + H_2O + O_2 \rightarrow RCHO + NH_3 + H_2O_2 \quad (1)$$

Several recent reviews provide comprehensive accounts of quinoproteins in general, and amine oxidases more specifically [1–6].

The redox-active cofactor in copper amine oxidases is the quinone form of 2,4,5-trihydroxyphenylalanine (TPQ) (Figure 1, top), a post-translationally modified tyrosine residue [7–9] that requires copper and oxygen for its formation [10]. The first X-ray crystallographic three-dimensional structure of an amine oxidase was of that from *Escherichia coli* (ECAO), solved to 2.4 Å resolution [11] and more recently improved to 2.04 Å [12]. A number of other crystal structures of amine oxidases have since been solved: *Pisum sativum* (pea seedling) [13], *Arthrobacter globiformis* [14] and *Hansenula polymorpha* [15]. ECAO is a homodimer of 80 kDa subunits, each containing an active site consisting of a TPQ moiety and a copper ion; an active-site base, Asp-383, has been shown to be essential for catalysis [12,16].

Substrate-like inhibitors have been employed extensively in studies of amine oxidases. For example, phenylhydrazine (PHZ) was employed in the structural identification of the TPQ cofactor in bovine serum amine oxidase [7]. Amine oxidases are known to be inhibited irreversibly by a number of such compounds, which include PHZ derivatives [17,18], isoniazid [19] and 2-hydrazinopyridine (2HP) [20]. The 2.0 Å crystal structure of 2HP-inhibited ECAO [16] shows the 2HP moiety to be bound covalently at the O-5 position of TPQ, mimicking the substrate–Schiff-base complex [21–23]. Such inhibitors become irreversibly attached to the TPQ cofactor. Adducts of amine oxidase with PHZs and related compounds have intense visible chromophores, and kinetic studies of adduct formation offer a route to understanding the initial stages of the catalytic cycle. A disadvantage of this class of inhibitor for mimicking the reactions involved in substrate binding is their irreversible nature. An inhibitor that more closely resembles substrate in its reversibility, together with giving an intense adduct chromophore, would provide an important additional tool. Amphetamines have been shown to act as classical competitive inhibitors of pig plasma amine oxidase [24]. One such compound, tranylcypromine (TCP; 2-phenylcyclopropylamine; Figure 1, middle), has been shown to be a reversible inhibitor of flavin-containing monoamine oxidase and is used clinically as an anti-depressant drug [25]. We therefore selected tranylcypromine for binding studies with ECAO. Crystallographic studies have shown that the 1*S*,2*R*-(+)-*trans* enantiomer of TCP [(+)TCP] forms an adduct with wild-type (WT) ECAO (Figure 1, bottom) in which the inhibitor is covalently bound at the O-5 position of

Abbreviations used: ECAO, *Escherichia coli* amine oxidase; 2HP, 2-hydrazinopyridine; PHZ, phenylhydrazine; TCP, tranylcypromine; TPQ, 2,4,5-trihydroxyphenylalanine quinone; WT, wild-type.

[1] Present address: Department of Biochemistry, Adrian Building, University of Leicester, University Road, Leicester LE1 7RH, U.K.

[2] Present address: Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, CT 06520, U.S.A.

[3] Present address: Department of Biochemistry, Molecular Biology and Biophysics, University of Minnesota, 6-155 Jackson Hall, 321 Church Street SE, Minneapolis, MN 55455, U.S.A.

[4] To whom correspondence should be addressed (e-mail p.f.knowles@leeds.ac.uk).

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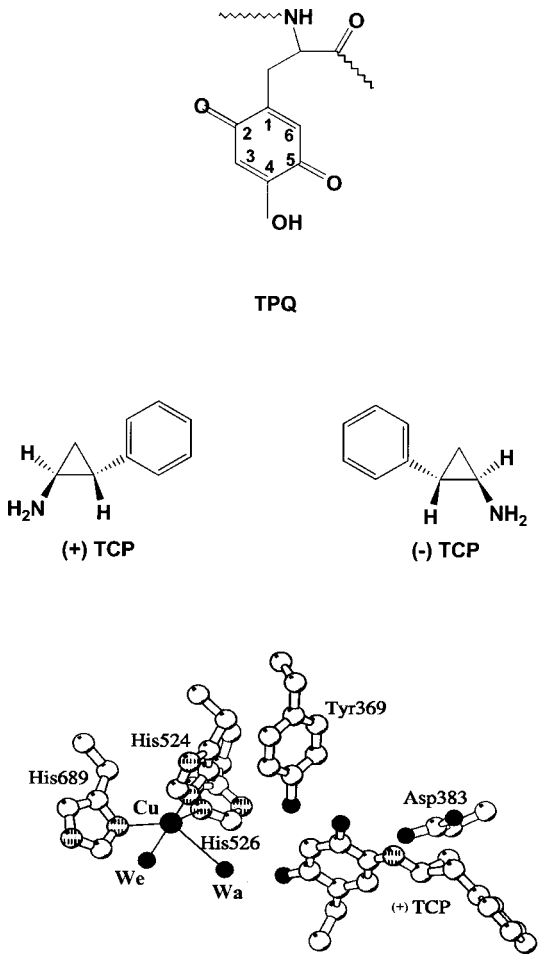

Figure 1 Structures of TPQ (top), TCP *trans* enantiomers (middle) and (+)-TCP in the active site of ECAO, showing the position of Asp-383 (bottom). Protein Data Bank code 1LVN.

