

Reduction of Dopamine β -Monooxygenase

A UNIFIED MODEL FOR APPARENT NEGATIVE COOPERATIVITY AND FUMARATE ACTIVATION*

(Received for publication, January 24, 1996, and in revised form, May 24, 1996)

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The interactions of reductants with dopamine β -monooxygenase (D β M) were examined using two novel classes of reductants. The steady-state kinetics of the previously characterized D β M reductant, *N,N*-dimethyl-1,4-*p*-phenylenediamine (DMPD), were parallel to the ascorbic acid-supported reaction with respect to pH dependence and fumarate activation. DMPD also displayed pH and fumarate-dependent apparent negative cooperativity demonstrating that the previously reported cooperative behavior of D β M toward the reductant is not unique to ascorbic acid. The 6-OH phenyl and alkylphenyl-substituted ascorbic acid derivatives were more efficient reductants for the enzyme than ascorbic acid. Kinetic studies suggested that these derivatives behave as pseudo bisubstrates with respect to ascorbic acid and the amine substrate. The lack of apparent cooperative behavior with these derivatives suggests that this behavior of D β M is not common for all the reductants. Based on these findings and additional kinetic evidence, the proposal that the apparent negative cooperativity in the interaction of ascorbic acid with D β M was due to the presence of a distinct allosteric regulatory site has been ruled out. In contrast to previous models, where fumarate was proposed to interact with a distinct anion binding site, the effect of fumarate on the steady-state kinetics of these novel reductants suggests that fumarate and the reductant may interact with the same site of the enzyme. In accordance with these observations and mathematical analysis of the experimental data, a unified model for the apparent negative cooperativity and fumarate activation of D β M in which both fumarate and the reductant interact with the same site of all forms of the enzyme with varying affinities under steady-state turnover conditions has been proposed.

Dopamine β -monooxygenase (D β M¹; EC 1.14.17.1), a copper containing tetrameric enzyme, catalyzes the conversion of dopamine to the neurotransmitter, norepinephrine, within the chromaffin granules of the adrenal medulla and the large dense-cored synaptic vesicles of the sympathetic nervous system (1–3). D β M in the adrenal chromaffin granules exists in

both soluble and membrane-bound forms with an approximately equal distribution (1–3). The observations that ascorbic acid (AscH₂) is an efficient *in vitro* reductant of D β M and that high levels of AscH₂ are present in D β M containing neurosecretory vesicles (4–5) appear to indicate that intragranular AscH₂ may provide the necessary reducing equivalents for the D β M reaction *in vivo*. However, recent studies with chromaffin granule ghosts suggest that extragranular (cytosolic) AscH₂ may be involved in the reduction of membrane-bound D β M directly (6–7).

Although the hydroxylation site of D β M has been studied in great detail (8–10), the specificity and chemistry of the reduction site of the enzyme is poorly understood. For example, AscH₂ has been considered to be the most efficient, structurally optimal reductant for the enzyme, but recent findings from our laboratory (11) have demonstrated that 6-*O*-phenyl and 6-*S*-phenyl-L-ascorbic acid derivatives are much more efficient reductants for the enzyme compared with AscH₂. In addition, the facile single electron donors such as *N,N,N',N'*-tetramethyl-1,4-*p*-phenylenediamine (TMPD) and *N,N*-dimethyl-1,4-*p*-phenylenediamine (DMPD), which have no structural resemblance to AscH₂ (Scheme I), are also well behaved efficient reductants for the enzyme (12–13). These findings appear to indicate that either the reduction site of the enzyme is not specific or the enzyme may possess multiple reduction sites with different specificity. The physiological significance of this highly nonspecific nature of the reductant site of the enzyme is not clearly understood at present.

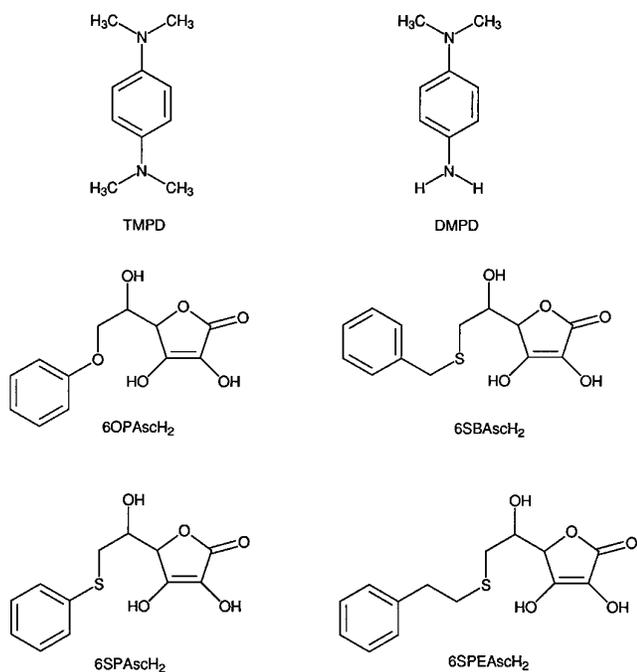
Although the precise geometry of the D β M active site is not known, the first evidence for the existence of distinct non-overlapping binding sites for the amine substrate and the reductant has come from the observation that the reduced enzyme-product complex (*E*_{rd}-P) is the predominant form under steady-state turnover conditions in the presence of excess AscH₂ (14). These results were used to propose that under steady-state conditions the *E*_{ox}-P complex is preferentially reduced prior to the release of the product requiring a distinct reductant binding site that is non-overlapping with the amine binding site (14). This information was incorporated into a working model suggesting that one of the two copper sites in the D β M active site functions strictly as the reduction site, whereas the other copper site is responsible for molecular oxygen activation and insertion into the organic substrate. In addition, the apparent negative cooperativity observed in the interaction of AscH₂ with D β M under a variety of experimental conditions was proposed to be due either to the interaction of AscH₂ with a specific allosteric regulatory site or due to the existence of multiple reducible forms of the enzyme in the catalytic cycle (15).

Halides and organic anions have long been known to activate D β M under steady-state conditions (16–18). The most efficient activator of the enzyme, the dicarboxylic acid, fumarate, was shown to exert its effect on the steady-state kinetics of the

* This work was supported by Grant GM45026 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¹ The abbreviations used are: D β M, dopamine β -monooxygenase; AscH₂, ascorbic acid; 6SAscH₂, 6-*S*-benzyl-L-ascorbic acid; DMPD, *N,N*-dimethyl-1,4-*p*-phenylenediamine; 6OPAscH₂, 6-*O*-phenyl-L-ascorbic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; 6SPEAscH₂, 6-*S*-phenylethyl-L-ascorbic acid; 6SPAscH₂, 6-*S*-phenyl-L-ascorbic acid; TMPD, *N,N,N',N'*-tetramethyl-1,4-*p*-phenylenediamine.

SCHEME I. Alternate D β M reductants.

enzyme by (a) increasing the pK_a of catalytically important active site residues leading to the optimal activity of the enzyme over a wide pH range and (b) pH-dependent decrease of the rate of dissociation of the amine substrate from the enzyme ternary complex (19). In addition to these effects, recent studies have shown that at low AscH₂ concentrations fumarate behaves as a reversible inhibitor for the enzyme which is believed to be due to the competitive binding of fumarate to the reduction site of the enzyme. The observation that fumarate is capable of activating D β M even in the presence of saturating levels of AscH₂ has been accepted as evidence for the existence of a distinct anion activation site in the enzyme. However, the molecular mechanism of fumarate activation of the enzyme is not clearly understood.

In the present study we have examined the interaction of the reductant with D β M using two novel classes of reductants. The steady-state kinetic behavior of the DMPD-mediated D β M reaction was found to parallel the AscH₂-supported reaction, especially with respect to pH dependence and fumarate activation. In addition, steady-state kinetics of the reaction with respect to DMPD displayed pH and fumarate-dependent apparent negative cooperative behavior that was parallel to the kinetics of AscH₂ demonstrating that the cooperative behavior of D β M is not unique to AscH₂. The 6-OH modified AscH₂ derivatives (Scheme I), 6-O-phenyl (6OPAscH₂), 6-S-phenyl (6SPAscH₂), 6-S-benzyl (6SBAscH₂), and 6-S-phenylethyl (6SPEAscH₂) were found to be much more efficient reductants for the enzyme than AscH₂. Detailed kinetic studies have revealed that these derivatives may behave as pseudo bisubstrates for the enzyme with respect to AscH₂ and the amine substrate. However, the initial rate kinetics of these AscH₂ derivatives (except 6OPAscH₂) did not display any significant cooperativity, suggesting that the cooperative behavior of D β M is not common for all of the reductants. Based on these findings the proposal that the observed apparent negative cooperativity with respect to the reductant was due to the presence of a distinct allosteric regulatory site in D β M has been ruled out in favor of the possibility that the observed cooperativity is due to the existence of two reducible steady-state forms of the enzyme

with different affinities for the reductants.² In contrast to previous models, where fumarate was proposed to interact with a distinct anion binding site in the enzyme, based on the above findings and the results of the mathematical analysis of the experimental data, we propose that fumarate exerts its effects by interacting with the same reduction site of various steady-state forms of the enzyme with varying affinities. The significance of these findings in relation to the *in vivo* reduction of D β M and modulation of norepinephrine biosynthesis is discussed.

MATERIALS AND METHODS

Tyramine hydrochloride, disodium fumarate, and MES were from Sigma. *N,N*-Dimethyl-1,4-*p*-phenylenediamine hydrochloride salt and AscH₂ were from Aldrich. Beef liver catalase (65,000 units/mg of protein) was from Boehringer Mannheim. The AscH₂ derivatives, 6OPAscH₂ and 6SPAscH₂, were synthesized as previously reported (8). 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, 13–30 units/mg) according to the procedures previously described (20) with minor modifications using freshly prepared bovine adrenal chromaffin granules (21–22). The concentration of purified enzyme was estimated spectrophotometrically using $E_{280} = 1.24 \text{ ml mg}^{-1} \text{ cm}^{-1}$. UV-visible spectroscopic measurements were carried out using an HP-8452A diode array 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-propanesulfonic acid sodium salt (when D₂O 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. All the kinetic parameters were apparent and were determined by computer fit of the initial rate data to the hyperbolic form of the Michaelis-Menten equation using the "Enzfit" program. The inhibition constants were determined by direct fit of the data to the Cleland programs (23). The pH dependence of the initial rates were analyzed by computer fitting the data to Equation 1.

$$v = \frac{c}{1 + [H^+]/K_a + K_f/[H^+]} \quad (\text{Eq. 1})$$

In cases where a significant deviation from the expected kinetic behavior (apparent cooperativity) was observed, the data were analyzed by the general model of Alberty (15, 24) that was developed for the analysis of non-Michaelis-Menten-type kinetics of enzymes due to the multiple interactions of the substrate with various steady-state forms of the enzyme. The corresponding four Michaelis constants V_1 and K_1 (at low substrate concentrations) and V_2 and K_2 (at high substrate concentrations) were estimated by computer fitting of the data to the general kinetic Equation 2.

