Plausible molecular mechanism for activation by fumarate and electron transfer of the dopamine β -mono-oxygenase reaction

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A series of fumarate analogues has been used to explore the molecular mechanism of the activation of dopamine β -mono-oxygenase by fumarate. Mesaconic acid (MA) and *trans*-glutaconic acid (TGA) both activate the enzyme at low concentrations, similar to fumarate. However, unlike fumarate, TGA and MA interact effectively with the oxidized enzyme to inhibit it at concentrations of 1–5 mM. Monoethylfumarate (EFum) does not activate the enzyme, but inhibits it. In contrast with TGA and MA, however, EFum inhibits the enzyme by interacting with the reduced form. The saturated dicarboxylic acid analogues, the geometric isomer and the diamide of fumaric acid do not either activate or inhibit the enzyme. The phenylethylamine–fumarate conjugate, *N*-(2-phenylethyl)fumaramide (PEA-Fum), is an ~ 600 -fold more potent inhibitor than EFum and behaves as a bi-substrate inhibitor for the reduced enzyme. The amide of PEA-Fum behaves similarly, but with an inhibition

potency ~ 20 -fold less than that of PEA-Fum. The phenylethylamine conjugates of saturated or geometric isomers of fumarate do not inhibit the enzyme. Based on these findings and on steady-state kinetic analysis, an electrostatic model involving an interaction between the amine group of the enzyme-bound substrate and a carboxylate group of fumarate is proposed to account for enzyme activation by fumarate. Furthermore, in light of the recently proposed model for the similar copper enzyme, peptidylglycine α -hydroxylating mono-oxygenase, the above electrostatic model suggests that fumarate may also play a role in efficient electron transfer between the active-site copper centres of dopamine β -mono-oxygenase.

Key words: ascorbic acid, catecholamine biosynthesis, enzyme activation, mono-oxygenation.

INTRODUCTION

Dopamine β -mono-oxygenase (D β M; EC 1.14.17.1), a copper-containing enzyme, catalyses the conversion of dopamine into noradrenaline (norepinephrine) in the chromaffin granules of the adrenal medulla and the large dense-cored synaptic vesicles of the sympathetic nervous system [1–8]. While the crystal structure of the D β M active site is not yet available, the existence of distinct binding sites for the amine substrate and the reductant is evident from the steady-state kinetics [9]. Based on this and other experimental evidence, a model in which one of the copper sites functions exclusively as the reduction site, while the other is responsible for molecular oxygen activation and insertion into the organic substrate, has been proposed for the D β M active site [9–12]. Although the chemistry of the hydroxylation site of the enzyme has been extensively studied, details of the reduction site are still poorly understood [6–8,13,14]. For example, 6-*O*- and 6-*S*-alkylphenyl-L-ascorbic acid derivatives were shown to be much more efficient reductants for the enzyme than the natural reductant, ascorbic acid (AscH₂) [10,15]. In addition, well-characterized outer sphere single-electron reductants such as *N,N,N',N'*-tetramethyl-1,4-*p*-phenylenediamine and *N,N*-dimethyl-1,4-*p*-phenylenediamine (DMPD), which have no structural resemblance to AscH₂, are also efficient reductants for the enzyme [16,17].

Halides and organic anions, especially the dicarboxylic acid fumarate, have long been known to activate D β M under

steady-state conditions [18–21]. The observation that fumarate is capable of activating D β M even in the presence of saturating levels of AscH₂ has been used as evidence for the existence of a distinct anion activation site in the enzyme [22]. Based on the effects of fumarate on the steady-state kinetics with two different classes of reductants, we have previously proposed a unified model for enzyme activation by fumarate in which both fumarate and the reductant interact with the reduction site of the various steady-state forms of the enzyme with different affinities [10]. However, the molecular details of the effect(s) of fumarate on the steady-state kinetics of the enzyme are still not understood.

In the present studies, a series of fumarate derivatives, analogues and their phenylethylamine conjugates have been used to examine the electronic and steric constraints of the fumarate, phenylethylamine and reductant binding regions, and to explore the molecular mechanism of activation of the enzyme by fumarate. Our findings strongly suggest that the electrostatic interaction between the amine group of the active-site-bound amine substrate and a carboxylate group of fumarate is primarily responsible for the observed effects of fumarate on steady-state kinetics. In addition, the remarkable structural similarity between *N*-(2-phenylethyl)fumaramide (PEA-Fum) and the high-affinity bi-substrate reductant 6-deoxy-*S*-ethylphenyl ascorbic acid (6SPEA) further supports the previous proposal that fumarate and the reductant interact with the same site of the enzyme. Furthermore, in light of the proposed model for the similar copper enzyme, peptidylglycine α -hydroxylating

Abbreviations used: AscH₂, ascorbic acid; DMPD, *N,N*-dimethyl-1,4-*p*-phenylenediamine; D β M, dopamine β -mono-oxygenase; EFum, monoethylfumarate; E_{rd}, reduced form of the enzyme; E_{rd} · Fum, reduced fumarate-bound form of the enzyme; E_{rd} · Fum · O₂, reduced fumarate- and oxygen-bound form of the enzyme; E_{rd} · O₂, reduced oxygen-bound form of the enzyme; E_{rd} · Tyr, reduced tyramine-bound form of the enzyme; E_{rd} · Tyr · Fum, reduced fumarate- and tyramine-bound form of the enzyme; E_{rd} · Tyr · O₂, reduced tyramine- and oxygen-bound form of the enzyme; MA, mesaconic acid; PEA-Fum, *N*-(2-phenylethyl)fumaramide; PEA-Fum-NH₂, *N*-(2-phenylethyl)fumaradiamide; PEA-Glu, *N*-(2-phenylethyl)glutaramide; PEA-Mal, *N*-(2-phenylethyl)maleamide; PEA-Suc, *N*-(2-phenylethyl)succinamide; 6SPEA, 6-deoxy-*S*-ethylphenyl ascorbic acid; TGA, *trans*-glutaconic acid.