Wa and We are axial and equatorial water molecules respectively.

TPQ, the site of substrate binding (C. M. Wilmot, C. G. Saysell, A. Blessington, D. A. Conn, M. J. McPherson, P. F. Knowles and S. E. V. Phillips, unpublished work).

Here we report kinetic studies of the reactions between ECAO, the substrate β-phenylethylamine and the inhibitor TCP. These studies have been carried out over a range of pH values and with variants mutated at the active-site base (Asp-383) to probe the role of this residue in the reactions leading to substrate Schiff-base formation. The results provide strong support for the proposal that the initial step in the catalytic cycle is optimized when the amine is protonated [3], but that an alternative reaction pathway involving deprotonated amine can also operate. This alternative pathway is much less favoured, and corresponds to the pathway when the active-site base, Asp-383, is inoperative.

EXPERIMENTAL

All UV–visible spectrophotometric absorbance measurements and enzyme activity assays were performed at 25.0 ± 0.1 °C on a Shimadzu UV-2401PC UV–visible recording spectrophotometer using masked quartz cells with a pathlength of 1 cm, or an Applied PhotoPhysics SX-17MV stopped-flow instrument. All studies were carried out under air saturating conditions.

Large-scale production and purification of ECAO

Conditions for WT and variant expression are given in [12].

Purification procedures were as described in [11]. The ECAO dimer concentration was determined from an absorbance reading at 280 nm, using a molar absorption coefficient of 2.1×10^5 $M^{-1} \cdot cm^{-1}$, based on gravimetric analysis [26].

Copper content was determined by inductively coupled plasma MS, as described in [12]. For the WT enzyme, the value found was 1.46 ± 0.15 copper atoms per protein dimer. Similar copper analyses for the D383E and D383N variants were not done, but the X-ray crystallographic results [12,16] show clearly that copper is present at levels comparable with those in the WT. Enzyme not used immediately was stored (20 mM Tris/HCl buffer, pH 7.2) as 1 ml aliquots in a liquid nitrogen store, and was thawed slowly on ice prior to use.

Chemicals

All chemicals were reagent grade. Buffers were made up to the desired concentration in deionized water (conductivity $\leqslant 10$ $\mu S \cdot cm^{-1}$) and adjusted to an ionic strength of 0.10 M using NaCl (Sigma). Buffers were selected to provide overlap of their pH ranges, to identify any buffer interactions with protein as have been observed with other enzymes [27]. Mes (Sigma) was used between pH 5.5 and 6.7; sodium phosphate (BDH) buffer, prepared according to the method of Gomori [28], was used between pH 5.7 and 8.0; Hepes (Sigma) was used between pH 6.8 and 8.2; Tris (Sigma) was used between pH 7.1 and 8.9; and 2-[*N*-cyclohexylamino]ethanesulphonic acid (Ches; Sigma) was used between pH 8.6 and 10.0. Buffers were prepared at room temperature (approx. 20 °C), and the pH was adjusted where necessary to compensate for the standard study temperature of 25.0 °C.

Enzyme sample preparation

Enzyme was used in the concentration range 1.0–6.0 μM for UV–visible and stopped-flow spectrophotometry. Typically, 2 ml of enzyme solution was dialysed against the appropriate buffer (20 mM) for 16 h at 4 °C. Any precipitate was removed by centrifugation (bench centrifuge) at 4 °C. Enzyme was diluted to the desired concentration by addition of an appropriate volume of buffer immediately prior to use.

Inhibitor preparation

TCP hemisulphate, 2HP dihydrochloride (Aldrich) and PHZ hydrochloride (Sigma) were used without further purification. Stock solutions of 20 mM of each reagent were made up in the desired buffer (100 mM) and diluted to the required concentration by addition of 100 mM buffer. All solutions were made up immediately prior to use. Solutions of TCP and 2HP were found to be stable at room temperature for more than 1 day, although those of PHZ were found to decay over a period of hours, and were used within 10 min of preparation and then discarded. TCP, as supplied, exists as an equal mixture of two *trans* enantiomers, only one of which, the 1*S*,2*R*-(+)-*trans*-2-phenylcyclopropylamine enantiomer, can adduct ECAO–TPQ, as shown by the crystal structure of the complex (C. M. Wilmot, C. G. Saysell, A. Blessington, D. A. Conn, M. J. McPherson, P. F. Knowles and S. E. V. Phillips, unpublished work). TCP

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concentrations were subject to a correction factor of 0.5 to allow for this fact.

Steady-state kinetics

Steady-state kinetic measurements were carried out with the substrate β-phenylethylamine (Sigma) using a coupled assay system [11]. The effects of varying the substrate concentration and pH on the rate of substrate turnover were determined.

