Characterization of Alternate Reductant Binding and Electron Transfer in the Dopamine β-Monooxygenase Reaction†

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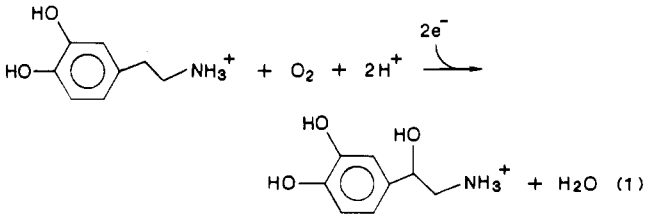
ABSTRACT: The steady-state limiting kinetic parameters V_{max}, V/K_{DA}, and V/K_{O_2}, together with deuterium isotope effects on these parameters, have been determined for the dopamine β-monooxygenase (DβM) reaction in the presence of structurally distinct reductants. The results show the one-electron reductant ferrocyanide to be nearly as kinetically competent as the presumed in vivo reductant ascorbate. Further, a reductant system of ferricyanide plus substrate dopamine yields steady-state kinetic parameters and isotope effects very similar to those measured solely in the presence of ferrocyanide, indicating a role for catecholamine in the rapid recycling of oxidized ferrocyanide. Use of substrate dopamine as the sole reductant is found to lead to a highly unusual kinetic independence of oxygen concentration, as well as significantly reduced values of V_{max} and V/K_{DA}, and we conclude that dopamine reduces enzymic copper in a rate-limiting step that is 40-fold slower than with ascorbate. The near-identical kinetic parameters measured in the presence of either ascorbate or ferrocyanide, together with markedly reduced rates with dopamine, are interpreted in terms of a binding site for reductant that is physically distinct from the substrate binding site. This view is supported by molecular modeling, which reveals ascorbate and ferrocyanide to possess an unexpected similarity in potential sites for interaction with enzymic residues. With regard to electron flux, identical values of V/K_{O_2} have been measured with [2,2-$^2H_2$]dopamine as substrate both in the presence and in the absence of added ascorbate. This key result unambiguously rules out an entry of electrons to enzyme forms leading from the enzyme–dopamine complex to enzyme-bound product and, hence, reaction mechanisms involving a reductive activation of the putative Cu(II)–OOH prior to substrate hydroxylation.

Dopamine β-monooxygenase (DβM)[1] is a copper-containing monooxygenase, catalyzing the introduction of a hydroxyl moiety to the β-carbon of dopamine concomitant with an overall four-electron reduction of oxygen to water:



(1)

As indicated in eq 1, DβM requires an exogenous electron donor such as ascorbate for the full expression of enzyme activity. In vivo, enzyme is observed to be compartmentalized to either chromaffin granules in the adrenal gland or synaptic vesicles in the sympathetic nervous system. This fact, together with the existence of high levels of endogenous ascorbate within adrenal chromaffin vesicles, implicates ascorbate as the physiologic electron donor for DβM (Terland & Flatmark, 1975). Although early investigators (Goldstein et al., 1968) proposed ascorbate to act as a two-electron donor toward enzyme, it has more recently been demonstrated that the enzyme undergoes reduction by two successive one-electron oxidations of ascorbate (Skotland & Ljones, 1980; Diliberto & Allen, 1981). Further support for sequential one-electron reductions of DβM comes from the finding that the one-electron reductant ferrocyanide can support enzymatic hydroxylation of dopamine (Diliberto & Kaufman, 1978; Ljones & Flatmark, 1974; Rosenberg & Lovenberg, 1980).

In spite of the existing evidence for one-electron reductions of DβM, many questions remain concerning both the nature of the reductant binding site and the exact point of entry of electrons in the catalytic cycle. For example, while the involvement of two copper atoms per enzyme subunit in DβM catalysis appears well established (Klinman et al., 1984; Ash et al., 1984), the distance between copper atoms and their precise role in catalysis remain to be elucidated. It is of particular interest whether a single active site is comprised of both copper atoms as opposed to distinct copper centers performing separate functions. Whereas EPR measurments of the resting form of DβM permit the full detection of Cu(II), eliminating significant spin coupling between metal centers (Villafranca, 1981) and suggesting a distance between coppers >6 Å (Klinman et al., 1984), rapid freeze–quench EPR studies indicate a very low level of Cu(II) under steady-state conditions (Skotland et al., 1980; Brenner & Klinman, 1987). Importantly, both rapid acid quench kinetic studies under similar conditions (Murray & Klinman, 1985) and deuterium isotope effects (Ahn & Klinman, 1983; Miller & Klinman, 1985) implicate the enzyme product complex (E·P) as the dominant form of DβM in the steady state. Thus, it has recently been proposed that E·P, rather than free enzyme, undergoes reduction by ascorbate (Brenner & Klinman, 1987).