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mono-oxygenase [23,24], the above electrostatic model suggests that fumarate may also play a role in efficient electron transfer between the active-site copper centres of D β M.

MATERIALS AND METHODS

Materials

Tyramine hydrochloride, disodium fumarate and Mes were from Sigma. Monoethylfumarate (EFum), *trans*-glutaconic acid (TGA), mesaconic acid (MA), AsCH₂ and DMPD were from Aldrich. DMPD was purified further by recrystallization. Beef liver catalase (65000 units/mg of protein) was from Boehringer-Mannheim. All other chemicals were of the highest purity available and were purchased from various sources. Bovine adrenal soluble D β M was isolated and purified (specific activity 23–30 units/mg) according to the procedures described previously [25], with minor modifications, using freshly prepared bovine adrenal chromaffin granules [26,27]. The concentration of purified enzyme was estimated spectrophotometrically using $\epsilon_{280} = 1.24 \text{ ml} \cdot \text{mg}^{-1} \cdot \text{cm}^{-1}$.

General methods

UV–visible spectroscopic measurements were carried out using a Varian Cary 300 Bio double-beam UV–visible spectrophotometer equipped with a temperature-controlled cell compartment. ¹H- and ¹³C-NMR spectra were recorded on a Varian XL-300 (300 MHz) NMR spectrometer using tetramethylsilane or 3-(trimethylsilyl)-1-propanesulphonic acid sodium salt (when ²H₂O was the NMR solvent) as internal standards. Initial rates of steady-state oxygen consumption were measured using a Yellow Springs Model YSI 5300 polarographic oxygen monitor.

Oxygen monitor assay of D β M activity

The initial rates of the D β M-catalysed reaction under various experimental conditions were determined by measuring the initial rate of oxygen consumption using a polarographic oxygen electrode at 37 °C as previously described [17]. All enzymic reactions were carried out in 50 mM Mes, pH 5.2. The standard reaction mixtures contained 100 $\mu\text{g}/\text{ml}$ catalase, 2 μM CuSO₄ and 10 mM AsCH₂, in the presence or absence of 10 mM fumarate depending on the experiment, unless otherwise stated, in a total volume of 2.6 ml. D β M concentrations were kept at a constant 2.8 μg or 5.6 μg of protein per assay in the presence and absence of fumarate respectively. The enzymic reactions were usually initiated by the addition of the desired concentration of tyramine, and the initial turnover rates were measured as the rate of oxygen consumption minus the small background rate due to auto-oxidation of AsCH₂. Stock solutions of sparingly water-soluble inhibitors were made in the buffer with 20 μl of 6 M NaOH, and the pH of the final solution was adjusted to 5.2 with dilute HCl.

Spectrophotometric assays of D β M activity

All enzymic reactions were carried out in 50 mM Mes, pH 5.2. The standard reaction mixtures contained 100 $\mu\text{g}/\text{ml}$ catalase and 0.5–2.0 μM CuSO₄ in the presence or absence of 10 mM fumarate depending on the experiment, unless otherwise stated, in a total volume of 1.0 ml. D β M concentrations were kept constant for each experiment, but varied between 0.6 and 1.2 μg of protein per assay depending on the experiment. The enzymic reactions were usually initiated with the reductant (DMPD),

unless otherwise stated. The rate of increase in absorbance at 515 nm due to the enzyme-mediated formation of the DMPD cation radical at 37 °C was measured against a reference solution identical to the enzymic reaction mixture, but without the enzyme, as previously described [17] (for further details see the corresponding Figure legends). In the enzyme inhibition experiments, when the reductant was the variable substrate, the tyramine concentration was kept at 10 mM (saturating) and the reductant, DMPD, was varied between 2 and 12.5 mM, unless otherwise stated. Similarly, when tyramine was the variable substrate, DMPD was kept at a constant concentration of 12.5 mM and tyramine was varied between 2 and 10 mM.

Data analysis and molecular modelling

All kinetic parameters were apparent, and were determined at a single concentration of 256 μM oxygen (atmospheric oxygen saturation). V_{max} values were calculated based on the monomer molecular mass of 70000 Da for D β M. The initial screening of substrates and inhibitors was carried out under two different assay conditions, i.e. high reductant/low amine (12.5 mM DMPD and 2 mM tyramine) and low reductant/high amine (2 mM DMPD and 10 mM tyramine) concentrations, in the presence and absence of fumarate. The activation or inhibition of the enzyme by various fumarate analogues and derivatives under these conditions was determined qualitatively by plotting the steady-state kinetic data in the form of v against activator or inhibitor concentration. All inhibition experiments were carried out using at least five different concentrations of the inhibitor in the desirable concentration ranges, which were determined by the above screening experiments (for further details see corresponding Figure legends).

All inhibition kinetic parameters were determined by direct fit of the steady-state kinetic data to Cleland's COMP, UNCOMP and NONCOMP programs, and the patterns of inhibition were determined by using the stated statistical criteria [28]. The quantitative analysis of enzyme activation by fumarate was carried out by standard fitting of the kinetic data to the desired equation (eqn 1; see below) using Sigma Plot (Jandel Scientific). Individual structures of bi-substrate reductants and bi-substrate inhibitors were minimized by using a MM2 parameter set and were overlaid for comparison purposes by using Chem3D Pro (Cambridge Software Corp.).

Syntheses

PEA-Fum

A solution of 2.54 g (17.6 mmol) of EFum and 4.05 g (19.6 mmol) of dicyclohexylcarbodi-imide in 20 ml of dichloromethane was stirred for 10 min, and then 2.02 g (16.7 mmol) of phenylethylamine was added. The reaction mixture was stirred for 4 h, then the white precipitate, dicyclohexylurea, was filtered and the filtrate was evaporated to dryness under reduced pressure. The crude product was purified by silica gel column chromatography (ethyl acetate/hexane, 3:7, v/v). The pure ester was suspended in 2 M NaOH and stirred overnight at room temperature. The homogeneous reaction mixture was extracted with chloroform and the aqueous layer was acidified with conc. HCl and refrigerated overnight. The white crystalline product was filtered and purified further by crystallization with hot water. The overall yield was 2.2 g (57%). ¹H-NMR (²H₂O): δ 2.72 (t, J = 6.9 Hz, 1 H), 3.42 (t, J = 6.9 Hz, 1 H), 6.41 (d, J = 15.7 Hz, 1 H), 6.50 (d, J = 15.7 Hz, 1 H), 7.15–7.27 (m, 5 H).