The concentration of substrate in the assay mixture was varied between 15 and 0.5 μM. The final ECAO dimer concentrations were 1.8 nM (WT) and 81 nM (D383E).

For determination of the K_i for TCP, *o*-dianisidine (Sigma) was used in the assay rather than 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid), which was found to react with TCP. TCP from a stock solution (100 mM) was added to 1 ml aliquots of assay buffer solutions to give final concentrations of 25, 50, 100, 200, 300 and 400 μM. The reaction was started by enzyme addition and followed by changes in absorbance at 460 nm. Activities were calculated using a molar absorption coefficient for the oxidized form of *o*-dianisidine of 11 300 $M^{-1} \cdot cm^{-1}$ ($\lambda = 460$ nm).

UV–visible titrations

ECAO was titrated aerobically with TCP, 2HP or PHZ in 100 mM buffer over the pH range 5.5–9.5, giving rise to a highly absorbing enzyme–inhibitor adduct with a λ_{max} in the region 350–360 nm (TCP) or 420–440 nm (2HP [20] and PHZ [7]). Buffered stock solutions of TCP, 2HP or PHZ were added in 2 μl aliquots to 1 ml of enzyme to give increments of ~ 0.15 mol/mol equivalents to enzyme per addition. Spectra were recorded in the range 200–700 nm after each addition of inhibitor. Incubation for between 5 and 10 min between aliquot additions was found to be sufficient time to allow the reaction to go to completion. Additions were continued until no further change in absorbance of the adduct occurred. In titrations where enzyme activity measurement was required during the titration, a 2 μl aliquot was taken from the reaction mixture. Samples were kept on ice and assayed by the coupled-assay method after completion of the titration. All UV–visible spectra were corrected for dilution effects.

Determination of the pK_a of TCP

The pK_a of TCP was studied by dilution of a 20 mM stock solution to a final concentration of 40–70 μM into 20 mM solutions of buffer covering the pH range 4.0–10.0. Spectra were recorded in the range 200–350 nm. The pHs of solutions were recorded before and after each spectrum was taken.

Stopped-flow spectrophotometry

Reactions of WT ECAO with TCP under pseudo-first-order conditions were monitored at 25.0 °C using an Applied Photophysics SX-17MV Stopped-Flow Reaction Analyzer at a fixed wavelength, corresponding to the maximal absorbance change, of 330 nm (TCP with D383E or D383N) or 365 nm (TCP with WT ECAO). At each pH, the TCP concentration was varied from a minimum of 10-fold excess over enzyme (typically 1.5–8.0 μM) to between 1 and 5 mM. Five absorbance–time traces were averaged to obtain a mean rate constant at each inhibitor concentration used. Absorbance–time traces were fitted using a single-exponential fitting function on the SX-17MV system software.

RESULTS

Absence of buffer effects

Kinetic and UV–visible measurements made at the same pH values using different buffers gave essentially identical results, indicating the absence of any specific buffer interactions.

UV–visible studies on WT, D383E and D383N ECAO

The spectra of WT ECAO, and of the D383E and D383N variants, have been published [12]. The visible spectrum of WT ECAO is characterized by a broad peak resulting from the TPQ cofactor, with a λ_{max} of 480 nm and a molar absorption coefficient (ϵ) of 3660 $M^{-1} \cdot cm^{-1}$ (pH 7.0), giving rise to the characteristic pink colour of the oxidized enzyme. The values for D383E are very similar to those of WT ECAO, with a $\lambda_{max} =$ 480 nm and $\epsilon = 3460\ M^{-1} \cdot cm^{-1}$ (pH 7.0). The D383N variant differs somewhat, with a $\lambda_{max} = 450$ nm and $\epsilon = 3460\ M^{-1} \cdot cm^{-1}$ (pH 7.1). No pH effects on enzyme absorbance were observed within the pH range 5.3–9.5. These results indicate that the TPQ contents of WT ECAO and of the D383E and D383N variants are closely similar.

TPQ cofactor titrations

2HP or PHZ adduct formation with the TPQ cofactor in ECAO was found to be irreversible, since extensive dialysis or heating (45–55 °C) failed to reverse the binding. As reported previously for WT ECAO [29], 1.3–1.4 equivalents per protein dimer of either inhibitor reacted rapidly to give the adduct, concomitant with loss of enzyme activity. This stoichiometry of adduct formation is observed over the entire pH range covered. As a control, 2HP titrations with pea seedling amine oxidase (results not shown) indicated 2 TPQs per dimer, in agreement with earlier reports [30]. Similar titrations with the ECAO variants D383N and D383E were attempted, but were found not to be reliable, owing to slow reaction with 2HP. Murray et al. [29] have shown that, under conditions of excess 2HP and longer reaction times, 2 TPQs per dimer could be demonstrated in WT ECAO. These results suggest that the environment and reactivity of TPQ in amine oxidases from different sources and in different mutational variants from one source (ECAO) vary. If we make the reasonable assumption that 2HP and TCP bind similarly, then the stopped-flow studies of TCP binding reported below involve only the rapidly reacting 1.3 TPQs per protein dimer.