The above observations suggest a model for DβM that involves separate binding sites for product and reductant and, by extension, the possibility of enzyme ternary complexes

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[1] Abbreviations: DβM, dopamine β-monooxygenase; EPR, electron paramagnetic resonance; DA, dopamine; HPLC, high-performance liquid chromatography.

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involving the simultaneous binding of substrate and reductant. Although an early study indicated ping-pong kinetics for the interaction of dopamine and ascorbate with DβM (Goldstein et al., 1968), it is very difficult to exclude electron-transfer step(s) subsequent to substrate binding from such steady-state patterns. We have, therefore, examined the relationship between structure and reactivity at the reductant site, for comparison with the geometrical constraints placed upon the substrate site by the stereochemistry and mechanism of the hydroxylation reaction itself (Miller & Klinman, 1985). As we show herein, using ascorbate, ferrocyanide, and catecholamines as reductants, the structural requirements at the reductant site provide support for a binding site distinct from substrate. Despite this finding, a comparison of kinetic parameters with slow reductants (catecholamines) vs. ascorbate unambiguously eliminates reaction mechanisms involving the reduction of enzyme forms leading from the binary enzyme–dopamine complex to enzyme-bound product.

Experimental Procedures

Materials. All chemicals were reagent grade unless otherwise noted. Dopamine hydrochloride, tyramine hydrochloride, and disodium fumarate were purchased from Sigma. (*R*)-Norepinephrine hydrochloride and (*R*)-epinephrine (free base) were purchased from Sigma and used without further purification on the basis of HPLC analysis; ascorbic acid was from Gallard-Schlesinger and catalase from Boehringer-Mannheim. Analytical grade cupric sulfate and potassium ferrocyanide were obtained from Mallinckrodt; potassium ferricyanide was from Allied Chemical. [2,2-$^2H_2$]Dopamine hydrochloride was synthesized as previously described (Ahn & Klinman, 1983); soluble dopamine β-monooxygenase was isolated from bovine adrenal glands as described previously (Krueger & Klinman, 1982).

Methods. Absorbance spectroscopy determinations were performed on a Cary 118 UV–visible spectrophotometer; oxygen consumption assays were carried out on a Yellow Springs Instrument Model 53 biological oxygen monitor. A Radiometer pH meter type pH M26 was utilized for determination of pH. Fluorometric assays were performed on a Perkin-Elmer MPF-44A fluorescence spectrophotometer. Computer graphics drawings of enzyme intermediates were generated with the FORTRAN program VIEW developed by Thomas Pollard at the University of California, Berkeley. VIEW, an upgraded version of SPACEFIL (Henry, 1983), featuring a command-style user interface, was run on a LSI-II computer fitted with a Tektronix 4025A display. Initial *xyz* coordinates were obtained graphically from scale models of each reductant.

Measurement of Enzyme Activity. During preparation of enzyme, activity was determined fluorometrically (Von Euler & Floding, 1955). For subsequent kinetic analyses, initial reaction rates were measured as the steady-state rate of oxygen consumption by using a polarographic oxygen electrode. Individual reaction mixtures were made within 15 min of completion of actual rate measurement by mixing appropriate aliquots of stock solutions. Reaction mixtures of a final volume of 1 mL were buffered with 50 mM sodium phosphate, pH 6.0, contained 10 mM disodium fumarate and 100 μg/mL catalase (sp act. = 5×10^4 units/mg), and were adjusted to a final ionic strength of 0.15 M with NaCl. Depending upon desired experimental conditions, reaction mixtures contained as added reductant either 10 mM ascorbate, 10 mM catecholamine, 2 mM potassium ferrocyanide, or a combination of 10 mM catecholamine and 2 mM potassium ferricyanide. Levels of added copper were dependent upon the reductant being used; 1 μM $CuSO_4$ was employed in the presence of ascorbate or catecholamines as reductants. In the case of 2 mM $K_4Fe(CN)_6$ as reductant, copper levels of 4 and 10 μM were examined. In all cases, final concentration of dopamine β-monooxygenase was between 4.95 and 9.9 μg/mL, unless otherwise noted. Initial velocities were all determined at 35 °C by varying both dopamine and oxygen concentrations. Oxygen and nitrogen (Ohio Chemical and Surgical Equipment Co.) were mixed so as to obtain desired oxygen concentrations, as determined by calibration against air-saturated distilled water. Concentrations of dopamine stock solutions were determined spectrophotometrically at 280 nm ($\epsilon = 2.7\ M^{-1}\ cm^{-1}$). Activities were determined by working at a constant oxygen concentration and alternating between protonated and deuteriated dopamine substrates. In cases where tyramine was employed as substrate, activities were measured only at an oxygen concentration of 0.2 mM.