Figure 2 Structures of fumarate analogues and phenylethylamine conjugates

inhibited by 100%, 94% and 36% in the presence of 10 mM, 5 mM and 1 mM concentrations respectively of TGA, the one-carbon-extended analogue of fumaric acid. Similarly, 10 mM and 5 mM concentrations of the 2-methyl derivative of fumarate, MA, inhibited the enzyme by 100% and 89% respectively. In addition, the enzyme was inhibited by 100%, 89% and 33% at 10 mM, 5 mM and 1 mM concentrations respectively of EFum under the same experimental conditions. However, the saturated dicarboxylic acid analogues (oxalic, malonic, succinic and glutaric acids), the geometric isomer of fumarate (maleic acid) and the diamide of fumaric acid (fumaradiamide) did not inhibit or activate the enzyme significantly at 10 mM under the same experimental conditions (for structures see Figure 2).

The qualitative examination of the effects of fumarate on the steady-state kinetics of D β M under two different sets of experimental conditions, i.e. low reductant/high amine (2.0 mM DMPD and 10 mM tyramine) and high reductant/low amine (12.5 mM DMPD and 2.0 mM tyramine) concentrations, demonstrated that fumarate behaves as an activator of the enzyme under both sets of conditions in the concentration range tested, i.e. 0.125–11 mM (Figure 3). While no enzyme inhibition was observed under the above conditions, at low reductant and high tyramine (10 mM) concentrations, high fumarate concentrations resulted in weak competitive inhibition with an estimated K_i of > 40 mM with respect to DMPD, suggesting weak competition for the reduction site of the enzyme (results not shown). These results demonstrate that fumarate behaves primarily as an activator of D β M at all tyramine concentrations when the DMPD concentration is > 2 mM and the fumarate concentration is < 20 mM.

Figure 3 v against [fumarate] plots for the steady-state kinetics of the D β M reaction at constant concentrations of tyramine and DMPD

The initial apparent rates of the DMPD-mediated D β M-catalysed reactions were measured as a function of fumarate concentration under the conditions described in the Materials and methods section, at constant concentrations of tyramine and the reductant, DMPD. Concentrations of DMPD and tyramine were: ○, 2 mM and 10 mM respectively; ●, 12.5 mM and 2 mM respectively.

Figure 4 shows that TGA possesses both enzyme activation and inhibition properties, depending on the experimental conditions and the concentration of TGA. TGA activates the enzyme in the concentration range 100 μ M–1.0 mM under low tyramine/high reductant concentration conditions (2 mM tyramine/12.5 mM DMPD) and in the absence of fumarate (Figure 4B). However, no enzyme activation was observed under high tyramine/low reductant concentration conditions (10 mM tyramine and 2 mM DMPD) (Figure 4A). In addition, in the presence of 10 mM fumarate, enzyme activation by TGA was not observable under either set of conditions. On the other hand, TGA at concentrations above 1.0 mM inhibited the enzyme under both low reductant/high tyramine and high reductant/low tyramine concentration conditions (Figures 4A and 4B). Enzyme inhibition was not significantly affected by the presence of 10 mM fumarate (Figures 4A and 4B). However, the data in Figure 3 show that fumarate only activated the enzyme in the same concentration range (125 μ M–11 mM) under both sets of experimental conditions, with no observable inhibition of the enzyme. The inhibition kinetic studies demonstrate that TGA is a reversible inhibitor of D β M that displays competitive inhibition kinetics with respect to the reductant (DMPD) and uncompetitive kinetics with respect to tyramine in the presence of fumarate (Table 2; accurate inhibition kinetic parameters could not be determined in the absence of fumarate under these conditions due to significant enzyme activation at low tyramine concentrations). In addition, K_i increased slightly with respect to DMPD in the presence of 10 mM fumarate (Table 2).

The behaviour of MA was observed to be parallel to that of TGA. However, MA is a good activator of D β M, with an activation potency similar to that of fumarate itself. The direct plot of the velocity data as a function of tyramine concentration at different concentrations of MA (Figure 5B) demonstrates that, under variable tyramine/high reductant concentration conditions, MA activates the enzyme at concentrations up to approx. 2 mM, similar to fumarate (see Figure 1), but inhibits the enzyme at higher concentrations. Furthermore, enzyme activation by MA is also apparent even under low reductant/high tyramine concentration conditions, although it is not as pronounced as

Table 2 Steady-state D β M inhibition kinetic parameters for various fumarate analogues and PEA-Fum conjugates under standard assay conditions

The reductants were AsCH₂ and DMPD in oxygen monitor and DMPD assays respectively. The inhibition kinetic parameters and the inhibition patterns were determined by the fit of the data to the Cleland's COMP, UNCOMP and NONCOMP programs as described in the Materials and methods section. (C), a competitive inhibition pattern was observed; (U), an uncompetitive inhibition pattern was observed; NA, not applicable; ND, not determined.