Enzyme activity and kinetic parameters at different pH values

The specific activity of purified WT ECAO at pH 7.0 with β-phenylethylamine as substrate was 11.0 ± 0.2 μmol/min per mg. The specific activity of the D383E variant was 0.0038 μmol/min per mg. The D383N variant was catalytically inactive, as reported previously [12]. The steady-state parameters k_{cat} and K_m were determined over a range of pHs for WT and D383E ECAO (Table 1) under conditions where the O_2 concentration was saturating. Both displayed typical Michaelis–Menten behaviour. k_{cat} and K_m were determined by fitting to the Michaelis–Menten equation using a least-squares method (Microcal Origin 4.1). The pH dependencies of $1/K_m$ and k_{cat}/K_m for WT and D383E ECAO are displayed in Figure 2. It was not possible to determine parameters accurately above pH 8 for either the WT or D383E, due to the very low enzyme activity. It is clear that at least one pK_a can be established in the WT/β-phenylethylamine system. Data points up to pH 7.5 were fitted using eqn (5) (see below), where k_{cat}/K_m values replaced K_b values, indicating a pK_a of 5.8 ± 0.15. The

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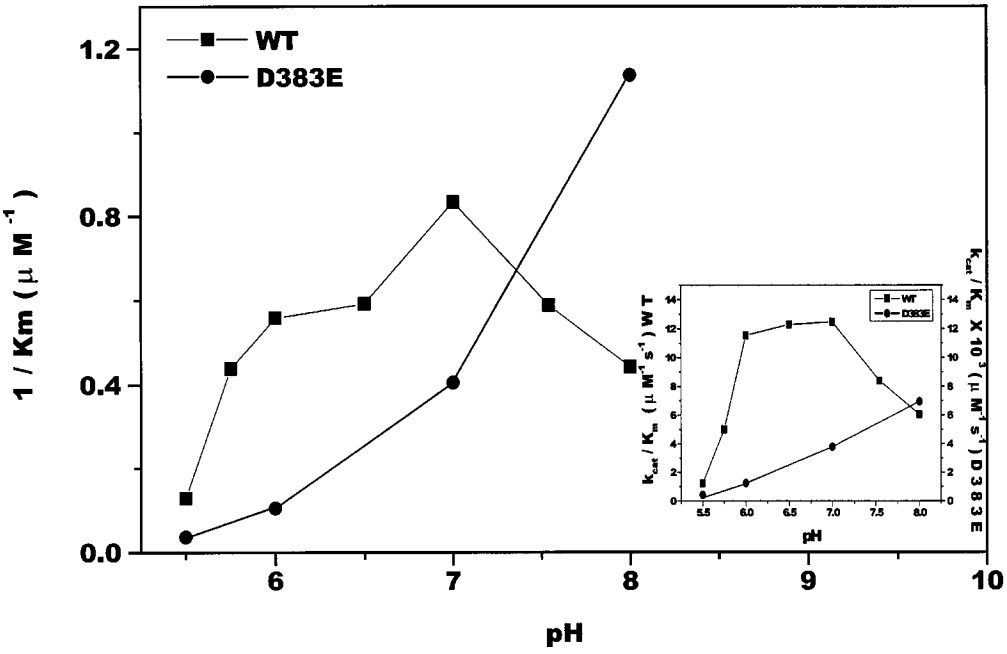

Figure 2 Dependence of $1/K_m$ on pH for catalytic turnover of β-phenylethylamine by WT (■) and D383E (●) ECAO

The inset shows similar data for the dependence of k_{cat}/K_m on pH.

Table 1 Kinetic parameters (K_m and k_{cat}) for WT ECAO and the D383E mutational variant

	K_m (μM)		k_{cat} (s^{-1})	
pH	WT	D383E	WT	D383E
5.5	7.79 ± 0.39	28 ± 3.3	9.6 ± 0.17	(11.63 ± 0.93) × 10^{-3}
5.75	2.28 ± 0.13		11.45 ± 0.21	
6.0	1.79 ± 0.08	9.62 ± 1.64	20.7 ± 0.26	(12.00 ± 1.08) × 10^{-3}
6.5	1.69 ± 0.10		20.77 ± 0.39	
7.0	1.2 ± 0.07	2.47 ± 0.27	14.98 ± 0.22	(9.37 ± 0.54) × 10^{-3}
7.5	1.7 ± 0.04		14.32 ± 0.36	
8.0	2.26 ± 0.06	0.88 ± 0.17	13.68 ± 0.11	(6.12 ± 0.31) × 10^{-3}

D383E/β-phenylethylamine system could not be fitted accurately in the same manner, although it is likely from the data that a pK_a of $\geqslant 8$ would result.

UV–visible determination of pK_a for TCP

The pK_a was determined (results not shown) by measuring the change in absorbance at 238 nm with pH. The pK_a of TCP was calculated to be 8.38 ± 0.03, which must correspond to the primary amine group (see Figure 1).