Analysis of Initial Velocity Data. For statistical analysis of our data, the nonlinear least-squares computer program HYPER was employed, which was written by Cleland (1979) and translated into BASIC for use on a Northstar Horizon computer (Ahn & Klinman, 1983). Enzyme activities vs. substrate concentrations (at a fixed concentration of alternate substrate) were fit to the expression

$$v = V_{max(app)}[E_T][S]/K_{m(app)} + [S] \qquad (2)$$

with weighting factors initially set to unity. Limiting kinetic constants were calculated by refitting $V_{max(app)}$ and $V_{max}/K_{m(app)}$ values vs. the alternative substrate concentrations to the same expression, using the weighting factors generated in the first cycle. Final kinetic parameters V_{max} and V/K_m were converted to rate constants of units of s^{-1} and $M^{-1}\ s^{-1}$ by dividing by the enzyme concentration, given a subunit molecular weight of 75000. Deuterium isotope effects were calculated as the ratios of the limiting kinetic parameters for protonated to deuteriated substrates.

Results

Reduction of Enzyme by Catecholamines. The rates of DβM turnover in the presence of dopamine, (*R*)-norepinephrine, or (*R*)-epinephrine as reductant were examined at a single, high catecholamine concentration (10 mM). These experiments utilized the nonoxidizable substrate tyramine (5 mM) and atmospheric oxygen. The net rates with norepinephrine and epinephrine as reductant, after correction for low background rates, were 0.19 ± 0.02 and 0.36 ± 0.03 $\mu mol \cdot min^{-1} \cdot mg^{-1}$, respectively. By contrast, initial velocities in the presence of dopamine were quite high (2.0 $\mu mol \cdot min^{-1} \cdot mg^{-1}$) and practically unchanged upon elimination of tyramine (1.6 $\mu mol \cdot min^{-1} \cdot mg^{-1}$), with the small difference (±tyramine) reflecting similar rates for substrate hydroxylation with either dopamine or tyramine (Miller & Klinman, 1985). From these results it is clear that both (*R*)-norepinephrine and (*R*)-epinephrine are very poor reductants relative to dopamine itself, an unexpected result on the basis solely of reduction potentials [0.46–0.51 V at pH 6 (Sternson et al., 1973)]. It should be noted that our data were collected at a single catecholamine concentration. Since DβM is subject to weak product inhibition, we considered the possibility that the low rates seen with (*R*)-norepinephrine and (*R*)-epinephrine were due to nonsaturating concentrations of these catecholamines. However, an increase in (*R*)-norepinephrine to 40 mM led to no significant increase in rate.

With regard to dopamine, the turnover rate is approximately 20% of the value observed with saturating ascorbate. Thus, the comparative ability of dopamine to serve as both reductant and substrate for DβM was examined in greater detail. Ob-

Table I: Initial Rate Parameters Determined with Ascorbate and Dopamine as Reductants[a]

reductant	$^{H}(V_{max})$ (s^{-1})	$^{D}(V_{max})$ (s^{-1})	$^{H}(V/K_{DA})$ (M^{-1} s^{-1})	$^{D}(V/K_{DA})$ (M^{-1} s^{-1})	$^{H}(V/K_{O_2})$ (M^{-1} s^{-1})	$^{D}(V/K_{O_2})$ (M^{-1} s^{-1})
ascorbate	20.5 ± 0.9	14.9 ± 0.8 [1.38 ± 0.09]	(1.77 ± 0.09) × 10^4	(1.54 ± 0.24) × 10^4 [1.15 ± 0.18]	(2.00 ± 0.02) × 10^5	(6.47 ± 0.33) × 10^4 [3.10 ± 0.33]
dopamine	4.78 ± 0.20	4.38 ± 0.51 [1.09 ± 0.02]	763 ± 50	750 ± 63 [1.01 ± 0.01]		(6.96 ± 1.04) × 10^4

[a] All assay conditions as described under Experimental Procedures. Numbers in brackets represent deuterium isotope effects on initial rate parameters.

