Reduction of Dopamine β-Monoxygenase: A Unified Model for Apparent Negative Cooperativity and Fumarate Activation

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

Kandatege Wimalasena, Silpadipathialage Dharmasena, D. Shyamali Wimalasena, and Dianna K. Hughs-Wheaton

From the Department of Chemistry, Wichita State University, Wichita, Kansas 67260-0051

The interactions of reductants with dopamine β-monoxygenase (DβM) were examined using two novel classes of reductants. The steady-state kinetics of the previously characterized DβM 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 DβM 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 substrates with respect to ascorbic acid and the amine substrate. The lack of apparent cooperative behavior with these derivatives suggests that this behavior of DβM 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 DβM 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 DβM 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-monoxygenase (DβM); 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). DβM 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 DβM and that high levels of AscH2 are present in DβM containing neurosecretory vesicles (4–5) appear to indicate that intragranular AscH2 may provide the necessary reducing equivalents for the DβM reaction in vivo. However, recent studies with chromaffin granule ghosts suggest that extragranular (cytosolic) AscH2 may be involved in the reduction of membrane-bound DβM directly (6–7).

Although the hydroxylation site of DβM 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,N,N- tetramethyl-1,4-p-phenylenediamine (TMFD) and N,N,dimethyl-1,4-p-phenylenediamine (DMPD), which have no structural resemblance to AscH2 (Scheme 1), 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 DβM 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 (Ered-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 Ered-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 DβM 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 DβM 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 DβM 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
A Model for Cooperativity and Fumarate Activation of DβM

Tyramine hydrochloride, disodium fumarate, and MES were from Sigma. N,N-Dimethyl-1,4-p-phenylenediamine hydrochloride salt and AscH$_2$ were from Aldrich. Beef liver catalase (65,000 units/mg of protein) was from Boehringer Mannheim. The AscH$_2$ derivatives, 6OPAscH$_2$, and 6SPAscH$_2$, were synthesized as previously reported (8). All other chemicals were of the highest purity available and were purchased from various sources. Bovine adrenal soluble DβM 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 $E_{280} = 1.24$ ml mg$^{-1}$ cm$^{-1}$. UV-visible spectroscopic measurements were carried out using an HP-8452A diode array spectrophotometer equipped with a temperature-controlled cell compartment. $^{1}$H and $^{13}$C NMR spectra were recorded on a Varian XL-300 (300 MHz) NMR spectrometer using tetramethylsilane or 3-(trimethylsilyl)-1-propane-sulfonic acid sodium salt (when D$_2$O 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.

\[
\nu = \frac{c}{1 + [H^+]K_a + K_b[H^+]} \quad (\text{Eq. 1})
\]

In cases where a significant deviation from the expected kinetic behavior (apparent cooperativity) was observed, the data were analyzed by the general model of Alberts (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.

\[
u = \frac{V_2 + V_1K_a(1 - V_1/V_0)[S]}{1 + K_a(1 - V_1/V_0)[S] + K_bK_a[1 - V_1/V_0][S]} \quad (\text{Eq. 2})
\]

**Oxygen Monitor Assay of DβM**—The initial rates of the DβM 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 6OPAscH$_2$, 6SPAscH$_2$, 6SBAascH$_2$, and 6SPEascH$_2$ 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 µg/ml catalase, 2 µM CuSO$_4$, 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 autoxidation of ascorbic acid (or AscH$_2$, derivative) concentration.

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 μM). The $V_{\text{max}}$ values were calculated based on the monomer molecular mass of 70,000 Da for DβM and normalized to a constant maximum specific activity of 30 μmol/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 AscH$_2$ 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 DβM—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 μg/ml catalase and 0.5–2.0 μM CuSO$_4$ in a total volume of 1.0 ml. DβM concentrations were kept constant for each experiment but varied between 1.2 and 3.2 μg 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 enzymic reaction mixture but without the enzyme as described previously (13). For further details see the corresponding figure legends.

$\text{C}_13\text{H}_14\text{O}_5\text{S}$

Calculated: C 55.30 H 4.99

Found: C 55.27 H 4.88

HPLC analysis, 98% pure with no trace of AscH$_2$.