TCP binding is reversible

TCP binds to the WT, D383E and D383N forms of ECAO, as shown by the adduct chromophore in the 300–500 nm region. Extensive dialysis of the adducted enzyme (3 × 1000-fold excess of buffer over 16 h) was found to remove the adducted TCP and to restore enzyme activity, indicating that binding is reversible. Heating the adducted enzyme solution to 50–55 °C resulted in loss of absorbance associated with the adduct, while re-cooling the solution to 25 °C restored the original absorbance, again demonstrating that TCP binding is fully reversible. Proton titration of adducted forms of the enzymes did not result in any significant change in absorbance within the pH range 5.3–9.2.

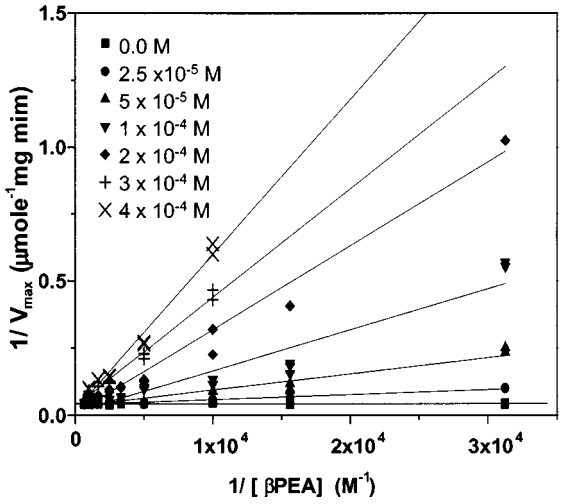

Figure 3 Dependence on substrate concentration of the initial rates of turnover of β-phenylethylamine (βPEA) by WT ECAO at different fixed concentrations of TCP shown in double-reciprocal form

The concentrations of TCP are given at the top left of the Figure. The data show that TCP is a competitive inhibitor.

TCP is a competitive inhibitor

TCP was found to be a competitive inhibitor of WT ECAO at pH 7 (Figure 3). Independent determinations of K_i from plots of

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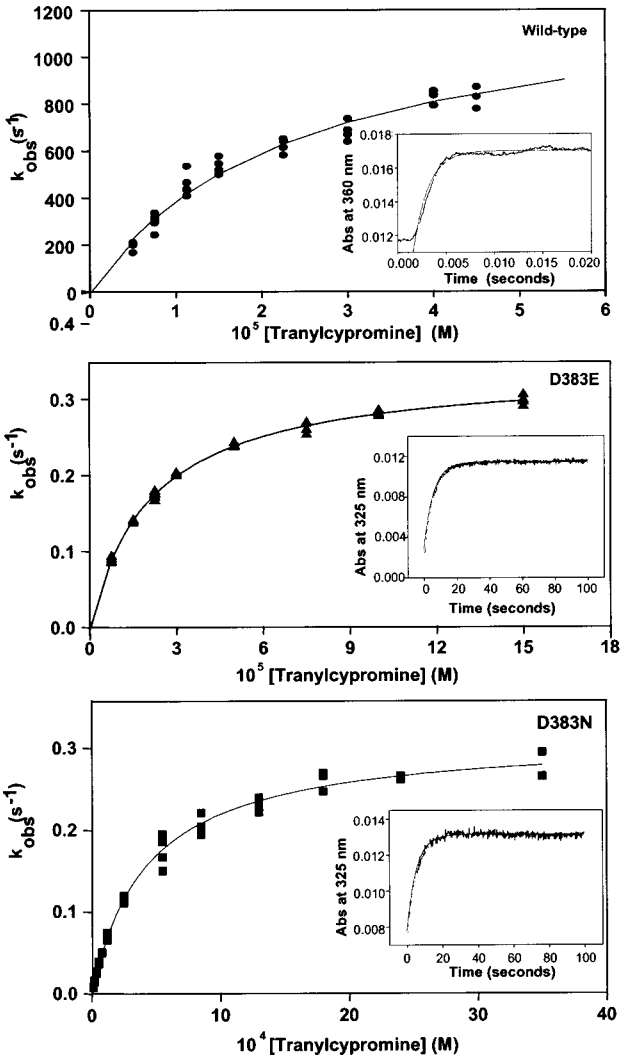

Figure 4 Stopped-flow studies of TCP binding to WT ECAO (top) and to the mutational variants D383E (middle) and D383N (bottom)

Shown is the dependence of k_{obs} on TCP concentration; the solid lines through the data are the fits according to eqns (3) and (4), yielding values for K_b and k_2. The insets show the raw stopped-flow data and the fit to give k_{obs}.

K_m^{app} against [TCP] and K_m^{app}/V_{max}^{app} against [TCP] gave values of 1.12 ± 0.2 μM and 1.31 ± 0.3 μM respectively. These values are in good agreement. K_i should be the dissociation constant for the ECAO/TCP adduct, and thus is comparable with $1/K_b$ determined by direct stopped-flow studies of TCP binding (see Figure 5, top panel). The value for $1/K_b$ of 13 μM at pH 7 is reasonably close to the above values for K_i, given the different reaction conditions for stopped-flow and coupled assay. Also, the pH dependence of $1/K_m$ and K_b for WT and D383E ECAO are qualitatively similar (compare Figure 2 with Figure 5).