Table II: Ferro- and Ferricyanide as Reductant for Dopamine β-Monooxygenase

experimental[a]	V_{max} (s^{-1})	$^{D}(V_{max})$	V/K_{DA} (M^{-1} s^{-1}) (×10^{-4})	$^{D}(V/K_{DA})$	V/K_{O_2} (M^{-1} s^{-1}) (×10^{-5})	$^{D}(V/K_{O_2})$
2 mM $K_4Fe(CN)_6$, 4 μM Cu^{2+}	16.5 ± 0.80	1.01 ± 0.25	1.21 ± 0.16	2.55 ± 0.38	1.05 ± 0.06	5.01 ± 0.32
2 mM $K_4Fe(CN)_6$, 10 μM Cu^{2+}	13.9 ± 0.20	1.06 ± 0.30	1.02 ± 0.10	1.81 ± 0.25	1.59 ± 0.04	5.48 ± 0.25
2 mM $K_3Fe(CN)_6$, 10 μM Cu^{2+}	9.99 ± 0.58	1.15 ± 0.16	1.65 ± 0.25	1.67 ± 0.42	1.79 ± 0.29	5.62 ± 0.82

[a] Assay conditions are described under Experimental Procedures. In assays of $K_3Fe(CN)_6$, dopamine and ferricyanide were incubated for 5 min, prior to addition of enzyme. Thus, a correction of dopamine concentrations due to oxidation by ferricyanide was applied prior to determination of apparent V_{max} and V/K_m parameters. The resulting concentrations were 0.80, 1.77, 3.72, 5.68, and 9.60 mM compared to initial concentrations of 1, 2, 4, 6, and 10 mM, respectively.

served velocities as a function of oxygen are shown in reciprocal form at a range of dopamine concentrations (Figure 1). The data in Figure 1A reveal a *hitherto unobserved property of dopamine hydroxylation—specifically, the absence of a dependence of rate on oxygen tension.* We attribute this phenomenon to a rate-limiting reduction of enzyme by dopamine, such that kinetic terms representing the ternary complex of enzyme–dopamine–O_2 have become relatively insignificant. This view is supported by the normal dependence of initial velocities on oxygen concentration when [2,2-$^2H_2$]dopamine is used as substrate (Figure 1B). Presumably, with the deuteriated substrate the chemical conversion step has been sufficiently reduced such that it has become comparable to the rate of enzyme reduction by dopamine. Previous studies have shown a very large intrinsic isotope effect for DβM, k_H/k_D = 9–11 for the C–H bond cleavage step with dopamine as substrate (Miller & Klinman, 1983).

Limiting parameters of V_{max} and V/K_{DA} and isotope effects on these parameters, $^{D}(V_{max})$ and $^{D}(V/K_{DA})$, have been summarized in Table I. Despite a reduction in rate, the magnitude of isotope effects for dopamine is seen to be similar to that for ascorbate. However, in all cases isotope effects are very small, indicating little or no contribution of substrate hydroxylation to the observed parameters. The near-unity values of $^{D}(V_{max})$ and $^{D}(V/K_{DA})$ for dopamine almost certainly indicate a rate limitation by enzyme reduction, whereas the small values for ascorbate have been previously ascribed to rate limitation by product and substrate release, respectively (Ahn & Klinman, 1983). In regard to V/K_{O_2}, it has only been possible to measure this parameter with deuteriated substrate, precluding a determination of $^{D}(V/K_{O_2})$. Nonetheless, a comparison of V/K_{O_2} values for [2,2-$^2H_2$]dopamine with either ascorbate or dopamine as reductant is possible, indicating essentially identical values. We believe this result has considerable mechanistic significance, as will be described under Discussion.

Enzyme Activity in the Presence of Ferrocyanide and Ferricyanide. As reported by Rosenberg and Lovenberg (1980), high concentrations of ferrocyanide can act in an inhibitory fashion toward DβM, with relief of this inhibition observed upon addition of exogenous copper. Prior to determining limiting kinetic parameters and isotope effects with high (2 mM) ferrocyanide as reductant, enzyme activity was determined as a function of added copper. At the level of 1 μM copper normally used to measure initial velocities with ascorbate as reductant, enzyme exhibits very low activity.