$$v = \frac{V_2 + V_1 K_2 [1 - V_1/V_2][S]}{1 + K_2 [1 - V_1/V_2][S] + K_1 K_2 [1 - V_1/V_2][S]^2} \quad (\text{Eq. 2})$$

Oxygen Monitor Assay of D β M—The initial rates of the D β M reaction under various experimental conditions were determined by the initial rate of oxygen consumption using a polarographic oxygen electrode at 37 °C as described previously (13). In these assays, 2–200 mM stock solutions of 6OPAscH₂, 6SPAscH₂, 6SBAscH₂, and 6SPEAscH₂ were prepared, and the pH values of 50–200 mM stocks were adjusted to 5.2 or 6.7 with NaOH. All enzymatic reactions were carried out in 50 mM MES buffer adjusted to either pH 5.2 or 6.7 depending on the experiment. The standard reaction mixtures contained 100 $\mu\text{g/ml}$ catalase, 2 μM CuSO₄, and 50 mM MES buffer. All the enzymatic reactions were initiated by the addition of tyramine to a final concentration of 10 mM, and the initial turnover rates were measured as the rate of oxygen consumption minus the small background rates due to autooxidation of ascorbic acid (or AscH₂ derivative). All kinetic parameters, V_{max} , K_m , etc. were apparent

² Cooperativity in enzymes has been defined as non-Michaelis-Menten sigmoidal kinetics due to the interaction of allosteric effectors that are generally structurally different from the substrate and binds at its own separate site away from the active site. Similar kinetic behavior could also be observed due to the interaction of substrate(s) with multiple binding sites (32) or with multiple steady-state forms of the enzyme (present study). In order to distinguish the latter from the first two we have used the phrase "apparent cooperativity."

and were determined at atmospheric oxygen saturation conditions (256 μ M). The V_{\max} values were calculated based on the monomer molecular mass of 70,000 Da for D β M and normalized to a constant maximum specific activity of 30 μ mol/min-mg for the purpose of internal comparison (see Table I). Stock solutions of sparingly water-soluble ascorbate derivatives were made in ethanol, and thus the equivalent amounts of ethanol were added to all the assay mixtures to maintain a constant ethanol concentration (maximum 7.5% v/v) throughout the experiment (control experiments indicated that the kinetic parameters or the apparent negative cooperative behavior of AscH₂ are not significantly altered by the presence of 7.5% of ethanol in the assay mixture). Further details of exact reaction conditions are given in the corresponding figure legends.

Spectrophotometric Assays of D β M—All enzymatic reactions (except pH dependence experiments) were carried out in 50 mM MES buffer adjusted to either pH 5.2 or 6.7 depending on the experiment. The pH dependence experiments were carried out in either 125 mM sodium acetate buffer or 100 mM potassium phosphate buffer depending on the pH. The standard reaction mixtures contained 100 μ g/ml catalase and 0.5–2.0 μ M CuSO₄ in a total volume of 1.0 ml. D β M concentrations were kept constant for each experiment but varied between 1.2 and 3.2 μ g of protein per assay depending on the experiment. The enzymatic reactions were usually initiated with the reductant 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 identical to the enzymatic reaction mixture but without the enzyme as described previously (13). For further details see the corresponding figure legends.

6-S-Benzyl-6-deoxy-L-Ascorbic Acid (6SBAscH₂)—6-Bromo-6-deoxy-L-ascorbic acid (2.0 g, 8.4 mmol) was added to a solution of Na₂CO₃ (3.2 g, 25.8 mmol) in water (10 ml) and stirred for 30 min. A solution of benzyl mercaptan (1.04 g, 8.4 mmol) in 0.5 M NaOH (16.8 ml) was added slowly to the reaction mixture and stirred at room temperature for 24 h under N₂. The reaction mixture was acidified with 1 M HCl to pH 1 and extracted with hexane (2 \times 100 ml), and the hexane layer was discarded. The aqueous layer was extracted with ethyl acetate (3 \times 100 ml), and the ethyl acetate layer was dried over anhydrous Na₂SO₄. Evaporation of the ethyl acetate layer under reduced pressure gave a yellow oily compound that was later crystallized from ethyl acetate and hexane. The resultant colorless crystals were dried in a vacuum desiccator. Second recrystallization of this material from ethyl acetate and hexane gave a colorless crystalline product. Yield 1.1 g (46.5%). ¹H NMR (D₂O): δ 2.76 (dd, J = 13.92 Hz, 7.32 Hz, 1H), 2.83 (dd, J = 13.92 Hz, 7.08 Hz, 1H), 3.74 (s, 2H), 4.14 (td, J = 7.11 Hz, 1.41 Hz, 1H), 4.88 (d, J = 1.27 Hz, 1H), 7.20–7.35 (m, 5H). ¹³C NMR (D₂O): δ 36.09 (t), 38.31 (t), 70.16 (d), 79.62 (d), 119.42 (s), 129.39 (d), 130.88 (d), 131.26 (d), 140.35 (s), 160.51 (s), 175.95 (s). m.p. 75–77 °C.



Calculated: C 55.30 H 4.99
Found: C 55.27 H 4.88

HPLC analysis, 98% pure with no trace of AscH₂.

6-S-Phenylethyl-6-deoxy-L-Ascorbic Acid (6SPEAscH₂)—6-Bromo-6-deoxy-L-ascorbic acid (1.0 g, 4.2 mmol) was added to a solution of Na₂CO₃ (1.56 g, 12.6 mmol) in water (10 ml) and stirred for 30 min at room temperature under N₂. A solution of phenylethyl mercaptan (0.58 g, 4.2 mmol) in 0.5 M NaOH (8.4 ml) was added slowly to the reaction mixture, and stirring was continued for another 18 h under N₂ at room temperature. The reaction mixture was acidified to pH 1 with 1 M HCl and extracted with hexane, and the hexane layer was discarded. The aqueous layer was extracted with diethyl ether (3 \times 100 ml). The ether layer was dried over anhydrous Na₂SO₄ and evaporated under reduced pressure to obtain a viscous oily product that was crystallized from ethyl acetate and hexane. Resultant crude crystals were collected and dried in a vacuum desiccator. This was further purified by recrystallization from ethyl acetate and hexane to yield a colorless crystalline product. Yield 350 mg (28.3%). ¹H NMR (CDCl₃): δ 2.68–2.89 (m, 6H), 4.01 (td, J = 6.32 Hz, 1.32 Hz, 1H), 4.83 (d, J = 1.47 Hz, 1H), 5.50 (br, disappeared with D₂O), 7.10–7.30 (m, 5H). ¹³C NMR (CDCl₃): δ 33.78 (t), 34.58 (t), 36.01 (t), 68.51 (d), 77.0 (d), 117.33 (s), 126.34 (d), 128.44 (d), 128.47 (d), 140.16 (s), 158.18 (s), 173.70 (s). m.p. 108–109 °C.



Calculated: C 56.74 H 5.44
Found: C 56.63 H 5.52

HPLC analysis, 97% pure with no trace of AscH₂.

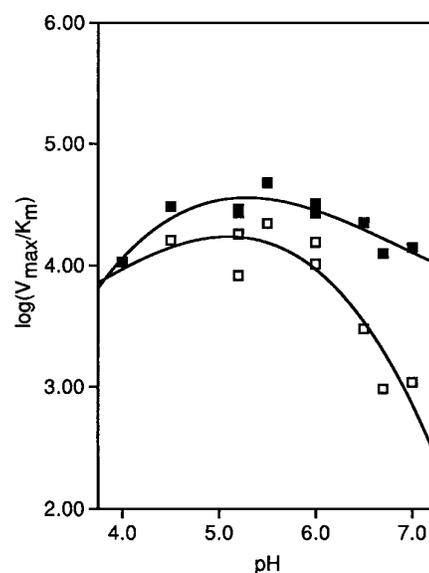


FIG. 1. The pH dependence of the DMPD-mediated D β M reaction. The initial apparent rates of the DMPD-mediated D β M reaction were measured as a function of tyramine using the previously described spectrophotometric assay (13), in the absence (□) or presence of 10 mM fumarate (■) in the pH range 4.0 to 7.0. All the reactions were carried out in the presence of a constant 12.5 mM concentration of DMPD, 0.5 μ M CuSO₄, 100 μ g/ml catalase, and 1.2 μ g/ml D β M in 125 mM sodium acetate buffer or 100 mM potassium phosphate buffer at 37 °C under atmospheric O₂ saturation conditions (256 μ M) as detailed under “Materials and Methods.” The apparent V_{\max} and K_m values were calculated by computer fitting the initial rate data to the hyperbolic form of the Michaelis-Menten equation.

RESULTS

The pH dependence of the apparent V_{\max}/K_m ratios for the DMPD-supported D β M hydroxylation of tyramine at a fixed 12.5 mM DMPD concentration in the presence and absence of fumarate is shown in Fig. 1. The analyses of these data by computer fitting to Equation 1 using the Bell program yielded apparent limiting values of $4.9 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ in the presence and $2.5 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ in the absence of fumarate for V_{\max}/K_m , demonstrating that 10 mM fumarate increases the apparent limiting V_{\max}/K_m for tyramine by about a factor of 2 similar to that observed for the AscH₂-mediated reaction (19). These analyses also revealed that at least two ionizable groups with apparent pK_a values of 5.3 and 5.9 in the presence and 4.8 and 5.4 in the absence of fumarate are involved in the catalytic cycle of the DMPD-mediated D β M reaction, again very similar to the AscH₂-supported reaction (19). The data presented in Fig. 2, A and B, demonstrate that the initial velocities of the DMPD-mediated D β M hydroxylation of tyramine as a function of DMPD, at a 0.1 to 50 mM concentration range, at pH 5.2, both in the presence and absence of 10 mM fumarate, display a significant deviation from the expected linearity in Eadie-Hofstee plots. At pH 6.7, this deviation was significantly pronounced both in the presence and absence of fumarate (Fig. 2, C and D) similar to the observed behavior for the AscH₂-supported D β M reaction. The analysis of the data by fitting to Equation 2 as described under “Materials and Methods” yielded low apparent K_m values of 0.70 mM in the absence and 1.56 mM in the presence of fumarate at pH 5.2 (Table I; at pH 6.7 accurate low K_m values could not be obtained due to very low turnover rates of the DMPD-mediated D β M reaction). The apparent K_m values at high DMPD concentrations were 5.95 mM at pH 5.2 and 47.1 mM at pH 6.7 in the absence of fumarate (Table I). In addition, the corresponding increase in V_{\max} values were in the 2–3-fold range depending on the experimental conditions (Table I).