Probing the initial substrate binding steps with TCP as a substrate analogue

Stopped-flow kinetic studies of TCP binding to WT ECAO allow the determination of binding constants and kinetic constants for adduct formation, which is analogous to substrate Schiff-base formation. Similar studies with mutational variants provide further insights into the role of the catalytic base Asp-383 in these early binding events.

Adduct formation between the WT, D383E and D383N forms of ECAO and TCP obeyed pseudo-first-order kinetics, showing saturation kinetics with respect to TCP concentration (Figure 4), and were fitted to a model, eqn (2), according to eqn (3):

$$E + TCP \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [E\text{–}TCP]^* \xrightarrow{k_2} \text{final adduct} \quad (2)$$

where E is enzyme and [E–TCP]* corresponds to an intermediate stage in adduct formation.

$$k_{obs} = \frac{K_b k_2 [TCP]}{1 + K_b [TCP]} \quad (3)$$

where $K_b = k_1/k_{-1}$.

Double-reciprocal plots give the kinetic parameters K_b and k_2 according to eqn (4):

$$\frac{1}{k_{obs}} = \frac{1}{K_b k_2} \frac{1}{[TCP]} + \frac{1}{k_2} \quad (4)$$

All reciprocal plots resulted in well defined linearity, and the resulting low degrees of error indicate that k_{obs} is hyperbolically dependent on inhibitor concentration.

Effects of pH on K_b and k_2 for TCP binding and adduct formation

The effects of pH on K_b and k_2 for the WT, D383N and D383E forms of ECAO are shown in Figure 5. For the WT ECAO/TCP system, K_b is affected significantly by pH, and the data are fitted according to eqn (5) using a least-squares program. The resulting 'bell-shaped' curve is indicative of two protonation events, where K_{a1} and K_{a2} represent the acid dissociation constants of two ionizing groups, and K_b^* corresponds to the maximum K_b value (Figure 5):

$$K_b = \frac{(K_b)^*}{\left(1 + \frac{[H^+]}{K_{a1}} + \frac{K_{a2}}{[H^+]}\right)} \quad (5)$$

where $pK_{a1} = 5.8 \pm 0.3$ and $pK_{a2} = 8.8 \pm 0.3$, for enzyme and TCP respectively. The value of pK_{a1} agrees with that obtained from the steady-state kinetic data (Figure 2) and is attributed to the catalytic base. The value of pK_{a2} agrees reasonably with the pK value for TCP determined experimentally (8.38 ± 0.03).

By comparison, the K_b versus pH data for mutants D383E and D383N (Figure 5, middle and bottom panels) showed only a single inflection point, and were fitted by least squares according to eqn (6):

$$K_b = \frac{(K_b)_o K_a + (K_b)_H [H^+]}{K_a + [H^+]} \quad (6)$$

where K_b corresponds to the experimentally determined values, and K_a, $(K_b)_o$ and $(K_b)_H$ can be determined from a fit of data to eqn (6). Here, $(K_b)_o$ and $(K_b)_H$ correspond to the estimated values of the high-pH and low-pH plateaus in Figure 5. D383E and D383N gave pK_a values of 8.0 ± 0.1 and 8.0 ± 0.3 respectively. The absence of the lower pK value seen with the WT enzyme is striking.

Clearly, there are differences in the magnitude of the K_b values between the WT enzyme and the catalytic base variants.

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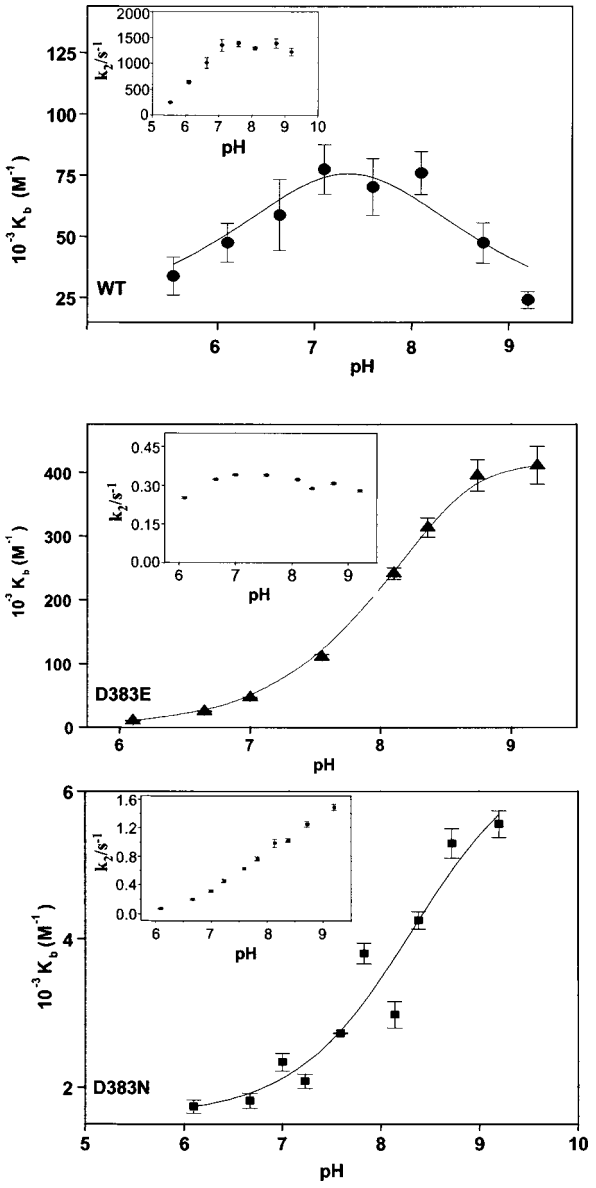