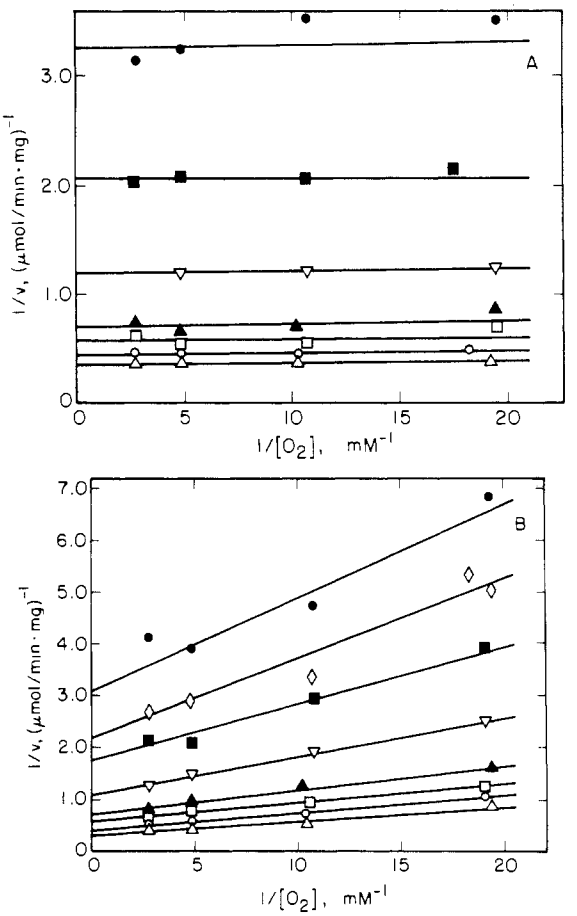


FIGURE 1: Dopamine as substrate and reductant for dopamine β-monooxygenase. All assay conditions as described under Experimental Procedures. Initial velocities were measured at a constant oxygen concentration by varying dopamine concentration. Data plotted as reciprocal velocities vs. reciprocal oxygen concentration for (A) [2,2-$^1H_2$]dopamine and (B) [2,2-$^2H_2$]dopamine as substrate/reductant: (●) 0.5, (◊) 0.75, (■) 1.0, (▽) 2.0, (▲), 4.0, (□) 6.0, (○) 10.0, and (△) 15.0 mM dopamine.

Activity increases dramatically at higher exogenous copper, with maximal activity arising between 10 and 100 μM copper (data not shown). Limiting kinetic parameters were therefore determined at several high copper levels, with the data for 4 and 10 μM copper summarized in Table II. It is noteworthy that limiting parameters at both copper levels are, on the average, 68% of the ascorbate values in Table I. The small

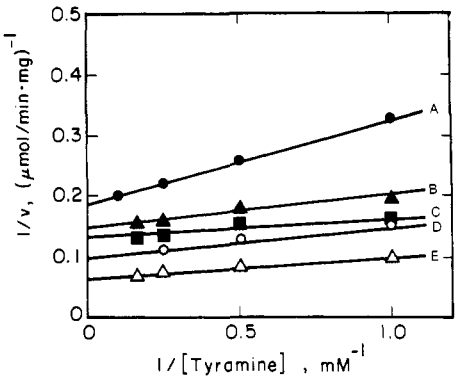


FIGURE 2: Catecholamine reduction of ferricyanide. Velocities measured at a single oxygen concentration of 0.2 mM as a function of substrate tyramine concentration in the presence of (A) (●) 10 mM (*R*)-norepinephrine and 2 mM $Fe(CN)_6^{3-}$; (B) (▲) 10 mM (*R*)-epinephrine and 2 mM $Fe(CN)_6^{3-}$; (C) (■) 10 mM dopamine and 2 mM $Fe(CN)_6^{3-}$; (D) (○) 2 mM $Fe(CN)_6^{4-}$; and (E) (△) 10 mM ascorbate. Reactions containing catecholamine plus $Fe(CN_6)^{3-}$ were preincubated for 5 min, prior to initiation with enzyme.

amount of inhibition seen with ferrocyanide may relate to the high levels of copper employed, since DβM has been observed to contain inhibitory copper site(s), in addition to two activator sites (Klinman et al., 1984).

Comparison of reduction potentials for catecholamines (~ 0.5 V at pH 6; Sternson et al., 1973) vs. that for ferrocyanide (0.36 V; Cotton & Wilkinson, 1972) suggests a possible coupling of dopamine turnover to ferrocyanide oxidation, specifically, a role for dopamine in the rapid cycling of ferricyanide back to its reduced form during the turnover. We therefore examined rates for dopamine hydroxylation in the presence of 2 mM ferricyanide. As summarized in Table II, limiting parameters and their isotope effects are increased, relative to dopamine alone, approaching values seen when the initial reductant is in its reduced form. We conclude that dopamine is capable of a rapid reduction of ferricyanide, producing a concentration of ferrocyanide in excess of its K_m of 0.1 mM.[2] A thermodynamic analysis using the available reduction potentials indicates that at equilibrium the concentration of ferrocyanide should exceed its K_m in the range of initial dopamine concentrations from 1.0 to 10 mM.