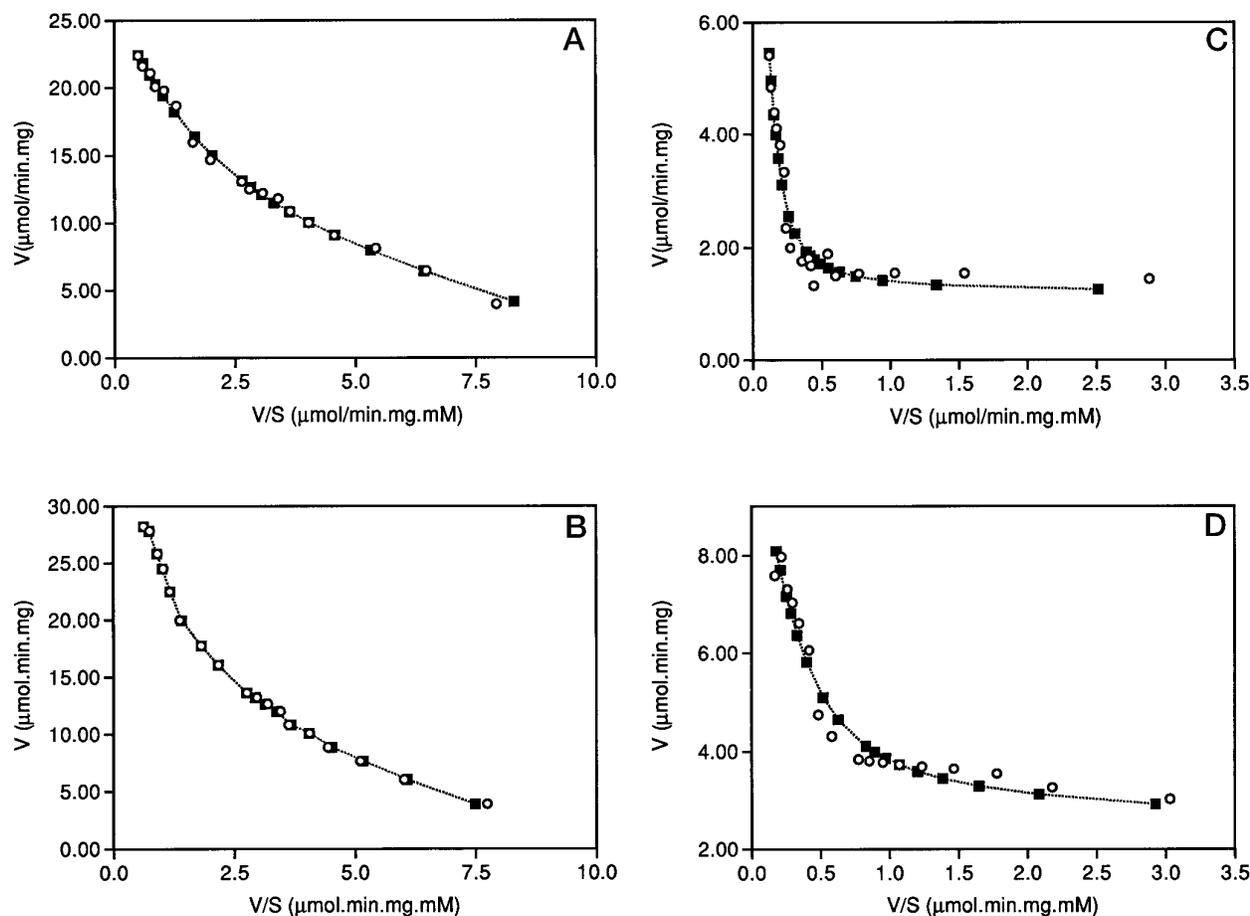


FIG. 2. The steady-state kinetic behavior of the DMPD-mediated D β M reaction as a function of DMPD. The initial apparent rates of the DMPD-mediated D β M reaction were measured as a function of DMPD using the previously described (13) spectrophotometric assay, in the absence (\circ , A and C) or in the presence of 10 mM fumarate (\circ , B and D) at pH 5.2 (A and B) and pH 6.7 (C and D). \blacksquare , the fit of the experimental data to the Equation 3. All the reactions were carried out in the presence of a constant 10 mM concentration of tyramine, 0.5 μ M CuSO₄, 100 μ g/ml catalase, and 1.6–3.2 μ g/ml D β M in 50 mM MES buffer at 37 °C under atmospheric O₂ saturation conditions (256 μ M) as detailed under “Materials and Methods.”

The Eadie-Hofstee plots of apparent initial rate kinetics of the DMPD-mediated D β M reaction with respect to tyramine, in the concentration range of 0.5 to 50 mM at pH 5.2 and 6.7 (data not shown), in the presence and absence of fumarate indicate that the deviation from the expected linearity is relatively small under all the conditions. As expected, fumarate exerts a significant pH-dependent effect on the apparent V_{\max}/K_m ratio that is more pronounced at pH 6.7 than at 5.2. For example, while the apparent V_{\max}/K_m ratio changed from $0.11 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ in the absence of fumarate to $0.49 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ in the presence of 10 mM fumarate at pH 6.7, the ratio changed from $1.10 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ in the absence to $2.2 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ in the presence of 10 mM fumarate at pH 5.2 (Table II; it should be noted that these values were somewhat lower than the values reported in the pH dependence studies possibly due to the difference in experimental conditions and the enzyme preparations used. However, this difference should not affect the relative dependence of V_{\max}/K_m on pH or fumarate since these experiments were carried out under identical conditions with the same preparation of the enzyme.). The results also show that the effect of fumarate on V_{\max} is relatively small at a constant pH (depending on the experimental conditions less than 15% increased), and the large effect on the ratio V_{\max}/K_m is mainly due to the increase in the apparent K_m for the substrate, tyramine, in the absence of fumarate (data not shown). Further confirmation of this increase in the apparent K_m for tyramine can be drawn by the observation that at very

high tyramine concentrations the initial rate of the reaction in the absence of fumarate approaches the rate of the reaction in the presence of 10 mM fumarate at both pH 5.2 and 6.7 (data not shown). These results also parallel the previous results of the AsC_H₂-supported D β M reaction (19).

The effect of fumarate on the rate of the DMPD-mediated D β M reaction was examined at a low DMPD concentration (near low K_m , 1.75 mM) at several different pH values using tyramine as the substrate. The results presented in Fig. 3 clearly demonstrate that fumarate inhibits the enzyme at all the fumarate concentrations tested (0.2–18 mM) at pH 5.2 in the presence of 1.75 mM DMPD. This behavior of fumarate is not significantly changed at pH 5.5 and appears to inhibit the enzyme again throughout the entire fumarate concentration range tested. However, fumarate behaves as an activator for the enzyme at pH 6.7 up to the concentration of 3.0 mM, but above this concentration it again inhibits the enzyme similar to the inhibition observed at pH 5.2 and 5.5. These results demonstrate that the inhibition of the enzyme by fumarate at low reductant concentrations is also common for both AsC_H₂ (15) and DMPD.

The initial rate kinetics of the AsC_H₂-mediated D β M reaction display significant deviation from the expected linearity in Eadie-Hofstee plots over the range of 0.02–55 mM AsC_H₂ concentration at pH 5.2 both in the presence and absence of fumarate at a constant tyramine concentration of 10 mM and at atmospheric oxygen saturation conditions (Fig. 4, A and B) in

TABLE I
 Steady state apparent kinetic parameters of various reductants

The initial apparent kinetic parameters were determined using various enzyme preparations with different specific activities (in the range of 13–30 $\mu\text{mol}/\text{min} \cdot \text{mg}$), and all the V_{max} values were normalized to the maximum specific activity of 30 $\mu\text{mol}/\text{min} \cdot \text{mg}$ for the purpose of internal comparison. All the reactions were carried out in 50 mM MES buffer containing a 10 mM constant concentration of tyramine either in the absence of fumarate (–F) or in the presence of 10 mM (+F) fumarate as described under “Materials and Methods.”

pH	Reductant	K_m (mM)		V_{max} (s^{-1})		V_{max}/K_m ($\text{s}^{-1} \text{mM}^{-1}$)	
		+F	–F	+F	–F	+F	–F
5.2	AscH ₂ ^a						
	Low	0.143 ± 0.009	0.065 ± 0.007	16.67 ± 0.34	10.26 ± 0.30	116.6	157.8
	High	— ^c	14.21 ± 2.39	— ^c	30.31 ± 1.21	—	2.13
	6OPAscH ₂ ^a	0.029 ± 0.003	0.034 ± 0.002	14.01 ± 0.28	12.95 ± 0.19	487.5	383.5
	6SPAscH ₂ ^a	0.020 ± 0.002	0.010 ± 0.001	11.2 ± 0.14	6.38 ± 0.11	554.1	665.1
	6SBAscH ₂ ^a	0.059 ± 0.004	0.023 ± 0.001	19.46 ± 0.28	11.86 ± 0.15	327.5	525.7
	6SPEAscH ₂ ^a	0.027 ± 0.004	0.009 ± 0.001	14.64 ± 0.51	7.12 ± 0.14	550.8	789.9
	DMPD ^b						
	Low	1.56 ± 0.17	0.70 ± 0.23	40.16 ± 2.89	24.36 ± 5.42	25.7	34.8
	High	— ^c	5.95 ± 0.94	— ^c	69.29 ± 1.63	—	11.6
6.7	6OPAscH ₂ ^a						
	Low	0.030 ± 0.002	0.008 ± 0.002	8.94 ± 0.09	6.86 ± 0.35	283.2	857.0
	High	— ^c	1.79 ± 0.57	— ^c	13.63 ± 0.55	—	7.6
	6SPAscH ₂ ^a	0.005 ± 0.0005	— ^c	4.70 ± 0.05	—	934.6	—
	6SBAscH ₂ ^a	0.013 ± 0.001	0.320 ± 0.16	12.79 ± 0.22	8.35 ± 0.12	965.9	26.1
	6SPEAscH ₂ ^a	0.026 ± 0.002	0.012 ± 0.002	11.41 ± 0.26	6.74 ± 0.18	442.2	545.0
	DMPD ^b						
	Low	— ^c	— ^c	— ^c	— ^c	—	—
	High	— ^c	47.11 ± 16.6	— ^c	28.71 ± 4.92	—	2.3

^a Determined using the oxygen monitor assay.

^b Determined using the previously described (13) spectrophotometric assay.

^c —, Accurate reproducible kinetic parameters could not be obtained either due to low turnover rates or the lack of a statistically significant number of data points in the respective concentration range.

 TABLE II
 Fumarate activation of D β M with various reductants

The initial apparent kinetic parameters were determined using various enzyme preparations with different specific activities (in the range of 13–30 $\mu\text{mol}/\text{min} \cdot \text{mg}$) and all the V_{max} values were normalized to the maximum specific activity of 30 $\mu\text{mol}/\text{min} \cdot \text{mg}$ for the purpose of internal comparison.

pH	Reductant	$(V_{\text{max}}/K_m)_{+F}$	$(V_{\text{max}}/K_m)_{-F}$	$(V_{\text{max}}/K_m)_{+F}/(V_{\text{max}}/K_m)_{-F}$
5.2	DMPD ^a	2.2×10^4	1.10×10^4	2.00
6.7	DMPD ^a	0.49×10^4	0.11×10^4	4.45
5.2	6OPEAscH ₂ ^b	2.6×10^4	0.35×10^4	7.43
5.2	AscH ₂ ^b	1.05×10^4	0.57×10^4	1.84

^a Determined using the previously described (13) spectrophotometric assay at a constant 12.5 mM concentration of DMPD in 50 mM MES buffer.