Figure 5 pH dependence of the binding parameters K_b and k_2 for binding of TCP to WT ECAO (top) and to the variants D383E (middle) and D383N (bottom)

For the K_b versus pH data, the solid line through the points is the fit according to eqn (5) for WT and eqn (6) for the variants. The insets show the dependence of k_2 on pH for the WT and variant forms.

Maximum binding occurs at pH 9 for D383E and D383N, and at pH 7.1 for WT. K_b values for WT and D383E are similar up to pH 7.5, but the K_b for D383E continues to increase further at higher pH values, whereas the K_b for WT decreases. The K_b versus pH profile for D383N mirrors that for D383E, yielding identical pK values, but the magnitude of K_b for D383N is two orders of magnitude lower than for D383E.

The saturating rate constants (k_2) as a function of pH for WT–, D383E– and D383N–TCP adduct formation are displayed in the insets to Figure 5. Both the WT and D383N systems showed variation with pH, indicative of a single pK_a, with a value of 6.2 ± 0.16 for WT and of 8.1 ± 0.2 for D383N. For D383E, variations in k_2 with pH are indicated at low pH (< 6) but did not allow a pK_a determination.

DISCUSSION

Consider first the effects of pH on the substrate binding and turnover parameters for ECAO. For the WT enzyme, the $1/K_m$ versus pH profile is bell-shaped, displaying pKs of 5.8 (attributed to Asp-383, the catalytic base) and ~ 8 (attributed to substrate amine). This behaviour is consistent with the dominant reactant species being the deprotonated base and the protonated amine, as proposed by Farnum et al. [31] from similar studies with bovine serum amine oxidase. The X-ray crystal structure of the D383E variant shows that there are minimal changes to the active site compared with the WT enzyme [12], with the base located close to O-5 of TPQ in both cases. In the present paper, we see that the optical spectra of WT ECAO and the D383E variant are closely similar, both showing λ_{max} at 480 nm, indicating that the base and its ability to ionize is having a similar effect on the TPQ chromophore; by contrast, the spectrum of the D383N variant, where this ionization cannot occur, shows λ_{max} at 450 nm. Based on these structural similarities of the active sites of WT and D383E ECAO, we would anticipate that the pH-dependence of $1/K_m$ for the two enzyme forms would be similar. In fact they are strikingly different, with D383E showing a steady increase in both parameters with pH over the pH range 6–8 and no maximum.

To explore the reasons for this distinct pH behaviour, we turned to studies with the inhibitor TCP. X-ray crystallography has shown (C. M. Wilmot, C. G. Saysell, A. Blessington, D. A. Conn, M. J. McPherson, P. F. Knowles and S. E. V. Phillips, unpublished work) that the 1*S*,2*R*-(+)-*trans* enantiomer of TCP binds to the carbonyl at the 5-position of TPQ and locates the bound inhibitor adjacent to Asp-383 (Figure 1, bottom). This is the site of substrate binding, as shown by studies on TPQ models by Mure and Klinman [32–34] and Lee and Sayre [35]. Stopped-flow kinetic studies [23] showed that the first intermediate in the catalytic cycle of bovine serum amine oxidase is a substrate Schiff base, with a λ_{max} of 340 nm. TCP binding to ECAO produces an adduct with a λ_{max} of 365 nm, which thus appears to correspond to the substrate Schiff base. In the present paper, TCP is shown to be a reversible competitive inhibitor of WT ECAO, and mechanistic studies of its binding should mimic the steps leading to substrate Schiff-base formation during the catalytic cycle. Studies of TCP binding also allow comparisons to be made between WT ECAO and the D383N variant, which could not be studied in the catalytic turnover experiments since it is inactive.

The λ_{max} for the TCP/WT ECAO chromophore is 365 nm, compared with 330 nm for the chromophore of the TCP adducts with D383E and D383N. This is an indication that the chemistry of the TCP adduct with WT ECAO on one hand, and with D383N and D383E on the other hand, is distinct. This changed chemistry may involve Asp-383 being unprotonated in the adduct with WT ECAO (final species in pathway I, Scheme 1), whereas it is protonated in D383E or blocked as the amide in D383N (final species in pathway II, Scheme 1). The kinetics for TCP binding to WT ECAO, D383N and D383E tend towards saturation at higher TCP concentrations, and can be analysed to give a binding constant K_b for the reaction between TCP and protein, and a rate constant k_2 for formation of the chromophore.