The possible interaction of either product (*R*)-norepinephrine or (*R*)-epinephrine with ferricyanide was also examined by using variable concentrations of tyramine at a single concentration of O_2 (Figure 2). As indicated earlier, attempts to employ either (*R*)-norepinephrine or (*R*)-epinephrine as sole reductants resulted in very low rates. When 2 mM ferricyanide was added, however, there was a marked increase in measured velocities, with (*R*)-epinephrine again found to be a more efficient reductant than (*R*)-norepinephrine. In the situation where dopamine and ferricyanide are present in the medium (Figure 2C) the rates closely approach those measured in the presence of ferrocyanide (Figure 2D). The reduced intercepts seen in Figure 2A–C, relative to Figure 2D, almost certainly reflect some competitive binding between the relatively inefficient catecholamine reductants and ferrocyanide at the reductant site. Although epinephrine and norepinephrine are seen to give lower limiting rates than dopamine, they are also believed to bind less effectively. Thus, the intrinsic rates at which the different catecholamines reduce ferricyanide may also contribute to the final velocities summarized in Figure 2.

Discussion

Nature of Reductant Binding Site. Although the ability of ferrocyanide to support DβM turnover has been studied, former investigations have been largely limited to single concentrations of substrate and oxygen (Rosenberg et al., 1980). In the present study, V_{max}, V/K_m, and isotope effects on these parameters were obtained by extrapolation to infinite concentrations of dopamine and oxygen. While enzyme activity at 1 μM Cu(II) is very low, an increase in exogenous copper to >4 μM leads to rates which are 68% of ascorbate (Tables I and II). As noted, the average 32% reduction of *all* rate parameters with ferrocyanide is ascribed to the higher levels of copper required in these assays, conditions leading to inhibitory binding of copper with ascorbate (Klinman et al., 1984).

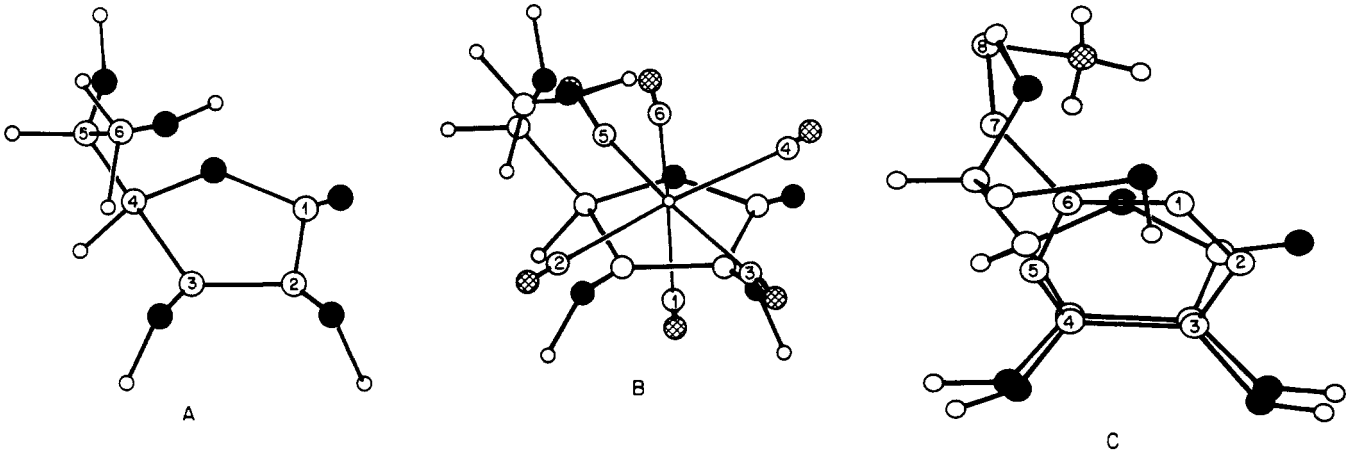
An examination of isotope effects indicates some differences between ascorbate and ferrocyanide, specifically, a small decrease in $^D(V_{max})$, together with significant increases in $^D(V/K_{DA})$ and $^D(V/K_{O_2})$. We attribute the former effect to experimental error, while the latter effects are reproducible. Some insight into the origin of increased isotope effects comes from earlier studies with ascorbate where, in contrast to catalytic copper (Klinman et al., 1984), inhibitory copper altered the magnitude of tritium isotope effects (Krueger and Klinman, unpublished results). Thus, it is possible that the trends in $^D(V/K_m)$ in Table II are the result of the presence of inhibitory copper. In any case, the larger $^D(V/K_m)$ effects with ferrocyanide indicate that the C–H bond cleavage step is *at least* as rate limiting with ferrocyanide as reductant. Overall, the data in Tables I and II support a sufficiently rapid reduction of enzyme such that this step does not limit turnover with ferrocyanide. While the direct reduction of DβM by ferrocyanide has not been studied, the data reported herein imply that this process occurs at least fivefold faster than product release, i.e., >60 s^{-1}. Freeze–quench studies of the reduction of enzyme-bound copper by ascorbate indicate a rapid process with k = 185 s^{-1} (Brenner & Klinman, 1987).