Inhibitor	Variable substrate	K_i (mM)			
		Oxygen monitor assay		DMPD assay	
		+ Fumarate (10 mM)	— Fumarate	+ Fumarate (10 mM)*	— Fumarate
EFum	Tyramine	1.24 $\pm$ 0.08 (C)	1.2 $\pm$ 0.1 (C)	1.5 $\pm$ 0.3 (C)	1.6 $\pm$ 0.2 (C)*
	DMPD	NA	NA	3.9 $\pm$ 0.5 (U)	4.4 $\pm$ 0.5 (U)*
TGA	Tyramine	ND	ND	1.8 $\pm$ 0.2 (U)	ND†
	DMPD	NA	NA	2.7 $\pm$ 0.2 (C)	1.5 $\pm$ 0.2 (C)
MA	Tyramine	ND	ND	5.5 $\pm$ 0.4 (U)	ND†
	DMPD	NA	NA	5.6 $\pm$ 0.7 (C)	ND†
PEA-Fum	Tyramine	(3.8 $\pm$ 0.5) $\times 10^{-3}$ (C)	(2.2 $\pm$ 0.2) $\times 10^{-3}$ (C)	(4.6 $\pm$ 0.4) $\times 10^{-3}$ (C)	(2.6 $\pm$ 0.3) $\times 10^{-3}$ (C)
	DMPD	NA	NA	(4.1 $\pm$ 0.5) $\times 10^{-3}$ (U)	(4.6 $\pm$ 0.4) $\times 10^{-3}$ (U)
	AsCH ₂	ND	(4.9 $\pm$ 0.7) $\times 10^{-3}$ (U)	NA	NA
PEA-Fum-NH ₂	Tyramine	(7.7 $\pm$ 1.0) $\times 10^{-2}$ (C)	(2.5 $\pm$ 0.3) $\times 10^{-2}$ (C)	(11.9 $\pm$ 0.7) $\times 10^{-2}$ (C)	(9.5 $\pm$ 0.7) $\times 10^{-2}$ (C)
	DMPD	NA	NA	(40.9 $\pm$ 3.2) $\times 10^{-2}$ (U)	(15.7 $\pm$ 1.3) $\times 10^{-2}$ (U)
	AsCH ₂	(11.1 $\pm$ 3.8) $\times 10^{-2}$ (U)	(11.8 $\pm$ 25) $\times 10^{-2}$ (U)	NA	NA

* No enzyme activation was observed under the experimental conditions.

† Could not be determined accurately due to enzyme activation.

Figure 4 v against [TGA] plots for the steady-state kinetics of the D β M reaction at constant concentrations of tyramine and DMPD

The initial apparent rates of the DMPD-mediated D β M-catalysed reactions were measured as a function of TGA concentration under the conditions described in the Materials and methods section, at constant concentrations of tyramine and the reductant DMPD. Concentrations of DMPD and tyramine were (A) 2 mM and 10 mM respectively, and (B) 12.5 mM and 2 mM respectively. ○, TGA; ●, TGA plus 10 mM fumarate.

with fumarate (Figure 5A). As expected, inclusion of 10 mM fumarate in the assay solution completely abolished the enzyme activation effect of MA (Figure 5A), suggesting that fumarate and MA activate the enzyme by a similar mechanism. Similar to TGA, enzyme inhibition by MA is competitive with respect to the reductant and uncompetitive with respect to tyramine in the presence of 10 mM fumarate (note that accurate kinetic constants could not be obtained in the absence of fumarate due to enzyme activation by MA under the experimental conditions). The comparison of K_i parameters indicates that MA is a somewhat weaker inhibitor than TGA under identical experimental conditions (Table 2).

In contrast with the behaviour of fumarate, TGA and MA, EFum did not show any detectable activation of D β M either at low DMPD/high tyramine (Figure 6A) or high DMPD/low tyramine (Figure 6B) concentration conditions in the concentration range 125 μ M–11 mM EFum. However, EFum

was an inhibitor of the enzyme under both sets of conditions (Figure 6). The inclusion of 10 mM fumarate in the assay solution did not affect the inhibition potency significantly under either set of conditions. The steady-state inhibition kinetic studies demonstrate that EFum is a kinetically well-behaved reversible inhibitor of the enzyme that displays competitive inhibition kinetics with respect to tyramine and uncompetitive kinetics with respect to the reductant, DMPD, in contrast with the behaviour of TGA and MA (Table 2). However, the inhibition potency of EFum is comparable with that of TGA (Table 2). The data presented in Table 2 also indicate that the inhibition constants for EFum with respect to tyramine or DMPD were not significantly affected by the presence of 10 mM fumarate in the assay solution. Furthermore, inhibition kinetic parameters determined by the oxygen monitor assay using AsCH₂ as reductant yielded similar K_i values, suggesting that enzyme inhibition by EFum is not significantly affected by the nature of the reductant.

Figure 5 Effects of MA on $D\beta M$ activity

(A) v against [MA] plots for the steady-state kinetics of the $D\beta M$ reaction at constant concentrations of tyramine and DMPD. The initial apparent rates of the DMPD-mediated $D\beta M$ -catalysed reactions were measured as a function of MA concentration under the conditions described in the Materials and methods section, at constant concentrations of tyramine (2 mM) and DMPD (10 mM). $\circ$, MA; $\bullet$, MA plus 10 mM fumarate. (B) Plots of the initial steady-state rates of the $D\beta M$ reaction as a function of tyramine concentration in the presence of MA and a constant high concentration of DMPD (12.5 mM). Concentrations of MA were 0.0 mM ($\circ$), 2.0 mM (∇), 3.0 mM ($\square$), 3.5 mM ($\blacksquare$) and 4.0 mM ($\diamond$).

Figure 6 v against [EFum] plots for the steady-state kinetics of the $D\beta M$ reaction at constant concentrations of tyramine and DMPD

The initial apparent rates of the DMPD-mediated $D\beta M$ -catalysed reactions were measured as a function of EFum concentration under the conditions described in the Materials and methods section, at constant concentrations of tyramine and the reductant DMPD. $\circ$, EFum; $\bullet$, EFum plus 10 mM fumarate. Concentrations of DMPD and tyramine were (A) 2 mM and 10 mM respectively and (B) 12.5 mM and 2 mM respectively.