^b Determined using the oxygen monitor assay at constant concentrations of 6OPEAscH₂ and AscH₂ 2.0 and 30.0 mM, respectively, in 50 mM MES buffer.

agreement with previous results (15). The analysis of the data by fitting to Equation 2 yielded low apparent K_m values of 0.143 mM in the presence and 0.065 mM in the absence of fumarate and a high K_m value of 14.2 mM in the absence of fumarate for AscH₂ (Table I, the calculated high K_m in the presence of fumarate was not reliable and was associated with a significantly high standard error). Furthermore, under these conditions the apparent V_{max} values were 16.7 s^{-1} in the presence and 10.3 s^{-1} in the absence of fumarate for the low K_m range and increased by about a factor of 3 at high K_m range both in the presence and absence of fumarate. These results confirm the previously proposed apparent cooperative behavior of D β M with respect to AscH₂ qualitatively and show that the experimental protocols we have used are comparable with those of Stewart and Klinman (15).

The initial rate of the 6OPAscH₂-mediated D β M monooxygenation of tyramine was examined as a function of 6OPAscH₂ in the concentration range of 0.005 to 33.0 mM, in the absence and in the presence of fumarate, at pH 5.2 and 6.7. In contrast to AscH₂, the initial rate of the reaction reached its maximum

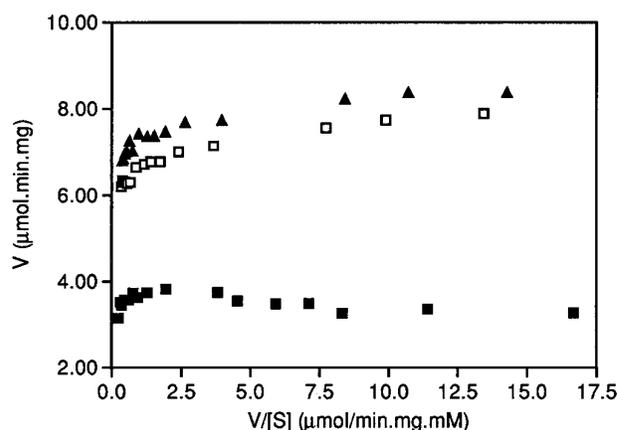


FIG. 3. The effect of fumarate on the turnover rate of the DMPD-mediated D β M reaction at a low DMPD concentration. The initial apparent rates of the DMPD-mediated D β M reaction were measured as a function of fumarate using the previously described (13) spectrophotometric assay, at pH values 5.2 (\square), 5.5 (\blacktriangle), and 6.7 (\blacksquare) at a constant 1.75 mM concentration of DMPD. All the reactions were carried out in the presence of a constant 10 mM concentration of tyramine, 0.5 μM CuSO₄, 100 $\mu\text{g}/\text{ml}$ catalase, and 2.6 $\mu\text{g}/\text{ml}$ D β M in 50 mM MES buffer at 37 °C under atmospheric O₂ saturation conditions (256 μM) as detailed under “Materials and Methods.”

at about 4 mM in the presence and in the absence of fumarate at both of the above pH values and thereafter decreased gradually with increasing concentration of 6OPAscH₂, suggesting an inhibition of the enzyme by 6OPAscH₂ at higher concentrations. This derivative exhibits normal linear Eadie-Hofstee kinetic plots up to about 4.0 mM concentration both in the presence and absence of fumarate at pH 5.2 (Fig. 5, A and B) and in the presence of fumarate at pH 6.7 (Fig. 5D). The apparent K_m values calculated for 6OPAscH₂ for the linear portions of the concentration ranges by direct fitting of the data to the hyperbolic form of the Michaelis-Menten equation were 0.029 mM in the presence and 0.034 mM in the absence of fumarate at pH 5.2 and 0.030 mM in the presence of fumarate at pH 6.7. These data

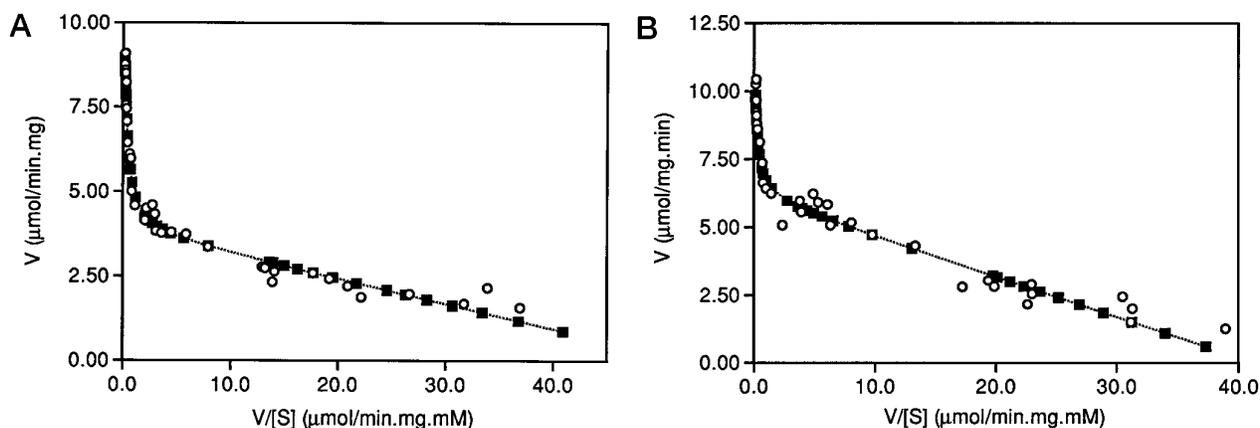


FIG. 4. The steady-state kinetic behavior of the AscH_2 -mediated $\text{D}\beta\text{M}$ reaction as a function of AscH_2 . The initial apparent rates of the AscH_2 -mediated $\text{D}\beta\text{M}$ reaction were measured as a function of AscH_2 using the oxygen monitor assay, in the absence (\circ , A) or in the presence of 10 mM fumarate (\circ , B) at pH 5.2. \blacksquare , the fit of the experimental data to Equation 3. All the reactions were carried out in the presence of a constant 10 mM concentration of tyramine, 2 μM CuSO_4 , 100 $\mu\text{g}/\text{ml}$ catalase, and 2.5 $\mu\text{g}/\text{ml}$ $\text{D}\beta\text{M}$ in 50 mM MES buffer at 37 $^\circ\text{C}$ under atmospheric O_2 saturation conditions (256 μM) as detailed under "Materials and Methods."

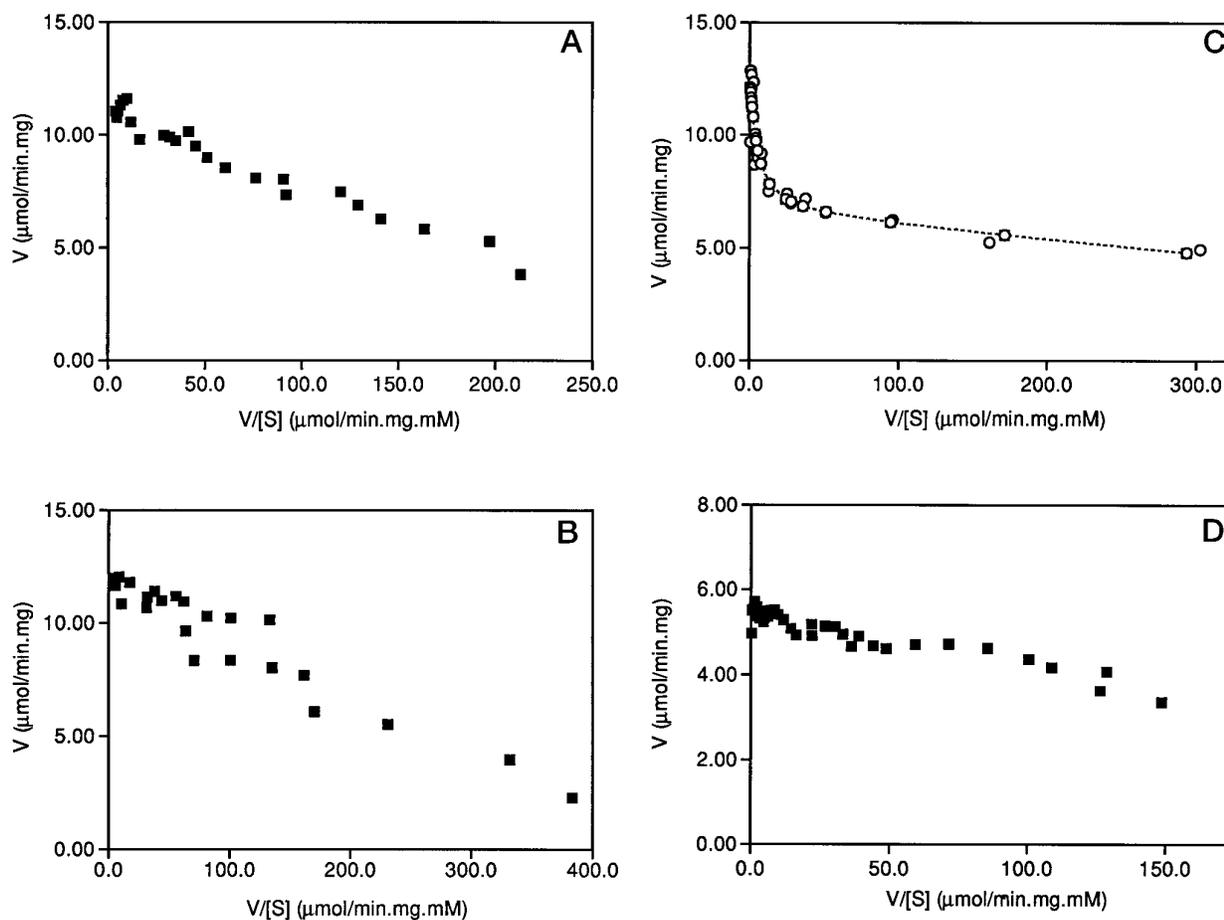


FIG. 5. The steady-state kinetic behavior of the 6OPAscH_2 -mediated $\text{D}\beta\text{M}$ reaction as a function of 6OPAscH_2 . The initial apparent rates of the 6OPAscH_2 -mediated $\text{D}\beta\text{M}$ reaction were measured as a function of 6OPAscH_2 using the oxygen monitor assay, in the absence (\blacksquare , A, and \circ , C) or in the presence of 10 mM fumarate (\blacksquare , B and D) at pH 5.2 (A and B) and pH 6.7 (C and D). \blacksquare , the fit of the experimental data in C to Equation 3. All the reactions were carried out in the presence of a constant 10 mM concentration of tyramine, 2 μM CuSO_4 , 100 $\mu\text{g}/\text{ml}$ catalase, and 2.5 $\mu\text{g}/\text{ml}$ $\text{D}\beta\text{M}$ in 50 mM MES buffer at 37 $^\circ\text{C}$ under atmospheric O_2 saturation conditions (256 μM) as detailed under "Materials and Methods."

clearly demonstrate that 6OPAscH_2 interacts with the enzyme much more efficiently than AscH_2 . In the absence of fumarate at pH 6.7, a significant deviation from the expected linearity was observed in Eadie-Hofstee plots for 6OPAscH_2 similar to that observed for AscH_2 (Fig. 5C). Analysis of the initial rate data at pH 6.7 in the absence of fumarate using Equation 2 yielded an apparent K_m of 0.008 mM at low concentrations and 1.79 mM at high 6OPAscH_2 concentrations. The increase in

apparent V_{max} at the high reductant concentration range was about 1.8-fold demonstrating that the apparent cooperativity in the interaction of 6OPAscH_2 with $\text{D}\beta\text{M}$ is somewhat less pronounced than that of the regular reductant, AscH_2 , under similar experimental conditions.