$\text{C}_13\text{H}_14\text{O}_5\text{S}$

Calculated: C 56.74 H 5.44

Found: C 56.63 H 5.52

HPLC analysis, 97% pure with no trace of AscH$_2$.

and were determined at atmospheric oxygen saturation conditions (256 μM). The $V_{\text{max}}$ values were calculated based on the monomer molecular mass of 70,000 Da for DβM and normalized to a constant maximum specific activity of 30 μmol/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 AscH$_2$ 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 DβM—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 μg/ml catalase and 0.5–2.0 μM CuSO$_4$ in a total volume of 1.0 ml. DβM concentrations were kept constant for each experiment but varied between 1.2 and 3.2 μg 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 enzymic reaction mixture but without the enzyme as described previously (13). For further details see the corresponding figure legends.

$\text{C}_13\text{H}_14\text{O}_5\text{S}$

Calculated: C 55.30 H 4.99

Found: C 55.27 H 4.88

HPLC analysis, 98% pure with no trace of AscH$_2$.

$\text{C}_13\text{H}_14\text{O}_5\text{S}$

Calculated: C 56.74 H 5.44

Found: C 56.63 H 5.52

HPLC analysis, 97% pure with no trace of AscH$_2$.
The Eadie-Hofstee plots of apparent initial rate kinetics of the DMPD-mediated DbM 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 $V_{\text{max}}/K_m$ ratio that is more pronounced at pH 6.7 than at 5.2. For example, while the apparent $V_{\text{max}}/K_m$ ratio changed from $0.11 \times 10^4$ M$^{-1}$ s$^{-1}$ in the absence of fumarate to $0.49 \times 10^4$ M$^{-1}$ s$^{-1}$ in the presence of 10 mM fumarate at pH 6.7, the ratio changed from $1.10 \times 10^4$ M$^{-1}$ s$^{-1}$ in the absence to $2.2 \times 10^4$ M$^{-1}$ 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 $V_{\text{max}}/K_m$ 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 $V_{\text{max}}$ is relatively small at a constant pH (depending on the experimental conditions less than 15% increased), and the large effect on the ratio $V_{\text{max}}/K_m$ 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 AscH$_2$-supported DbM reaction (19).

The initial rate kinetics of the AscH$_2$-mediated DbM reaction display significant deviation from the expected linearity in Eadie-Hofstee plots over the range of 0.02–55 mM AscH$_2$ 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
The initial apparent kinetic parameters were determined using various enzyme preparations with different specific activities (in the range of 13–30 μmol/min · mg), and all the V_max values were normalized to the maximum specific activity of 30 μmol/min · 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."

The apparent K_m values for the high (4-10 μM) 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 DβM with respect to AscH_2 qualitatively and show that the experimental protocols we have used are comparable with those of Stewart and Kliman (15).

The initial rate of the 60PAAscH_2-mediated DβM monooxygenation of tyramine was examined as a function of 60PAAscH_2 in the concentration range of 0.005 to 33.0 μM, in the absence and in the presence of fumarate, at pH 5.2 and 6.7. In contrast to AscH_2, the initial rate of the reaction reached its maximum at about 4 μM in the presence and in the absence of fumarate at both of the above pH values and thereafter decreased gradually with increasing concentration of 60PAAscH_2, suggesting an inhibition of the enzyme by 60PAAscH_2 at higher concentrations. This derivative exhibits normal linear Eadie-Hofstee kinetic plots up to about 4 μmol 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 60PAAscH_2 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 μM in the presence and 0.034 μM in the absence of fumarate at pH 5.2 and 0.030 μM in the presence of fumarate at pH 6.7. These data
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. 5). 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 D$_b$M is somewhat less pronounced than that of the regular reductant, AscH$_2$, under similar experimental conditions.
A Model for Cooperativity and Fumarate Activation of DβM

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 DβM. The present pH dependence studies of the DMPD-mediated DβM reaction demonstrate that two ionizable residues with pKa 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 pKa values of these two residues shifted to higher values by about 0.5 pH units, the limiting Vmax/Km 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 DβM 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 DβM 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 DβM reaction (15, 19), although AscH2 and DMPD are vastly structurally different reductants of the enzyme.

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/Km, 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/Km, 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.

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/Km, 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/Km, 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.

The initial apportion reaction as a function of 6SPAscH2. The initial apparent rates of the 6SPAscH2-mediated DβM 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 μg/ml catalase, and 2.5 μg/ml DβM in 50 mM MES buffer at 37 °C under atmospheric O2 saturation conditions (256 μM) as detailed under “Materials and Methods.”