Interaction of phenylethylamine–fumarate conjugates with $D\beta M$

In order to examine further the interaction of fumarate and its analogues with $D\beta M$, a series of phenylethylamine conjugates, including PEA-Fum, PEA-Fum- NH_2 , PEA-Suc, PEA-Mal and PEA-Glu (Figure 2), were synthesized, characterized, and their effects on the enzyme examined. Initial screening experiments revealed that $D\beta M$ was almost completely inhibited by 100 μM PEA-Fum, suggesting that it is a much more potent inhibitor of the enzyme than EFum, TGA and MA. Similarly, the amide derivative of PEA-Fum, PEA-Fum- NH_2 , inhibited the enzyme by approx. 50% at 100 μM . Interestingly, PEA-Suc, PEA-Mal and PEA-Glu displayed no significant enzyme inhibition, even at concentrations as high as 500 μM . In addition, none of these compounds were activators, substrates or irreversible inhibitors of the enzyme under standard assay conditions.

Steady-state kinetic studies indicated that, under standard assay conditions, PEA-Fum inhibits the enzyme competitively with respect to tyramine and uncompetitively with respect to the reductant, DMPD. These studies also revealed that K_i for PEA-Fum with respect to tyramine was $2.6 \pm 0.3 \mu\text{M}$ in the absence of fumarate when DMPD was the reductant (Table 2), suggesting

that it is a potent inhibitor of $D\beta M$ under these experimental conditions. The results shown in Table 2 also suggest that 10 mM fumarate decreases the inhibition potency with respect to tyramine noticeably under these conditions. However, no significant effect of fumarate was detectable with respect to DMPD (Table 2). A similar trend was observed with respect to tyramine when AscH_2 was the reductant (Table 2; oxygen monitor assay). Similar to PEA-Fum, PEA-Fum- NH_2 inhibits the enzyme competitively with respect to tyramine and uncompetitively with respect to DMPD, but with a much less pronounced inhibition potency (Table 2). However, the decreased inhibition potency of PEA-Fum- NH_2 in the presence of 10 mM fumarate was significant with respect to DMPD, but not with respect to tyramine. On the other hand, when AscH_2 was the reductant, 10 mM fumarate significantly decreased the inhibition potency of PEA-Fum- NH_2 with respect to tyramine, but not with respect to AscH_2 .

DISCUSSION

The steady-state kinetics of $D\beta M$ in the presence of fumarate at variable tyramine/high constant reductant concentrations could

only be satisfactorily fitted to a minimum kinetic scheme in which fumarate interacts exclusively with the reduced forms of the enzyme (Figure 1). The kinetic parameters (Table 1) obtained from these fits demonstrate that fumarate has a high affinity for the reduced form of the enzyme ($K_m = 0.18 \pm 0.02$ mM). Interestingly, the magnitude of the modulation factor α is 0.23 ± 0.03 (Scheme 1 and Table 1), suggesting that the tyramine-bound reduced enzyme, $E_{rd} \cdot \text{Tyr}$ (and/or the oxygen-bound form $E_{rd} \cdot O_2 \cdot \text{Tyr}$), has a higher affinity for fumarate than the reduced free enzyme, E_{rd} (and/or $E_{rd} \cdot O_2$) itself. Similarly, the fumarate-bound reduced enzyme, $E_{rd} \cdot \text{Fum}$ (and/or $E_{rd} \cdot O_2 \cdot \text{Fum}$), has a higher affinity for tyramine than the reduced free enzyme, E_{rd} (and/or $E_{rd} \cdot O_2$), itself. Consequently, the K_m for tyramine is decreased from 6.1 ± 0.6 mM in the absence of fumarate to 1.4 mM in its presence, which is in agreement with previous kinetic studies of the enzyme [21]. Similarly, the K_m for fumarate is decreased from 0.18 ± 0.02 mM in the absence of tyramine to 0.04 ± 0.002 mM in its presence. These results demonstrate that tyramine and fumarate modulate each other's binding affinities for E_{rd} (and/or $E_{rd} \cdot O_2$) in a mutually facilitative fashion. Furthermore, the magnitude of the second modulation factor, β (Scheme 1 and Table 1), which is close to unity (0.94 ± 0.05), demonstrates that fumarate has no significant effect on the combined chemical and product release steps of the enzyme, which is also in agreement with previous kinetic studies [21]. Therefore the kinetic mechanism shown in Scheme 1 describes the activation of $D\beta M$ by fumarate quantitatively under the described experimental conditions.

The fumarate analogues TGA and MA behave similarly to fumarate, but with some interesting subtle differences. Similar to fumarate, low concentrations of both of these derivatives activate $D\beta M$, especially at low tyramine concentrations (Figures 4 and 5). However, unlike fumarate, these compounds inhibit the enzyme strongly under standard steady-state conditions, even in the presence of high concentrations of reductant. The quantitative kinetic analyses demonstrate that the inhibitions are competitive with respect to the reductant and uncompetitive with respect to tyramine, suggesting that they compete for the reduction site of the oxidized enzyme. On the other hand, the ability of these analogues to activate the enzyme, especially at low tyramine concentrations, suggests that they also interact with the reduced enzyme, similar to fumarate. Therefore, while the interaction of fumarate and its analogues with the reduced enzyme leads to enzyme activation, especially at low tyramine concentrations, their interaction with the oxidized enzyme leads to enzyme inhibition at low reductant concentrations. While the affinities of TGA and MA for the reduced enzyme were similar to that of fumarate, their affinities for the oxidized enzyme were significantly higher (K_i values in the range 2–7 mM) than that of fumarate ($K_i \sim 40$ mM) under similar experimental conditions, indicating the subtle differences between the fumarate sites of the reduced and oxidized forms of the enzyme.

The contrasting behaviour of EFum in comparison with fumarate, TGA and MA is intriguing. While EFum possesses no enzyme activation properties under any experimental conditions, it still is a good inhibitor of $D\beta M$, with a potency comparable with those of TGA and MA. However, unlike TGA and MA, enzyme inhibition by EFum is competitive with respect to tyramine and uncompetitive with respect to the reductant, suggesting that inhibition by EFum is due to competition with tyramine for the reduced enzyme. Since the interaction of fumarate or fumarate analogues such as TGA and MA with the reduced enzyme usually activates the enzyme (see above), the ineffectiveness of EFum as an activator could be due to the lack of a second free carboxylate group in the molecule. Similarly, if

EFum interacts with the reduced enzyme in a manner similar to fumarate, its competition with tyramine could be due to the overlap of the ethyl functionality of the enzyme-bound EFum with the amine-binding site of the reduced enzyme (see below).