Examination of the steady-state kinetics of 6SPAscH_2 in the concentration range 0.006–9.00 mM revealed that the turnover rates reached their maximum at 1.2 mM, and beyond this

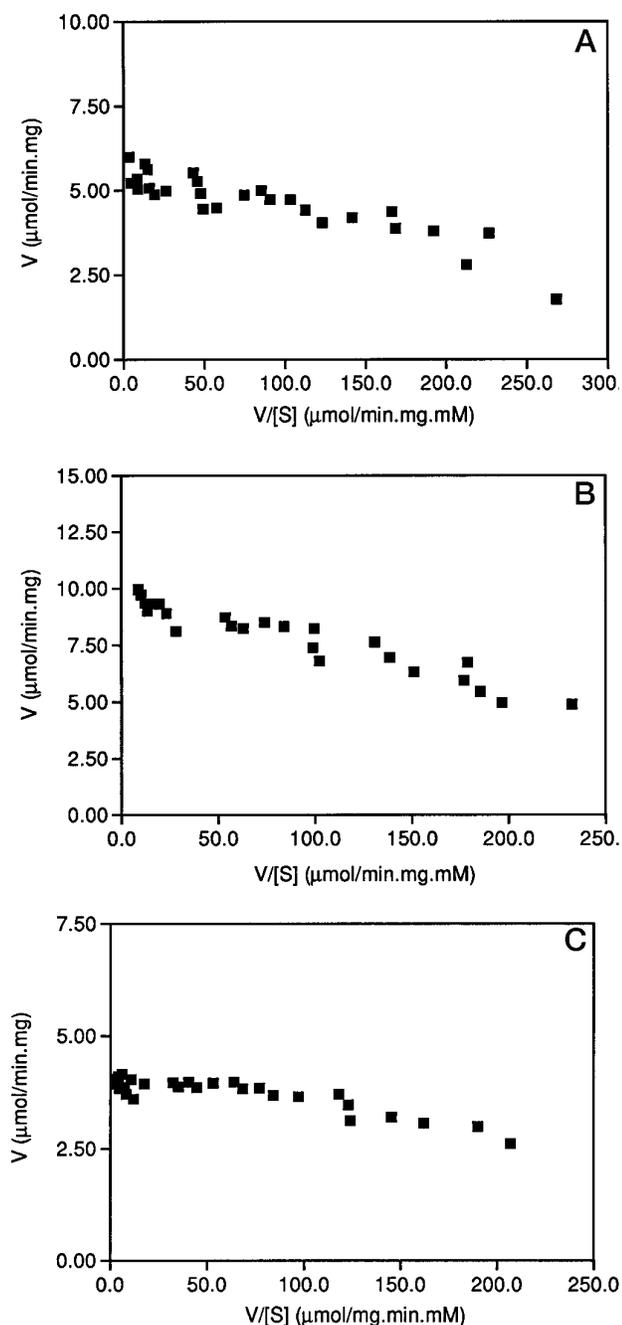


FIG. 6. The steady-state kinetic behavior of the 6SPAsCH₂-mediated $D\beta M$ reaction as a function of 6SPAsCH₂. The initial apparent rates of the 6SPAsCH₂-mediated $D\beta M$ reaction were measured as a function of 6SPAsCH₂ using the oxygen monitor assay, in the absence (A) or in the presence of 10 mM fumarate (B and C) at pH 5.2 (A and B) and pH 6.7 (C). All the reactions were carried out in the presence of a constant 10 mM concentration of tyramine, 2 μM CuSO₄, 100 $\mu g/ml$ catalase, and 2.5 $\mu g/ml$ $D\beta M$ in 50 mM MES buffer at 37 °C under atmospheric O₂ saturation conditions (256 μM) as detailed under "Materials and Methods."

concentration the rates were decreased under all the assay conditions similar to that observed for 6OPAsCH₂. Furthermore, as shown in Fig. 6, A and B, the Eadie-Hofstee plots exhibit normal linear kinetic patterns with 6SPAsCH₂ concentrations in the range of 0.01–1.2 mM at pH 5.2 in the presence and in the absence of fumarate. Whereas relatively linear plots were observed at pH 6.7 in the presence of fumarate (Fig. 6C), in the absence of fumarate at pH 6.7, the initial rates could be

determined only in a narrow substrate range (0.2–2.6 mM) with low reproducibility and accuracy due to the extremely slow turnover rate of the reaction. The kinetic behavior of 6SBAsCH₂ and 6SPEAsCH₂ derivatives was also examined in the concentration ranges 0.006–36.0 and 0.005–7.00 mM, respectively, in the presence and absence of fumarate at both pH 5.2 and 6.7. The Eadie-Hofstee plots of the apparent initial turnover rates were linear up to 3 mM for 6SBAsCH₂ (Fig. 7, A–D) and 1.5 mM for 6SPEAsCH₂ (Fig. 8, A–D) under all the experimental conditions. However, above these concentrations the initial velocities of the reactions were decreased with increasing reductant concentrations similar to that observed for other 6-OH-substituted AsCH₂ derivatives.

The observation that 6-substituted AsCH₂ derivatives, such as 6OPAsCH₂, 6SPAsCH₂, 6SBAsCH₂, and 6SPEAsCH₂, inhibit the enzyme turnover at higher concentrations was further examined using 6SPEAsCH₂ as a representative. The double-reciprocal plots of the data presented in Fig. 9A show that the inhibition of the enzyme turnover by 6SPEAsCH₂, at the concentration range 1.4 to 8.3 mM, is competitive with tyramine in the presence of 10 mM fumarate at pH 5.2 under standard experimental conditions. The inhibition ratio, K_i/K_m , for 6SPEAsCH₂ and tyramine was calculated by fitting the apparent initial rate data to Cleland's "COMP" program yielding a value of 0.87. In the presence of a large excess of AsCH₂ (100 mM), 6SPEAsCH₂ was still found to inhibit the enzyme competitively with respect to tyramine (Fig. 9B). The inhibition ratio, K_i/K_m , under these conditions was found to be 0.45, which is in the same range as the ratio determined in the absence of excess AsCH₂. These results clearly demonstrate that the inhibition of the enzyme at higher concentrations of 6SPEAsCH₂ is due to its efficient competition for the amine binding site of the enzyme with tyramine. Since the presence of a large excess of AsCH₂ changed neither the inhibition pattern nor the potency significantly, we conclude that 6SPEAsCH₂ interacts only with the amine binding site of the enzyme under these conditions.

DISCUSSION

We have previously reported (12–13) that the well characterized chromophoric single electron reductants, TMPD and DMPD (25–26), are well behaved efficient electron donors for $D\beta M$. The present pH dependence studies of the DMPD-mediated $D\beta M$ reaction demonstrate that two ionizable residues with pK_a values of 4.8 and 5.4 are essential for the catalytic cycle of the enzyme in the absence of the anion enzyme activator, fumarate (Fig. 1). In the presence of fumarate, while the pK_a values of these two residues shifted to higher values by about 0.5 pH units, the limiting V_{max}/K_m ratio increased by about a factor of 2 (Fig. 1). The Eadie-Hofstee plots of initial rate kinetics with respect to a wide range of tyramine concentrations were linear both in the presence and absence of fumarate and at both pH 5.2 and 6.7 for the DMPD-mediated $D\beta M$ reaction as expected (data not shown). The initial rate kinetics of the reaction with respect to DMPD showed a significant apparent negative cooperative behavior in the interaction of DMPD with $D\beta M$ both in the absence and presence of fumarate at pH 5.2 as well as 6.7 (Fig. 2, A–D) as indicated by the positive curvature of the Eadie-Hofstee plots. The magnitude of the apparent cooperativity was highly dependent on the pH and fumarate. Normally, at higher pH values in the absence of fumarate the cooperativity was found to be more pronounced (Fig. 2, A–D). These results demonstrate that the kinetics of DMPD, including the observed apparent negative cooperativity, very closely parallel the previously reported (that we also have confirmed (Fig. 4, A and B)) behavior of AsCH₂ in the $D\beta M$ reaction (15, 19), although AsCH₂ and DMPD are vastly structurally different reductants of the enzyme.