Fig. 6. The steady-state kinetic behavior of the 6SPAscH2-mediated DβM reaction as a function of 6SPAscH2. The initial apparent rates of the 6SPAscH2-mediated DβM 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 μg/ml catalase, and 2.5 μg/ml DβM in 50 mM MES buffer at 37 °C under atmospheric O2 saturation conditions (256 μM) as detailed under “Materials and Methods.”
The initial rate kinetic parameters of 6-O-or 6-S-substituted
A.sH2 derivatives indicate that the introduction of a hydropho-
bic phenyl or alkylphenyl substituent at the 6 position of A.sH2
significantly enhances the affinity toward the enzyme without
affecting the turnover rate significantly (Table I). The apparent
Km values determined for all these derivatives were 2–7-fold
less than the low Km values of A.sH2 under similar experimental
conditions. However, in contrast to the behavior of A.sH2
and DMPD, the steady-state initial rate kinetics of 6-O-phenyl,
6-S-phenyl, 6-S-benzyl, and 6-S-phenylethyl ascorbate deriva-
tives at pH 5.2, both in the presence and absence of fumarate,
displayed no observable apparent cooperativity 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 curva-
ture 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 DβM reaction with re-
spect to the reductant is not common for all the reductants and
also appear to be not strictly confined to the structural features
of A.sH2.

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 concentra-
tions that appears to be unique for these derivatives. The
steady-state inhibition kinetics of the representative derivat-
ive, 6SPEAscH2, at the inhibition concentration range, reveal-
ted that the inhibition was competitive with respect to ty-
ramine (Fig. 9A). In addition, the inhibition potency or the
pattern was not changed in the presence of a high concentra-
tion of A.sH2 (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 concen-
trations of A.sH2 (Fig. 9B). However, this mode of interaction
of 6SPEAscH2 with the enzyme must be different from its inter-
action 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 A.sH2 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 concentra-
tions and/or in the presence of high A.sH2 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. 7. The steady-state kinetic behavior of the 6SBAascH2-mediated DβM reaction as a function of 6SBAascH2. The initial apparent
rates of the 6SBAascH2-mediated DβM reaction were measured as a function of 6SBAascH2 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 μM CuSO4, 100 μg/ml catalase, and 2.5 μg/ml DβM in 50 mM MES buffer at 37 °C under
atmospheric O2 saturation conditions (256 μM) as detailed under “Materials and Methods.”](image-url)
strates for the enzyme mimicking ascorbate and phenylethylamine substrates.

The apparent negative cooperativity observed in the interaction of AscH₂ with DβM 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 DβM. 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 DβM 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 DβM 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 DβM (E₉⁻), (8–10, 27) freeze-quench kinetic studies by Klinman et al. (14) have demonstrated that in the presence of high concentrations of AscH₂, the E₉⁻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 DβM active site. Therefore, since the existence of a separate regulatory allosteric site for AscH₂ in DβM 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. E₉⁻ and E₉⁻P with different affinities for the reductant. At low AscH₂ concentrations the preferential reduction of the E₉⁻ under the conditions where the rate of the product release from the E₉⁻⁻P is rate-limiting in the overall reaction, leads to the low Kₐ for the overall reaction. At higher AscH₂ concentrations predominantly E₉⁻⁻P is reduced to produce E₉⁻⁻⁻P that presumably releases the product faster than E₉⁻⁻⁻P leading to a severalfold increase in the V₉⁻⁻⁻. The very high Kₐ observed for the E₉⁻⁻⁻, in contrast to the relatively low Kₐ for E₉⁻⁻⁻, 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 DβM 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

![Graphs showing steady-state kinetic behavior](image-url)
modified AsCH$_2$ derivatives must therefore be a consequence of their behavior as pseudo bi substrates mimicking AsCH$_2$ 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 60PAAsCH$_2$ 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. Therefore, our results strongly suggest that the observed apparent negative cooperativity in the interaction of the reductant with D$\beta$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$_{ir-P}$.