Turning to catecholamines as reductant, these have been assayed either alone or in the presence of ferricyanide. As shown in Table II and Figure 2, the combination of ferricyanide plus catecholamine approximates ferrocyanide. Among the catecholamines themselves, (*R*)-norepinephrine and (*R*)-epinephrine are very ineffective relative to dopamine itself. Since reduction potentials are similar, it appears that norepinephrine and epinephrine encounter steric hindrance at the reductant site.

A full analysis of enzyme turnover in the presence of dopamine as reductant has been carried out (Figure 1 and Table I). The absence of a dependence on oxygen concentration (Figure 1A) is of considerable mechanistic significance, indicating a marked decreased in the rate of enzyme reduction, such that this step has become slow relative to the subsequent binding of oxygen and hydroxylation of substrate. This lack of oxygen dependence allows us to use the measured V/K_{DA} parameter as an estimate of the second-order rate constant for dopamine reduction of enzyme. The measured V/K_{DA} of 763 s^{-1} represents a *40-fold decrease* from the values of 3.2 × 10^4 M^{-1} s^{-1} measured in the presence of ascorbate as reductant (Brenner & Klinman, 1987). An estimate of the first-order rate constant for enzyme reduction by dopamine can also be obtained from the measured V_{max} values of 4.4–4.8 (Table I). The reduction in these parameters relative to ascorbate indicates the presence of an additional step (enzyme

[2] Whereas the data in Table II involved 5-min preincubations between dopamine and ferrocyanide prior to addition of enzyme, separate kinetic experiments with (0.25–1 mM) dopamine and (1 mM) ferricyanide indicated an instantaneous reduction of ferri- to ferrocyanide.

Chart I: Structural Similarities between Ascorbate, Ferrocyanide, and Dopamine as Reductants for Dopamine β-Monooxygenase[a]



[a] All atom types are coded as follows: ○, carbon; ●, oxygen; cross-hatched circles, nitrogen; small open circles, hydrogen atoms. Although not pictured, numbering scheme for carbon skeleton of ascorbate in panels B and C remains identical with that of panel A. Note that oxygen and nitrogen lone electron pairs have been omitted from all models. Panel A: Molecular structure of ascorbate. Panel B: Superposition of ferrocyanide upon the ascorbate structure of panel A. Although the nitrogen atom of cyano group 5 is partially obscured by the C-6 oxygen of ascorbate, its proximity in space to the C-5 oxygen of ascorbate can be visualized. The proximity of N-6 of ferrocyanide to the O-6 of ascorbate involves overlap of lone pairs of electrons (not shown). Panel C: Superposition of dopamine upon the ascorbate structure of panel A. C–H bonds have been omitted from the dopamine structure (as well as those to C-6 of ascorbate), and the numbering scheme refers to the carbon skeleton of dopamine. Note the proximity in space of the protonated amino group of dopamine to the C-6 hydroxyl moiety of ascorbate.