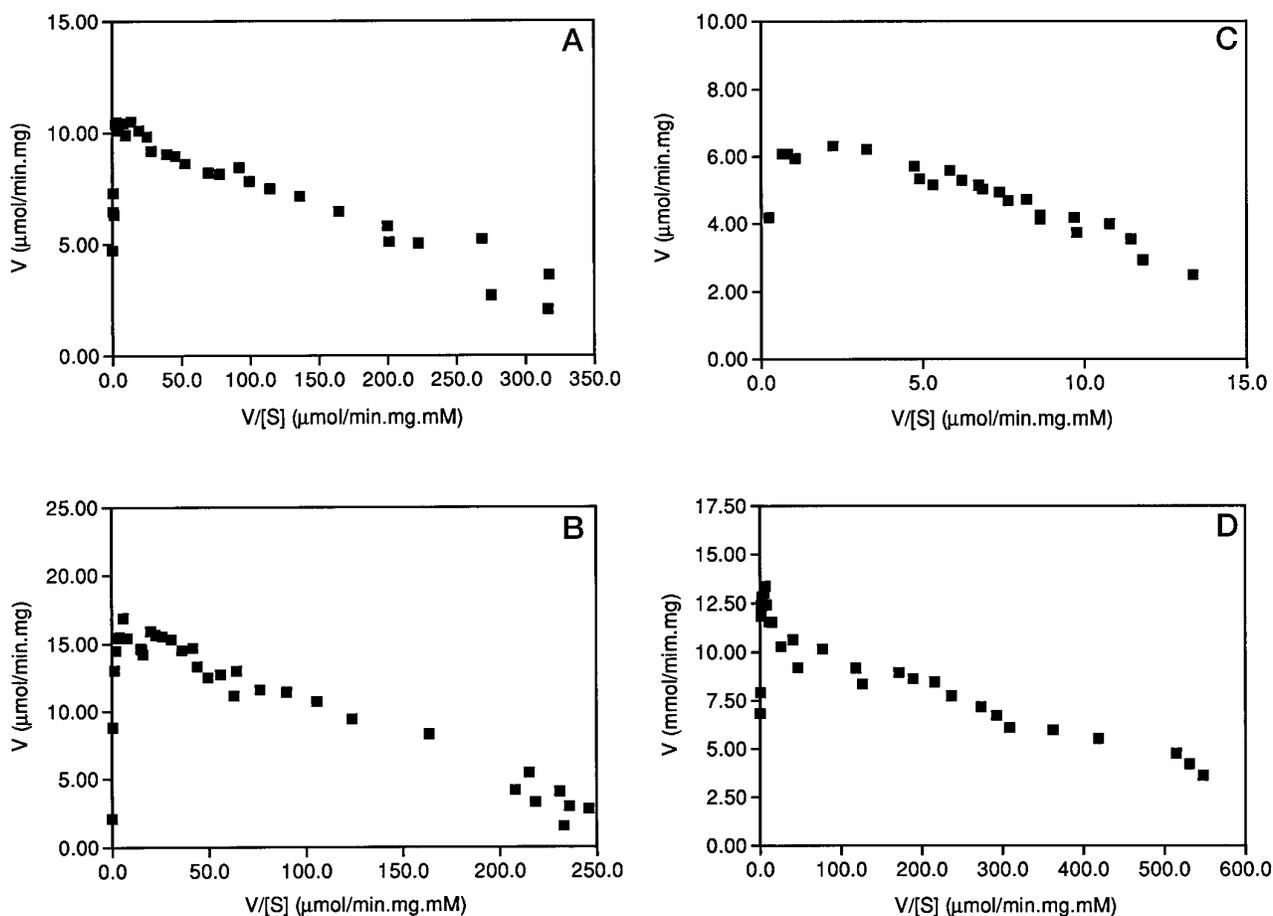


FIG. 7. The steady-state kinetic behavior of the 6SBAscH₂-mediated D β M reaction as a function of 6SBAscH₂. The initial apparent rates of the 6SBAscH₂-mediated D β M reaction were measured as a function of 6SBAscH₂ using the oxygen monitor assay, in the absence (A and C) or in the presence of 10 mM fumarate (B and D) at pH 5.2 (A and B) and pH 6.7 (C and D). All the reactions were carried out in the presence of a constant 10 mM concentration of tyramine, 2 μ M CuSO₄, 100 μ g/ml catalase, and 2.5 μ g/ml D β M in 50 mM MES buffer at 37 °C under atmospheric O₂ saturation conditions (256 μ M) as detailed under "Materials and Methods."

The initial rate kinetic parameters of 6-O- or 6-S-substituted AscH₂ derivatives indicate that the introduction of a hydrophobic phenyl or alkylphenyl substituent at the 6 position of AscH₂ significantly enhances the affinity toward the enzyme without affecting the turnover rate significantly (Table I). The apparent K_m values determined for all these derivatives were 2–7-fold less than the low K_m values of AscH₂ under similar experimental conditions. However, in contrast to the behavior of AscH₂ and DMPD, the steady-state initial rate kinetics of 6-O-phenyl, 6-S-phenyl, 6-S-benzyl, and 6-S-phenylethyl ascorbate derivatives at pH 5.2, both in the presence and absence of fumarate, displayed no observable apparent cooperativity² under the standard experimental conditions (Figs. 5–8, A and B). In addition, all these reductants except 6OPAscH₂ displayed no cooperative behavior even at pH 6.7 either in the presence or absence of fumarate (Figs. 6–8, C and D). While 6OPAscH₂ displayed no significant deviation from the expected linearity in the presence of fumarate at pH 6.7 (Fig. 5D), in the absence of fumarate at pH 6.7 it displayed significant positive curvature in the Eadie-Hofstee plots (Fig. 5C); the behavior of 6SP-AscH₂ under these conditions could not be confirmed with certainty due to the significantly low turnover rate of this compound at pH 6.7. These results establish that the observed apparent negative cooperativity in the D β M reaction with respect to the reductant is not common for all the reductants and also appear to be not strictly confined to the structural features of AscH₂.

Although, 6-OH-modified ascorbate derivatives possess un-

expected high affinity toward the reduction site of the enzyme, they also inhibit the enzyme reversibly at higher concentrations that appears to be unique for these derivatives. The steady-state inhibition kinetics of the representative derivative, 6SPEAscH₂, at the inhibition concentration range, revealed that the inhibition was competitive with respect to tyramine (Fig. 9A). In addition, the inhibition potency or the pattern was not changed in the presence of a high concentration of AscH₂ (100 mM) suggesting that 6SPEAscH₂ is capable of interacting with the amine site without interacting with the reductant site of the enzyme in the presence of high concentrations of AscH₂ (Fig. 9B). However, this mode of interaction of 6SPEAscH₂ with the enzyme must be different from its interaction as a reductant, since in the reductant binding mode, the heterocyclic ring system of the molecule must interact with the reduction site of the enzyme. Therefore, we conclude that these 6-OH-modified AscH₂ derivatives are capable of interacting with the enzyme by two different binding modes, *i.e.* at low concentrations they effectively interact with the reduction site of the enzyme with very high affinity and at high concentrations and/or in the presence of high AscH₂ concentrations they also interact with the amine site of the enzyme. The extreme high affinity of all these molecules toward the reduction site of the enzyme, which would appear to be restricted to the bulky hydrophobic phenyl substituents at the 6 position, must at least be partly due to the interaction of the phenyl group of the reductants with the phenyl binding region of the enzyme. Therefore, these derivatives appear to behave as pseudo bisub-

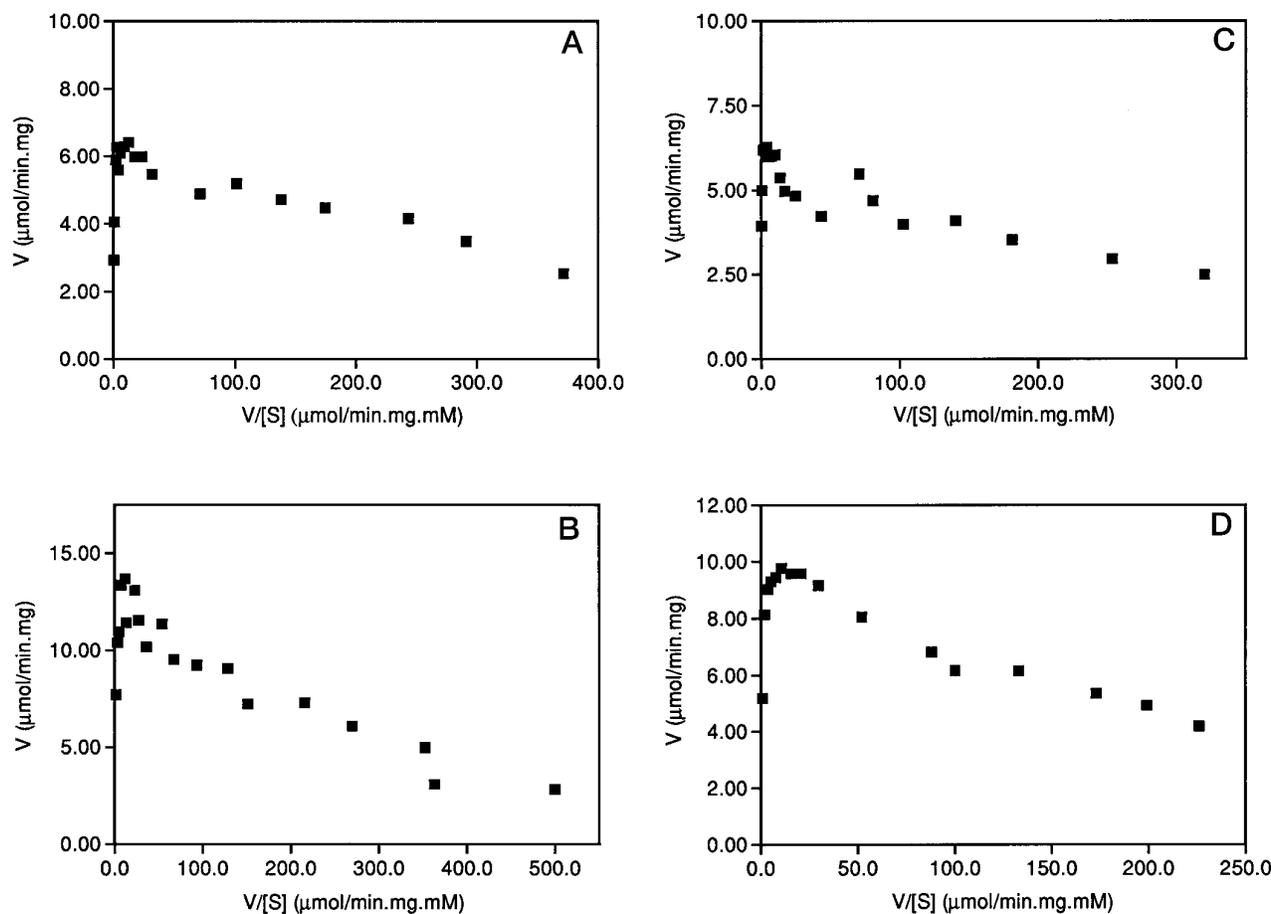


FIG. 8. The steady-state kinetic behavior of the 6SPEAscH₂-mediated D β M reaction as a function of 6SPEAscH₂. The initial apparent rates of the 6SPEAscH₂-mediated D β M reaction were measured as a function of 6SPEAscH₂ using the oxygen monitor assay, in the absence (A and C) or in the presence of 10 mM fumarate (B and D) at pH 5.2 (A and B) and pH 6.7 (C and D). All the reactions were carried out in the presence of a constant 10 mM concentration of tyramine, 2 μ M CuSO₄, 100 μ g/ml catalase, and 2.5 μ g/ml D β M in 50 mM MES buffer at 37 °C under atmospheric O₂ saturation conditions (256 μ M) as detailed under “Materials and Methods.”

strates for the enzyme mimicking ascorbate and phenylethylamine substrates.

The apparent negative cooperativity observed in the interaction of AscH₂ with D β M was proposed to be due to either the interaction of AscH₂ with a specific allosteric regulatory site in the enzyme or due to the existence of multiple reducible forms of the enzyme in the catalytic pathway (15). However, careful inspection of the above results with DMPD suggests that the apparent negative cooperativity with respect to AscH₂ could not be due to the presence of a specific allosteric regulatory site for AscH₂ in D β M. DMPD that is structurally vastly different from AscH₂ would not be expected to efficiently interact with the AscH₂ allosteric site because generally allosteric sites in enzymes are specific and structurally restrictive. Our observation that the interaction of DMPD with D β M also displays significant apparent cooperativity (Figs. 2, A–D) that is kinetically similar to that of AscH₂ (Fig. 4, A and B) strongly suggests that if such an allosteric site exists then DMPD should also interact with this same site with a similar affinity as AscH₂. Moreover, the inspection of the apparent kinetic parameters shown in Table I clearly indicates that if the observed cooperative behavior was due to the existence of an allosteric site, DMPD would interact approximately 2 times stronger with this site and 10 times weaker with the reduction site compared with AscH₂. Therefore, the observed apparent cooperativity of D β M with respect to the reductant is very unlikely to be due the presence of a separate allosteric regulatory site in the enzyme.