The kinetic parameters listed in Table 2 demonstrate that the conjugate PEA-Fum is a potent inhibitor of $D\beta M$, with a potency 600-fold greater than that of EFum and other fumarate analogues. The inhibition is competitive with respect to tyramine and uncompetitive with respect to the reductant, demonstrating exclusive interaction of PEA-Fum with the reduced form of the enzyme (Table 2). In contrast, the other structurally similar phenylethylamine conjugates PEA-Suc, PEA-Mal and PEA-Glu showed little or no inhibition of the enzyme under similar conditions. Although the amide of PEA-Fum, PEA-Fum-NH₂, is also a potent inhibitor of the enzyme and displays similar inhibition kinetics, its inhibition potency is about 20-fold lower than that of PEA-Fum, implying that the free carboxylate group of PEA-Fum may play a critical role in inhibiting the enzyme. In addition, the findings that saturated dicarboxylic acid analogues (oxalic, malonic, succinic and glutaric acids), the geometric isomer of fumarate (maleic acid), and the diamide of fumaric acid (fumaradiamide) are not inhibitors or activators of the enzyme demonstrate that the interaction of fumarate and its analogues with $D\beta M$ is highly specific. Therefore the very high affinity of PEA-Fum towards the enzyme must be due to its efficient interaction with both the amine- and fumarate-binding sites of the reduced enzyme as an apparent bi-substrate with respect to amine and fumarate.

The mode of interaction of PEA-Fum with $D\beta M$ proposed above may provide a plausible molecular mechanism for the activation of the enzyme by fumarate. The apparent bi-substrate behaviour of PEA-Fum with respect to fumarate and the amine suggests that the amine group of the enzyme-bound substrate must be within interacting distance of the carboxylate group of the enzyme-bound fumarate. Therefore the ionic interaction between the amine group of the amine substrate and the carboxylate group of fumarate may play a significant role in the affinities of the amine substrate and fumarate for the reduced enzyme. [We note that the ionic complex of phenylethylamine-fumarate could be somewhat more extended than the PEA-Fum conjugate in the fully extended form. However, our results indicate that the phenylpropylamine-fumarate conjugate does not interact with the enzyme as efficiently as the PEA-Fum conjugate (results not shown). Therefore the distance between the phenyl group and the free carboxylate group of PEA-Fum is optimal for efficient interaction with the enzyme. This may suggest that the proposed electrostatic complex between the amine and fumarate may not be linear.] The observed 4–5-fold higher affinity of the amine substrate for $E_{rd} \cdot \text{Fum}$ (and/or $E_{rd} \cdot \text{Fum} \cdot O_2$) in comparison with the E_{rd} form is in good agreement with the above proposal. Similarly, the observed high affinity of fumarate for the $E_{rd} \cdot \text{Tyr}$ (and/or $E_{rd} \cdot O_2 \cdot \text{Tyr}$) form in comparison with the E_{rd} form is also in good agreement with this proposal. Therefore we propose that the electrostatic interaction between fumarate and the amine substrate mutually facilitates their interactions with the reduced enzyme. This proposal is also consistent with the observation that fumarate causes a decrease in the rate of dissociation of the amine substrate from the $E_{rd} \cdot \text{Tyr}$ (and/or $E_{rd} \cdot \text{Tyr} \cdot O_2$) complex under steady-state conditions [21]. In addition, the apparent bi-substrate behaviour of PEA-Fum could be due to its close resemblance to the enzyme-bound amine-fumarate ionic complex.

We have shown previously that 6SPEA is a high-affinity reductant of $D\beta M$ ($K_m = 9.0 \pm 1.0$ μM in the absence of fumarate [10]), and proposed that the unexpected high affinity of this and

Figure 7 Overlay of the MM2 energy-minimized structures of 6SPEA and PEA-Fum

Initially, the energies of both molecules were minimized individually using an MM2 parameter set, and optimal structures were overlaid by using the overlay mode of Chem 3D (Cambridge Soft Corp.). C-1 and C-4 of the aromatic rings, the benzylic carbons of the two molecules, the carboxylate group of fumarate and the 3'-OH of AscH₂ were set during the overlay procedure.

other similar derivatives for the enzyme is due to their behaviour as pseudo-bi-substrates for the oxidized enzyme, mimicking AscH₂ and phenylethylamine substrates [10]. Comparative structural analysis of 6SPEA and PEA-Fum (MM2-optimized) reveals that the phenylethylamine portions and the carboxylate group of PEA-Fum and the 3'-OH group of 6SPEA could be overlapped with each other with distances less than 1 Å (Figure 7). Therefore we believe that these two molecules interact with the enzyme in a similar fashion and that fumarate and AscH₂ interact with the same (reduction) site of the enzyme. However, the difference between these two series of molecules is that, while 6SPEA interacts preferentially with the oxidized form with very high affinity, PEA-Fum interacts exclusively with the reduced form with a similar high affinity. Clearly, the differential behaviour of these compounds must be associated with the fumarate and AscH₂ portions of the molecules. Since AscH₂ has a high affinity for the oxidized enzyme ($K_m = 65 \pm 7 \mu\text{M}$ [10]) and a low affinity for the reduced enzyme, the AscH₂ conjugate 6SPEA interacts preferentially with the oxidized enzyme as a bi-substrate reductant, resulting in the observed kinetic behaviour. On the other hand, since fumarate interacts efficiently with the reduced enzyme ($K_m = 180 \pm 20 \mu\text{M}$) and weakly with the oxidized enzyme, PEA-Fum interacts preferentially with the reduced form of the enzyme as a bi-substrate, resulting in the observed kinetic behaviour. (According to the proposed model, both PEA-Fum and 6SPEA interact with the enzyme in a similar fashion. The observed preference of PEA-Fum for the reduced enzyme and of 6SPEA for the oxidized enzyme follow the specificities of AscH₂ and fumarate. Although we have no clear explanation for this difference, one possibility is that the reduction site may be relatively more open in the oxidized form of the enzyme than in the reduced form.) According to this model, the similar high affinities of 6SPEA for the oxidized enzyme and PEA-Fum for the reduced enzyme further suggest that the amine sites of the reduced and oxidized enzyme are similar, which is also in agreement with previous spectroscopic studies [7].