Examination of the effect of fumarate on the DMPD-mediated D$\beta$M reaction at a constant DMPD concentration in the presence of 10 mM fumarate at pH 5.2. All the reactions were carried out in the presence of 0.5 mM CuSO$_4$, 100 $\mu$g/ml catalase, and 2.5 $\mu$g/ml D$\beta$M in 50 mM MOPS buffer at 37°C under atmospheric O$_2$. The low affinity of DMPD to the enzyme mainly due to its compact structure. The lack of observable apparent cooperativity with the 6-OH-DMPD that are structurally vastly different from AsCH$_2$. The parallel kinetic behavior between DMPD and AsCH$_2$ could be explained by assuming that the reduction site of D$\beta$M is relatively nonspecific, and both AsCH$_2$ and DMPD are able to interact with the E$_{ox-P}$ form efficiently, and they interact weakly with the E$_{ir-P}$ 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 AsCH$_2$ under these conditions. On the other hand, the observed apparent high affinity of DMPD toward the E$_{ir}$ form of the enzyme in comparison to AsCH$_2$ (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.

Fig. 9. A, double-reciprocal plots of the competitive inhibition of D$\beta$M by 6SPEAsCH$_2$ at higher concentrations. The initial apparent rates of the 6SPEAsCH$_2$-mediated D$\beta$M reaction were measured at varying concentrations of tyramine and 6SPEAsCH$_2$ 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 of 0.5 mM CuSO$_4$, 100 $\mu$g/ml catalase, and 2.5 $\mu$g/ml D$\beta$M in 50 mM MOPS buffer at 37°C under atmospheric O$_2$. b, double-reciprocal plots of the competitive inhibition of D$\beta$M by 6SPEAsCH$_2$ at higher concentrations in the presence of 100 mM AsCH$_2$. Reaction conditions were same as for A except that constant 100 mM concentration of AsCH$_2$ was present in all the reaction mixtures. Concentrations of 6SPEAsCH$_2$ were (A) 1.4 mM, (B) 2.8 mM, (C) 5.5 mM, (D) 8.3 mM.

3 A possibility exists that the apparent lack of negative cooperativity with the 6-OH-modified AsCH$_2$ 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" determined for 6OPAsCH$_2$ at pH 6.7 in the absence of fumarate was 1.5 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.

The high degree of negative cooperativity and smaller high K$_m$ observed for 6OPAsCH$_2$ in comparison to AsCH$_2$ (Table I) suggest that 6OPAsCH$_2$ possesses an unexpected affinity to the E$_{ir}$ 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 AsCH$_2$ site, since AsCH$_2$ is expected to interact stronger with such a site than the structurally altered 6OPAsCH$_2$. Therefore, a possible explanation for this observation is that the phenyl binding region of the E$_{ir}$ form of the enzyme is somewhat loose and allows slight interaction of the phenyl group of the structurally compact 6OPAsCH$_2$, in contrast to the other 6-OH derivatives of AsCH$_2$ that are structurally extended.
affinities of these various forms of the enzyme are represented by the competition of sterically less bulky fumarate for the same site with the reductant and oxygen. Note that both reductant and fumarate compete for the same site of the enzyme with different affinities, and the relative rate can be explained by assuming that fumarate preferentially interacts with the reduction site of the enzyme in comparison to the reductant (either AscH₂ or DMPD). Based on this model, the enzyme activation 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 substrates with respect to the reductant and the amine substrate. Therefore, as stated above, the observed apparent negative cooperation 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 \( E_{ox} \)-Tyr (or \( E' \)-Tyr) by efficient competition with the reductant leading to the decrease of the rate of amine dissociation and alteration of the \( k_{a} \) values of active site residues, as previously suggested (19). This possibility is also strongly supported by the observation that fumarate activation of the DβM 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 \( E_{ox} \)-Tyr (or \( E' \)-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 the enzyme activation by fumarate even at saturating concentrations of 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 \( E_{s1} \), \( E_{s2} \), and \( E_{s1}s_{2} \) forms of the enzyme are not considered for simplicity) should describe the kinetics of the interaction of the reductant with the enzyme.

\[
V' = \frac{V_1}{1 + \frac{K_{m}}{K_{a}}} + \frac{V_2}{1 + \frac{K_{m}}{K_{a}}}
\]

where

\[
V_1 = \frac{k_{h}k_{r}k_{a}}{k_{r} + k_{h} + k_{c}h_{2}} \quad V_2 = \frac{k_{h}k_{r}k_{a}}{k_{r} + k_{h} + k_{c}h_{2}}
\]