In contrast to the previous proposal that AscH₂ interacts exclusively with the reduced free form of D β M (E_{ox}), (8–10, 27) freeze-quench kinetic studies by Klinman *et al.* (14) have demonstrated that in the presence of high concentrations of AscH₂, the E_{ox} -P complex is also reduced under steady-state turnover conditions. These results strongly suggest the existence of a dual pathway for the reduction of the enzyme and two distinct binding sites for the amine substrate and the reductant in the D β M active site. Therefore, since the existence of a separate regulatory allosteric site for AscH₂ in D β M is very unlikely (as argued above), the observed enzyme activation by the reductant at higher concentrations could be due to the existence of two reducible steady-state forms of the enzyme, *i.e.* E_{rd} and E_{rd} -P with different affinities for the reductant. At low AscH₂ concentrations the preferential reduction of the E_{ox} , under the conditions where the rate of the product release from the E_{ox} -P is rate-limiting in the overall reaction, leads to the low K_m and low V_{max} of the overall reaction. At higher AscH₂ concentrations predominantly E_{ox} -P is reduced to produce E_{rd} -P that presumably releases the product faster than E_{ox} -P leading to a severalfold increase in the V_{max} . The very high K_m observed for the E_{ox} -P, in contrast to the relatively low K_m for E_{ox} , may be a consequence of the partial overlap of the product in the amine binding site with the reductant binding site.

The above proposal that the apparent negative cooperativity observed in the interaction of AscH₂ with D β M is due to the existence of two reducible steady-state forms of the enzyme was consistent with the observation that this behavior is not limited

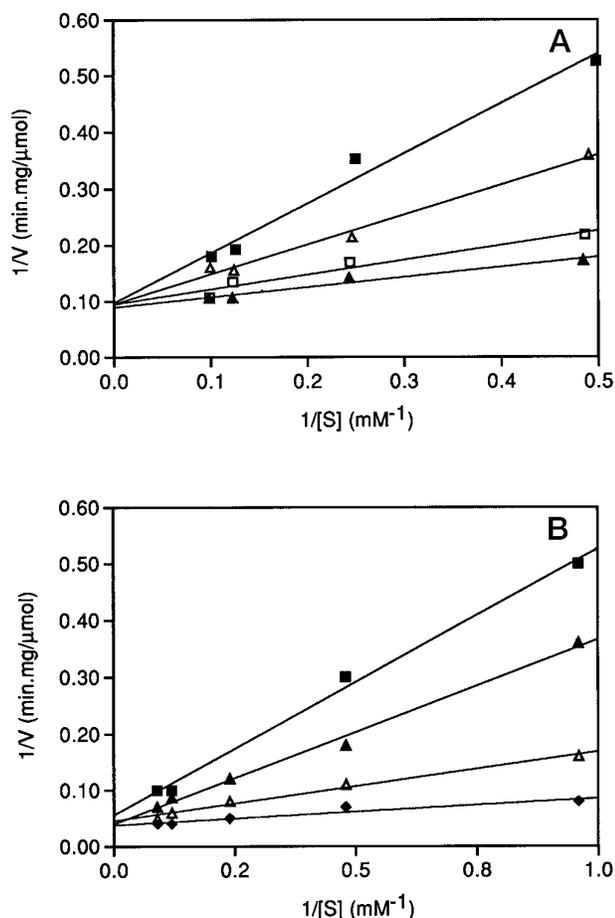


FIG. 9. *A*, double-reciprocal plots of the competitive inhibition of D β M by 6SPEAscH₂ at higher concentrations. The initial apparent rates of the 6SPEAscH₂-mediated D β M reaction were measured at varying concentrations of tyramine and 6SPEAscH₂ using the oxygen monitor assay in the presence of 10 mM fumarate at pH 5.2. All the reactions were carried out in the presence 0.5 μ M CuSO₄, 100 μ g/ml catalase, and 2.5 μ g/ml D β M in 50 mM MES buffer at 37 $^{\circ}$ C under atmospheric O₂ saturation conditions (256 μ M) as detailed under "Materials and Methods." Concentrations of 6SPEAscH₂ were (\blacktriangle) 1.4 mM, (\square) 2.8 mM, (\triangle) 5.5 mM, (\blacksquare) 8.3 mM. *B*, double-reciprocal plots of the competitive inhibition of D β M by 6SPEAscH₂ at higher concentrations in the presence of 100 mM AscH₂. Reaction conditions were same as for *A* except that constant 100 mM concentration of AscH₂ was present in all the reaction mixtures. Concentrations of 6SPEAscH₂ were (\blacklozenge) 0.0 mM, (\triangle) 1.8 mM, (\blacktriangle) 4.3 mM, (\blacksquare) 6.3 mM.

to AscH₂ but also observed with other reductants such as DMPD that are structurally vastly different from AscH₂. The parallel kinetic behavior between DMPD and AscH₂ could be explained by assuming that the reduction site of D β M is relatively nonspecific, and both AscH₂ and DMPD are able to interact with the E_{ox} form efficiently, and they interact weakly with the E_{ox} -P form due to the steric and/or electronic constraints exerted by the product in the proximal amine site. Furthermore, the somewhat less pronounced curvature observed in Eadie-Hofstee plots for DMPD at pH 5.2 both in the presence and absence of fumarate may be due to the relatively low affinity of DMPD to the E_{ox} form of the enzyme in comparison to AscH₂ under these conditions. On the other hand, the observed apparent high affinity of DMPD toward the E_{ox} -P form of the enzyme in comparison to AscH₂ (Table I) may be due to the relatively small interference of DMPD with the amine site of the enzyme mainly due to its compact structure. The lack of observable apparent cooperativity with the 6-OH-

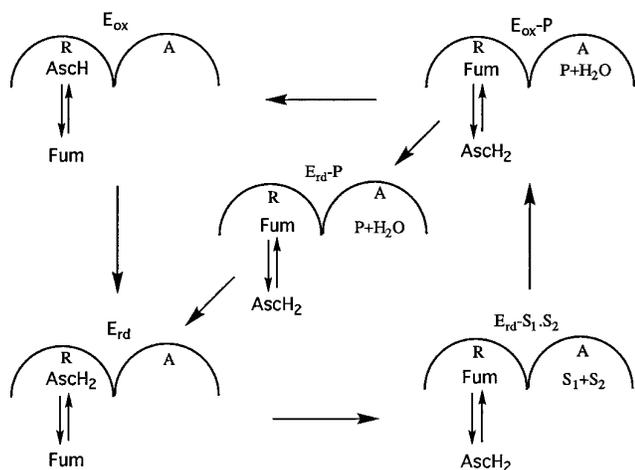
modified AscH₂ derivatives³ must therefore be a consequence of their behavior as pseudo bisubstrates mimicking AscH₂ and the amine substrate. The interaction of these reductants with the reduction site of E_{ox} -P is disfavored by the presence of the product in the amine site, resulting in the overlapping of the phenyl groups of these reductants with the occupied phenyl binding region of the amine site of the enzyme as mentioned above. The observation that 6OPAscH₂ displays apparent negative cooperativity at higher pH values and in the absence of fumarate may simply be due to the interaction of this molecule with the E_{ox} -P form of the enzyme under those conditions probably due to the more compact nature of the molecule.⁴ Therefore, our results strongly suggest that the observed apparent negative cooperativity in the interaction of the reductant with D β M is most probably due to the existence of two reducible steady-state forms of the enzyme with different affinities for the reductants together with the favorable release of the product from the E_{ox} -P form of the enzyme in comparison to E_{rd} -P.

Examination of the effect of fumarate on the DMPD-mediated D β M reaction at a constant DMPD concentration (near the low K_m) demonstrates that fumarate competes with DMPD and inhibits the enzyme under these conditions (Fig. 3), similar to (but in a somewhat more pronounced manner) that of the AscH₂-mediated reaction (15). Inspection of the kinetic data presented in Table I also demonstrates that 10 mM fumarate increases the low apparent K_m for most of the reductants (with few exceptions) under a variety of experimental conditions, suggesting that fumarate successfully competes with the reductants for the reduction site of the enzyme that appears to be independent of the nature of the reductant. However, the extent of the enzyme activation by fumarate appears to be dependent on the nature of the reductant (see Table II). For example, while 10 mM fumarate increased the apparent V_{max}/K_m for tyramine by about 180% in the AscH₂ (30 mM)-supported D β M reaction, 10 mM fumarate increased this ratio by about 750% in the 6SBAscH₂ (2.0 mM)-supported D β M reaction under similar conditions (Table II). These findings clearly demonstrate that fumarate activation of the enzyme is somehow associated with the nature of the reductant and may not simply be explained by the generally accepted hypothesis that D β M possesses a specific anion activating site for fumarate independent from the reductant binding site.

In order to accommodate the above observations, we propose a unified model in that both fumarate and the reductant interact with the same site of all forms of the enzyme with varying

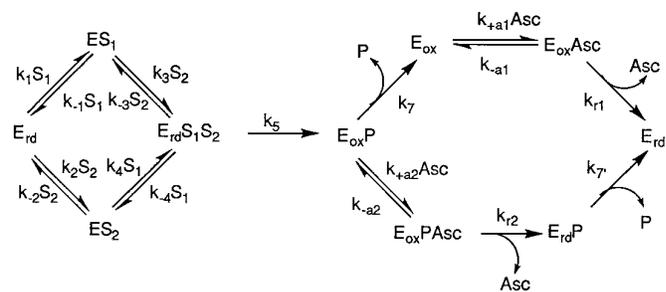
³ A possibility exists that the apparent lack of negative cooperativity with the 6-OH-modified AscH₂ derivatives is due to the masking of the enzyme activity by the enzyme inhibition at higher concentrations of these derivatives. However, since these derivatives are relatively weak inhibitors and all the experiments were carried out in the presence of 10 mM tyramine, we do not believe the inhibition is strong enough to completely mask the negative cooperativity (if present) of these molecules. Furthermore, the "high K_m " determined for 6OPAscH₂ at pH 6.7 in the absence of fumarate was 1.8 mM (see Footnote 4) that is lower than the inhibition range of most of these derivatives, and therefore, the cooperativity, if present, must be experimentally observable for all these derivatives.

⁴ The high degree of negative cooperativity and smaller high K_m observed for 6OPAscH₂ in comparison to AscH₂ (Table I) suggest that 6OPAscH₂ possesses an unexpected affinity to the E_{ox} -P form of the enzyme that appears to be inconsistent with the proposed model. These observations are also clearly inconsistent with the existence of a specific regulatory AscH₂ site, since AscH₂ is expected to interact stronger with such a site than the structurally altered 6OPAscH₂. Therefore, a possible explanation for this observation is that the phenyl binding region of the E_{ox} -P form of the enzyme is somewhat loose and allows slight interaction of the phenyl group of the structurally compact 6OPAscH₂, in contrast to the other 6-OH derivatives of AscH₂ that are structurally extended.