Previous steady-state kinetic studies have shown that fumarate increases the pH optimum of the enzyme by shifting the pK_a of one of the catalytically important residues from approx. 5.5 to 6.5. The pK_a value and other literature evidence [29] have led to speculation that this residue could be a protonated histidine [21]. Since no conserved histidines are present in the vicinity of the active site, except for the five histidines that are involved in the copper co-ordination in the recently proposed active-site

model of D β M [24,30], we believe that one of the histidines of the reduction site [24] may be associated with the fumarate-mediated pH perturbation of the enzyme. An attractive hypothesis would be that the second carboxylate group of fumarate interacts with this protonated histidine residue, resulting in an increase of its pK_a from 5.5 to 6.5. Since the 3'-OH group of AscH₂ and the carboxylate group of fumarate appear to occupy similar positions in the active site, the 3'-OH group of AscH₂ may interact with the same histidine residue of the oxidized enzyme. Although previous studies have speculated that the 3'-OH group of AscH₂ interacts directly with the reduction-site Cu of the enzyme, the demonstration that the outer-sphere single electron donors DMPD and *N,N,N',N'*-tetramethyl-1,4-*p*-phenylenediamine [31,32] are excellent reductants for the enzyme suggests that direct interaction of the reductant with the Cu centre is not mandatory for efficient reduction of the enzyme. In fact, according to the above model, the efficient reduction of D β M by structurally unrelated DMPD could be attributed to the ability of unprotonated DMPD ($pK_a = 6.4$ [33]) to interact with the same protonated histidine residue in the enzyme.

Structural studies by Amzel and colleagues [23,24,34] have shown that the active-site coppers of peptidylglycine α -hydroxylating mono-oxygenase are held 11 Å apart on either side of a solvent-filled inter-domain cleft, and that electron transfer between the two copper centres must be mediated by the substrate itself. The similarity between peptidylglycine α -hydroxylating mono-oxygenase and D β M with respect to the mechanism of catalysis [35], cofactor dependence and sequence similarities have led these authors to propose a plausible structural model for the active site of D β M based on the features of the peptidylglycine α -hydroxylating mono-oxygenase active site [24]. In this model, the two copper centres of D β M are also approx. 11 Å apart and are separated by a solvent-filled inter-domain cleft requiring a similar substrate-mediated electron transfer. Since the interaction of fumarate and the amine substrate bridges the two copper centres of the enzyme in the model proposed above, we believe that this model may also provide a short and efficient pathway for electron transfer between the two copper centres of D β M for the reduction of molecular oxygen. Therefore activation of D β M by fumarate, especially the decrease in the rate of dissociation of the amine substrate from the $E_{rd} \cdot \text{substrate} \cdot O_2$ complex, could also be partly due to efficient electron transfer between the two coppers through the amine-fumarate ionic complex in comparison with the amine substrate itself. Studies to provide additional evidence for the above proposals are currently under way in our laboratory.

This work was supported by grant GM45026 from the National Institutes of Health.

REFERENCES

- 1 Kaufman, S., Bridges, W. F., Eisenberg, F. and Friedman, S. (1962) The source of oxygen in the phenylalanine hydroxylase- and dopamine β -hydroxylase-catalyzed reactions. *Biochim. Biophys. Res. Commun.* **9**, 497–502
- 2 Goldstein, M., Lauber, E. and McKereghan, M. R. (1965) The purification and characterization of 3,4-dihydroxyphenylethylamine β -hydroxylase. *J. Biol. Chem.* **240**, 2066–2072
- 3 Kaufman, S. and Friedman, S. (1965) Dopamine β -hydroxylase. *Pharmacol. Rev.* **17**, 71–100
- 4 Taylor, K. B. (1974) Dopamine β -hydroxylase: stereochemical course of the reaction. *J. Biol. Chem.* **249**, 454–458
- 5 Rosenberg, R. C. and Lovenberg, W. (1980) Dopamine β -hydroxylase. *Essays Neurochem. Neuropharmacol.* **4**, 163–209
- 6 Ljones, T. and Skotland, T. (1984) Dopamine β -monooxygenase. In *Copper Proteins and Copper Enzymes* (Lontie, R., ed.), vol. 2, pp. 131–157. CRC Press, Boca Raton
- 7 Stewart, L. C. and Klinman, J. P. (1988) Dopamine β -hydroxylase of adrenal chromaffin granules: structure and function. *Annu. Rev. Biochem.* **57**, 551–592