C is a function of \( k_{1}, k_{-1}, k_{2}, k_{-2}, k_{3}, k_{-3}, k_{4}, k_{-4}, k_{5}, k_{S1}, k_{S2} \) 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, \( k_{5} = 580 \text{ s}^{-1}, k_{-5} = k_{2} = 185 \text{ s}^{-1} \) in the presence and \( k_{5} = 1200 \text{ s}^{-1}, k_{-5} = k_{2} = 185 \text{ s}^{-1} \) in the absence of fumarate (19), 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 \( E_{ox} \)-P, \( k_{7} \), is about three times faster than the rate of product release from \( E_{ox} \)-Tyr, \( k_{7} \), 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 \( k_{2} \) and \( k_{7} \) parameters are consistently increased in both AscH₂- and DMPD-mediated reactions.

\[\text{Scheme II A model proposed for the interaction of the reductant and fumarate with DβM.} \]

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 substrates with respect to the reductant and the amine substrate. Therefore, as stated above, the observed apparent negative cooperation 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 \( E_{ox} \)-Tyr (or \( E' \)-Tyr) by efficient competition with the reductant leading to the decrease of the rate of amine dissociation and alteration of the \( k_{a} \) values of active site residues, as previously suggested (19). This possibility is also strongly supported by the observation that fumarate activation of the DβM 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 \( E_{ox} \)-Tyr (or \( E' \)-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...
suggested that fumarate accelerates the product release slightly from both E\text{ox-P} and E_{red-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 DβM 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 AscH_2 for the E_{red} form is much higher in comparison to the E_{ox-P} form, whereas this difference is less contrasting for DMPD as we have predicted. Moreover, the magnitudes of both K_s1 and K_s2 values were increased significantly for both DMPD and AscH_2 in the presence of fumarate especially at pH 5.2 indicating that fumarate competes with the reductant for both E_{ox} and E_{red-P} forms of the enzyme that are as predicted by the proposed model. An estimation of the K_s values of fumarate with respect to the reductant for E_{ox} and E_{ox-P} forms yielded 45–36 and 12–25 mM, respectively, indicating that fumarate interacts with the E_{ox-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 DβM 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 DβM in chromaffin granule ghosts is not directly interacting with the internal AscH_2 and is exclusively reduced from the external AscH_2 probably through the transmembrane electron transport protein cytochrome b_{561} (28–31). However, since cytochrome b_{561} and membrane-bound DβM do not appear to be directly coupled reductively, there may be other redox cofactors and/or proteins involved in the \textit{in vivo} reduction of DβM. Therefore, the anion binding, reduction site of DβM may function as a reduction, recognition, and/or regulation site for the redox cofactors and/or proteins \textit{in vivo}. Complete elucidation of the molecular mechanism of the \textit{in vivo} reduction of both soluble and membrane-bound forms of the enzyme may finally resolve some of these issues.

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

\section*{REFERENCES}


\begin{table}
\centering
\begin{tabular}{|c|c|c|c|c|}
\hline
pH & Parameter & AscH_2 & DMPD & 60ASECH_2 \\
\hline
5.2 & $k_s$ (s^{-1}) & 17.3 ± 0.7 & 10.6 ± 0.4 & 16.5 ± 2.2 & 13.2 ± 2.3 & a & a \\
      & $k_{s1}$ & 40.7 ± 0.9 & 32.9 ± 1.9 & 68.2 ± 4.7 & 37.7 ± 1.2 & a & a \\
      & $K_{s1}$ & 611 ± 236 & 332 ± 52 & 115.9 ± 1.5 & 13.0 ± 1.5 & a & a \\
      & $K_{s2}$ & a & a & 99 ± 16 & 71 ± 15 & a & a \\
6.7 & $k_s$ (s^{-1}) & a & a & 3.3 ± 0.5 & 1.4 ± 0.2 & a & 6.5 ± 0.8 \\
      & $k_{s1}$ & a & a & 14.2 ± 1.6 & 14.5 ± 3.5 & a & 12.7 ± 0.7 \\
      & $K_{s1}$ & a & a & 342 ± 87 & 167 ± 19 & a & 67 ± 35 \\
      & $K_{s2}$ & a & a & b & b & b & b \\
\hline
\end{tabular}
\caption{Kinetic parameters of various reductants}
\end{table}