SCHEME II. A model proposed for the interaction of the reductant and fumarate with D β M. E_{ox} , oxidized free enzyme; E_{rd} , reduced free enzyme; E_{ox-P} , oxidized enzyme product complex; E_{rd-P} , reduced enzyme product complex; and $E_{rd-S_1-S_2}$, reduced enzyme, oxygen, amine ternary complex; R, reduction site; A, amine binding site; Fum, fumarate. Note that both reductant and fumarate compete for the same site of all the forms of the enzyme with different affinities, and the relative affinities of these various forms of the enzyme are represented by placing the high affinity agent *in* and low affinity agent *out* of the reduction site.

affinities under steady-state turnover conditions (Scheme II). The inhibition of the enzyme by fumarate at low reductant concentrations and the increase of K_m for most of the reductants in the presence of 10 mM fumarate (Table I) must therefore be due to the efficient competition of fumarate with the reductant for the reduction site of the E_{ox} form of the enzyme. While both AscH₂ and DMPD interact relatively efficiently with the E_{ox} form, both interact weakly with the other forms of the enzyme due to steric and/or electronic constraints imposed by the presence of the amine substrate or the product in the amine site of the enzyme as mentioned above. All the 6-OH derivatives of AscH₂ interact very efficiently and exclusively with the E_{ox} form but not with the other forms, due to their behavior as pseudo bisubstrates with respect to the reductant and the amine substrate. Therefore, as stated above, the observed apparent negative cooperativity with respect to AscH₂ and DMPD may be due to their interaction with the E_{ox-P} form of the enzyme at higher concentrations, and the lack of cooperativity with 6-OH-modified derivatives of AscH₂ could be primarily due to their inability to interact with this form of the enzyme. Furthermore, the decrease of cooperativity in the presence of fumarate could be a result of the pH-dependent efficient competition of sterically less bulky fumarate for the E_{ox-P} form of the enzyme in comparison to the reductant (either AscH₂ or DMPD). Based on this model, the enzyme activation by fumarate can be explained by assuming that fumarate preferentially interacts with the reduction site of $E\cdot O_2\cdot$ Tyr (or $E\cdot$ Tyr) by efficient competition with the reductant leading to the decrease of the rate of amine dissociation and alteration of the pK_a values of active site residues, as previously suggested (19). This possibility is also strongly supported by the observation that fumarate activation of the D β M reaction-mediated by 6-OH derivatives of AscH₂ is about four times more pronounced than that of the AscH₂-mediated reaction (Table II). When 6-OH derivatives of AscH₂ are the reductants, fumarate may interact much more freely and efficiently with the $E\cdot O_2\cdot$ Tyr (or $E\cdot$ Tyr) form of the enzyme due to the lack of competition from these derivatives for the reduction site (Scheme II). Finally, fumarate activation of the enzyme even at saturating concentrations of AscH₂ must be due to the relative high affinity of fumarate to



SCHEME III

the $E\cdot O_2\cdot$ Tyr (or $E\cdot$ Tyr) form of the enzyme in comparison to AscH₂, again due to steric and/or electronic factors as mentioned above.

In order to test and further substantiate the above proposed model, the experimental data were further analyzed mathematically. Based on the proposed model, the kinetic expression shown in Equation 3 that was derived for the kinetic scheme (Scheme III) shown below (in the absence of fumarate) to include the interaction of the reductant with both E_{ox} and E_{ox-P} forms of the enzyme (possible interactions of the reductant with ES_1 , ES_2 , and ES_1S_2 forms of the enzyme are not considered for simplicity) should describe the kinetics of the interaction of the reductant with the enzyme.

$$\frac{v}{E_t} = \frac{V_1 + \left[\frac{Asc}{K_{m1}} \right] V_2}{1 + \frac{K_{m1}}{[Asc]} + \frac{[Asc]}{K_{m2}}} \quad (\text{Eq. 3})$$

where

$$V_1 = \frac{k_5 k_7 k_{r1}}{k_5 k_7 + k_5 k_{r1} + C k_7 k_{r1}} \quad V_2 = \frac{k_5 k_7 k_{r2}}{k_5 k_7 + k_5 k_{r2} + C k_7 k_{r2}}$$

$$K_{m1} = [K_{S1}] \left[\frac{V_1}{k_{r1}} \right] \quad K_{m2} = [K_{S2}] \left[\frac{V_2}{V_1} \right] \left[\frac{k_7}{k_{r2}} \right]$$

$$K_{S1} = \frac{k_{-a1} + k_{r1}}{k_{+a1}} \quad K_{S2} = \frac{k_{-a2} + k_{r2}}{k_{+a2}}$$

C is a function of k_1 , k_{-1} , k_2 , k_{-2} , k_3 , k_{-3} , k_4 , k_{-4} , k_5 , S_1 , and S_2 and when S_1 and S_2 are saturating approaches 1. S_1 and S_2 are either oxygen or tyramine.

Inspection of Equation 3 indicates that it is similar to Equation 2 except the definition of K_{m2} . The fits shown in Figs. 2, 4, and 5C that were obtained by the direct fitting of the experimental data to Equation 3 using the previously determined kinetic parameters, $k_5 = 580 \text{ s}^{-1}$, $k_{r1} = k_{r2} = 185 \text{ s}^{-1}$ in the presence and $k_5 = 1200 \text{ s}^{-1}$, $k_{r1} = k_{r2} = 185 \text{ s}^{-1}$ in the absence of fumarate (19),⁵ demonstrate that the experimental data are in excellent agreement with Equation 3 in all cases. In addition, the kinetic parameters shown in Table III that were extracted from these fits show that the rate of product release from E_{rd-P} , $k_{7'}$, is about three times faster than the rate of product release from E_{ox-P} , k_7 , for both AscH₂ and DMPD-mediated reactions at pH 5.2 in the absence of fumarate as predicted by the proposed model. In the presence of fumarate, especially at pH 5.2, both k_7 and $k_{7'}$ parameters are consistently increased in both AscH₂- and DMPD-mediated reactions,

⁵ We have used the kinetic parameters that are reported for pH 6.0 for dopamine since a complete set of these parameters is not available for pH 5.2 for tyramine (19). Since we are only interested in the relative magnitude of these parameters and not absolute values, the absolute variations of these parameters due to small pH changes (5.2–6.0) or change in the substrate (from dopamine to tyramine) should not significantly affect our major conclusions.

TABLE III
 Kinetic parameters of various reductants

These parameters were obtained by fitting the experimental data directly to Equation 3 as described in the text. The kinetic parameters were normalized to the maximum specific activity of 30 $\mu\text{mol}/\text{min} \cdot \text{mg}$ for the purpose of internal comparison. For experimental details see Table II.

pH	Parameter	AsCH ₂		DMPD		6OPAsCH ₂	
		+F	-F	+F	-F	+F	-F
5.2	k_7 (s ⁻¹)	17.3 ± 0.7	10.6 ± 0.4	16.5 ± 2.2	13.2 ± 2.3	^a	^a
	$k_{7'}$ (s ⁻¹)	40.7 ± 6.9	32.9 ± 1.9	68.2 ± 4.7	37.7 ± 1.2	^a	^a
	K_{s1}	3.8 ± 0.4	3.0 ± 0.3	15.9 ± 1.5	13.0 ± 1.5	^a	^a
	K_{s2}	611 ± 236	332 ± 52	99 ± 16	71 ± 15	^a	^a
6.7	k_7 (s ⁻¹)	^a	^a	3.3 ± 0.5	1.4 ± 0.2	^a	6.5 ± 0.8
	$k_{7'}$ (s ⁻¹)	^a	^a	14.2 ± 1.6	14.5 ± 3.5	^a	12.7 ± 0.7
	K_{s1}	^a	^a	^b	^b	^a	0.4 ± 0.3
	K_{s2}	^a	^a	342 ± 87	167 ± 19	^a	67 ± 35

^a Not determined.

^b Accurate kinetic parameters could not be obtained.

suggesting fumarate accelerates the product release slightly from both $E_{\text{ox}}\text{-P}$ and $E_{\text{rd}}\text{-P}$ complexes under the experimental conditions. This observation appears to contrast with the previously reported effect of fumarate on the product release step of the D β M reaction (19). However, we believe this apparent discrepancy may be due to the extreme sensitivity of fumarate toward experimental conditions, *i.e.* pH, reductant concentration, etc. as predicted by the above model. Inspection of the K_s values reported in Table III demonstrates that the affinity of AsCH₂ for the E_{ox} form is much higher in comparison to the $E_{\text{ox}}\text{-P}$ form, whereas this difference is less contrasting for DMPD as we have predicted. More importantly, the magnitudes of both K_{s1} and K_{s2} values were increased significantly for both DMPD and AsCH₂ in the presence of fumarate especially at pH 5.2 indicating that fumarate competes with the reductant for both E_{ox} and $E_{\text{ox}}\text{-P}$ forms of the enzyme that are as predicted by the proposed model. An estimation of the K_f values of fumarate with respect to the reductant for E_{ox} and $E_{\text{ox}}\text{-P}$ forms yielded 45–36 and 12–25 mM, respectively, indicating that fumarate interacts with the $E_{\text{ox}}\text{-P}$ form more strongly than the E_{ox} form. These results strongly suggest that the product in the amine site facilitates fumarate interaction with the reductant site while strongly disfavoring the reductant interaction, which is also consistent with the proposed model. Therefore, we conclude that the proposed model successfully explains the observed apparent negative cooperativity and the effect of the anion activator, fumarate, on the steady-state kinetics of the D β M reaction qualitatively and quantitatively.

Finally, not only does the proposed model adequately explain all the experimental observations, it is much simpler and more reasonable than the previous models where amine substrate, fumarate, reductant, and the allosteric modulators were proposed to interact with four distinct sites of the enzyme. The physiological significance of this intricate mechanism for the modulation of the enzyme activity by both anion activators and the reductant itself is not clear at present. Increasing recent evidence (6–7) suggests that the membrane-bound D β M in chromaffin granule ghosts is not directly interacting with the internal AsCH₂ and is exclusively reduced from the external AsCH₂, probably through the transmembrane electron transport protein cytochrome b_{561} (28–31). However, since cytochrome b_{561} and membrane-bound D β M do not appear to be directly coupled reductively, there may be other redox cofactors and/or proteins involved in the *in vivo* reduction of D β M. Therefore, the anion binding, reduction site of D β M may func-

tion as a reduction, recognition, and/or regulation site for the redox cofactors and/or proteins *in vivo*. Complete elucidation of the molecular mechanism of the *in vivo* reduction of both soluble and membrane-bound forms of the enzyme may finally resolve some of these issues.

Acknowledgment—We thank Donovan C. Haines, Department of Chemistry, Wichita State University for the assistance in the mathematical analysis of the experimental data.

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