- 8 Fitzpatrick, P. F. and Villafranca, J. J. (1987) Mechanism-based inhibitors of dopamine β -hydroxylase. *Arch. Biochem. Biophys.* **257**, 231–250
- 9 Brenner, M. C. and Klinman, J. P. (1989) Correlation of copper valency with product formation in single turnovers of dopamine β -mono-oxygenase. *Biochemistry* **28**, 4664–4670
- 10 Wimalasena, K., Dharmasena, S., Wimalasena, D. S. and Hughbanks-Wheaton, D. K. (1996) Reduction of dopamine β -mono-oxygenase: a unified model for negative cooperativity and fumarate activation. *J. Biol. Chem.* **271**, 26032–26043
- 11 Klinman, J. P., Krueger, M., Brenner, M. and Edmondson, D. E. (1984) Evidence for two copper atoms/subunit in dopamine β -mono-oxygenase catalysis. *J. Biol. Chem.* **259**, 3399–3402
- 12 Ash, D. E., Papadopoulos, N. J., Colombo, G. and Villafranca, J. J. (1984) Kinetic and spectroscopic studies of the interaction of copper with dopamine β -hydroxylase. *J. Biol. Chem.* **259**, 3395–3398
- 13 Skotland, T. and Ljones, T. (1979) Dopamine β -mono-oxygenase: structure, mechanism and properties of the enzyme-bound copper. *Inorg. Prospect. Biol. Med.* **2**, 151–180
- 14 Klinman, J. P. (1996) Mechanisms whereby mononuclear copper proteins functionalize organic substrates. *Chem. Rev.* **96**, 2541–2561
- 15 Wimalasena, K., Dharmasena, S. and Wimalasena, D. S. (1994) Ascorbate based novel high affinity alternate reductants and competitive inhibitors of dopamine β -mono-oxygenase. *Biochem. Biophys. Res. Commun.* **200**, 113–119
- 16 Wimalasena, K. and Wimalasena, D. S. (1991) N,N,N',N'-Tetramethyl-1,4-phenylenediamine: a facile electron donor and chromophoric substrate for dopamine β -mono-oxygenase. *Biochem. Biophys. Res. Commun.* **175**, 920–927
- 17 Wimalasena, K. and Wimalasena, D. S. (1991) Continuous spectrophotometric assays for dopamine β -mono-oxygenase based on two novel electron donors: N,N-dimethyl-1,4-phenylenediamine and 2-aminoascorbic acid. *Anal. Biochem.* **197**, 353–361
- 18 Goldstein, M., Joh, H. T. and Garvey, III, T. Q. (1968) Kinetic studies of the enzymatic dopamine β -hydroxylation reaction. *Biochemistry* **7**, 2724–2730
- 19 Levin, E. Y., Lovenberg, B. and Kaufman, S. (1960) The enzymatic conversion of 3,4-dihydroxyphenylethylamine to norepinephrine. *J. Biol. Chem.* **235**, 2080–2086
- 20 Craine, J. E., Daniels, G. H. and Kaufman, S. (1973) Dopamine β -hydroxylase: the subunit structure and anion activation of the bovine adrenal enzyme. *J. Biol. Chem.* **248**, 7838–7844
- 21 Ahn, N. and Klinman, J. P. (1983) Mechanism of modulation of dopamine β -mono-oxygenase by pH and fumarate as deduced from initial rate and primary deuterium isotope effect studies. *Biochemistry* **22**, 3096–3106
- 22 Stewart, L. C. and Klinman, J. P. (1991) Cooperativity in the dopamine β -mono-oxygenase reaction: evidence for ascorbate regulation of enzyme activity. *J. Biol. Chem.* **266**, 11537–11543
- 23 Prigge, S. T., Kolhekar, A. S., Eipper, B. A., Mains, R. E. and Amzel, L. M. (1999) Substrate-mediated electron transfer in peptidylglycine α -hydroxylating mono-oxygenase. *Nat. Struct. Biol.* **6**, 976–983
- 24 Prigge, S. T., Mains, R. E., Eipper, B. A. and Amzel, L. M. (2000) New insights into copper mono-oxygenases and peptide amidation: structure, mechanism and function. *Cell. Mol. Life Sci.* **57**, 1236–1259
- 25 Ljones, T., Skotland, T. and Flatmark, T. (1976) Purification and characterization of dopamine β -hydroxylase from bovine adrenal medulla. *Eur. J. Biochem.* **6**, 525–533
- 26 Kirshner, N. (1962) Uptake of catecholamines by a particulate fraction of the adrenal medulla. *J. Biol. Chem.* **237**, 2311–2317
- 27 Njus, D. and Radda, G. K. (1979) A potassium ion diffusion potential causes adrenaline uptake in chromaffin-granule ghosts. *Biochem. J.* **180**, 579–585
- 28 Cleland, W. W. (1979) Statistical analysis of enzyme kinetic data. *Methods Enzymol.* **63**, 103–138
- 29 Aunis, D., Miras-Portugal, M. T. and Mandel, P. (1973) Bovine adrenal medullary dopamine β -hydroxylase: purification by affinity chromatography, kinetic studies and presence of essential histidyl residues. *Biochim. Biophys. Acta* **327**, 313–327
- 30 Robertson, J. C., Desai, P. R., Kumar, A., Farrington, G. K., Fitzpatrick, P. F. and Villafranca, J. J. (1990) Primary amino acid sequence of bovine dopamine β -hydroxylase. *J. Biol. Chem.* **265**, 1029–1035
- 31 Nishida, Y., Takeuchi, M., Oishi, N. and Kida, S. (1983) Catalytic activity of copper (II) complexes with a flexible polyatomic bridging group for the oxidation of TMPD by O₂. *Inorg. Chim. Acta* **75**, 169–171
- 32 Nishida, Y., Takeuchi, M., Oishi, N. and Kida, S. (1985) Effect of coordination structure on the redox reactions of mononuclear copper (II) complexes. *Inorg. Chim. Acta* **96**, 81–85
- 33 Wikstrom, M. and Penttila, T. (1982) Critical evaluation of the proton-translocating property of cytochrome oxidase in rat liver mitochondria. *FEBS Lett.* **144**, 183–189
- 34 Prigge, S. T., Kolhekar, A. S., Eipper, B. A., Mains, R. E. and Amzel, L. M. (1997) Amidation of bioactive peptides: the structure of peptidylglycine α -hydroxylating mono-oxygenase. *Science* **278**, 1300–1305
- 35 Francisco, W. A., Merkler, D. J., Blackburn, N. J. and Klinman, J. P. (1998) Kinetic mechanism and intrinsic isotope effects for the peptidylglycine α -amidating enzyme reaction. *Biochemistry* **37**, 8244–8252

Received 4 February 2002/29 May 2002; accepted 31 May 2002

Published as BJ Immediate Publication 31 May 2002, DOI 10.1042/BJ20020216