For WT ECAO, the pH dependence of K_b reveals a maximum at pH 7, similar to that observed in the $1/K_m$ versus pH behaviour already discussed. The pH dependence of k_2 suggests that a reaction step involving the unprotonated base (Asp-383) is rate

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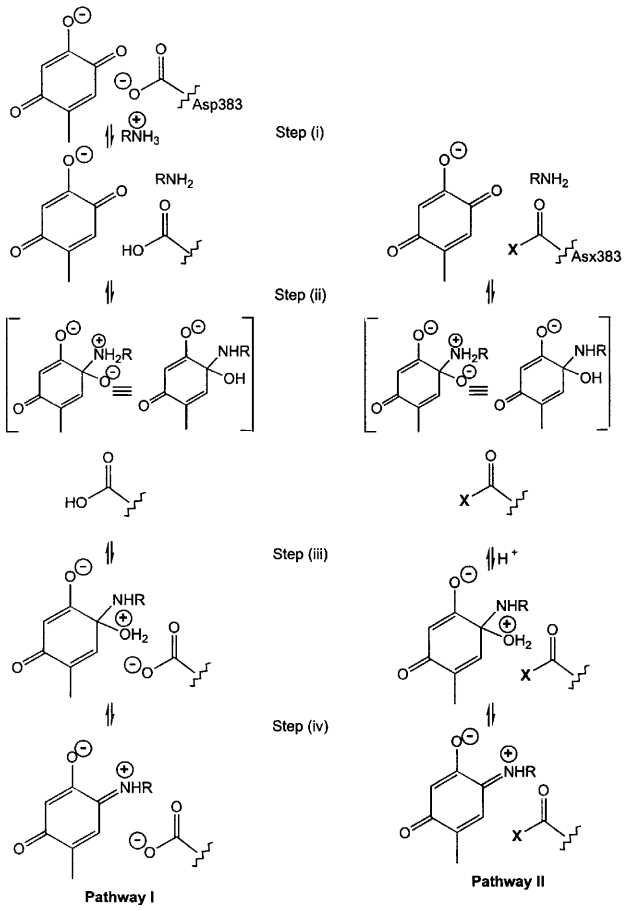

Scheme 1 Alternative mechanistic pathways for formation of the TCP adduct of ECAO

O═C–X represents either O═C–OH or O═C–NH_2 at position 383 for WT and D383N respectively. Asx represents either Asp or Asn. TCP is represented by RNH_2. In pathway I (WT enzyme), Asp-383 facilitates TCP binding by accepting a proton from the protonated amine in step (i) and donating a proton back to the unstable charge-distributed oxyanion species in step (iii), thus enhancing the rate of formation of the substrate Schiff base. In pathway II, which is a less favoured pathway in the WT enzyme but the only possible pathway in D383N, deprotonated amine binds in step (ii) and a proton, presumably from water, is required to stabilize the oxyanion in the charge-distributed species (step iii).

limiting, consistent with pathway I in Scheme 1, with step (i) being the probable rate-limiting step in TCP chromophore formation.

Consider next the TCP binding data for D383N. The pH dependence of K_b indicates an increase with increasing pH, and a pK of ~ 8 points to unprotonated TCP (pK 8.3) being the reacting species. This suggests a reaction by pathway II (see Scheme 1), which can be regarded as the pathway followed in the absence of rate enhancement from the catalytic base. The pH dependence of k_2 for D383N suggests that step (ii) may be the rate-limiting step in pathway II. The pH profile for K_b for the binding of TCP by D383E mirrors qualitatively that with D383N, suggesting that pathway II is followed with this variant. However, quantitatively K_b is 1–2 orders of magnitude greater for D383E than for D383N. The explanation for this might lie in the ordered nature of the TPQ in D383E compared with D383N [12] and its orientation, which could position it well for reaction with the deprotonated TCP. The low value of k_2 for D383E and its pH independence is suggested to be due to steric crowding in the active site preventing water from leaving from the carbinolamine intermediate at step (iv) of pathway II.

Based on this analysis of the TCP binding data for D383E, we suggest that substrate binding to D383E also follows pathway II. For some reason not obvious from the structure, the carboxylate side chain of Glu-383 is unable to accept a proton from the protonated amine and thus to follow the favourable pathway (pathway I) used exclusively in the WT enzyme with aspartate at this site. Evidence has been presented previously that Asp-383 in ECAO and its counterpart in *Hansenula polymorpha* amine oxidase (Asp-319) perform multiple roles in the catalytic cycle of amine oxidases [12,36], and the present paper defines the role in the early steps leading to substrate Schiff-base formation in more detail. Schiff-base formation in WT ECAO does not follow the chemically intuitive route of nucleophilic attack by free amine at the 5-position of TPQ (i.e. pathway II in Scheme 1), but rather has evolved to bind protonated amine initially, then to pass the proton to the proximal Asp-383 base.

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