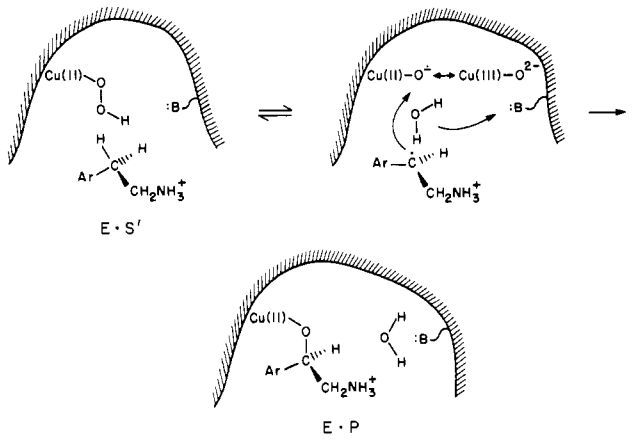
reduction). Employing values of 580 s^{-1} and 13 s^{-1} (Ahn & Klinman, 1983) for k_5 and k_7, respectively, and eq 4 of Scheme II, we estimate $k_R = 4.5$ s^{-1} for dopamine reduction of enzyme. Once again this represents a 40-fold decrease relative to the first-order rate constant of 185 s^{-1} for enzyme reduction by ascorbate (Brenner & Klinman, 1987).

In attempting to provide a basis for the relative rates of reduction of enzyme-bound copper by ascorbate, ferrocyanide, and catecholamines, two major factors have been considered. First, the rate of enzyme reduction will reflect, in part, the magnitude of reduction potentials. Several studies of DβM containing a single copper per subunit have provided reduction potentials of 0.31 (Walker et al., 1977) to 0.37 V (Ljones et al., 1978) at pH 7. Although no data are available regarding potentials for reductants bound to DβM, fairly extensive studies of free species provide a frame of reference (Sternson et al., 1973; Dryhurst et al., 1982). Inspection of these values indicates similar one-electron potentials for ascorbate [0.42 V, pH 6 (Sternson et al., 1973)] and ferrocyanide [0.36 V (Cotton & Wilkinson, 1972)], consistent with similar rates for reduction of enzyme-bound copper. Turning to dopamine, only a single (two-electron) potential has been reported (Sternson et al., 1973). The absence of two waves in cyclic voltammetry reflects either similar values of ~0.45 V for each of the one-electron oxidation steps leading from oxidized to fully reduced dopamine or a significantly larger thermodynamic driving force for the second electron transfer. The latter situation would imply a potential ≫0.45 V for the reverse, one-electron oxidation of dopamine, whereas the data in Table II and Figure 2 show similar reaction kinetics for dopamine hydroxylation beginning with either ferricyanide or ferrocyanide as reductant. This result implicates a rapid reduction of ferricyanide by dopamine and, hence, a possible similarity in one-electron potentials.

We have, therefore, investigated the nature of possible binding interactions at the DβM reductant binding site. Scale models of ascorbate and ferrocyanide indicate them to be of similar size on the basis of van der Waals atomic radii. Given the low first pK_a for ascorbic acid ($pK_a \sim 4.14$; Dryhurst, 1982), ascorbate almost certainly binds to the enzyme as the negatively charged monoanion and is proposed to interact with copper through the (ionized) 3-hydroxyl and 2-hydroxyl functionalities. The structure of ascorbate is shown in Chart I-A. We note the presence of additional hydroxyl groups at the 5- and 6-positions of the side chain, together with the carbonyl group at the 1-position, as potential sites for hydrogen-bonding interactions. As shown in Chart I-B, the relatively short iron–copper bond distances (ca. 1.91–1.93 Å; Sharpe, 1976) of the ferrocyanide molecule should facilitate the interaction of the π electrons of the 2- and 3-cyano groups with enzymic copper, analogous to the proposed chelation of copper by ascorbate. Additionally, three of the four remaining cyano groups of ferrocyanide are found to be correctly aligned for interactions with active site residues, as described for ascorbate. Since ferrocyanide can only serve as a hydrogen bond acceptor, it appears likely that the 5- and 6-position functional groups of both ascorbate and ferrocyanide function in this manner. Additionally, the nearly identical alignment of the 1-carbonyl moiety of ascorbate and the 4-cyano group of ferrocyanide suggests a fifth site of interaction, which may involve hydrogen bonding or possibly an electrostatic interaction. Thus, the similarity of ferrocyanide and ascorbate as reductants toward DβM is supported by both their reduction potentials and an unexpected, but remarkably close, similarity in potential binding interactions.

Turning to dopamine as reductant, precise correlations are hampered by the absence of one-electron reduction potentials in the literature (vide supra). As noted, however, the facile reduction of ferri- to ferrocyanide by dopamine may indicate similar potentials (Table II and Figure 2). We have therefore examined binding interactions in a manner analogous to ferrocyanide and ascorbate. Although dopamine will fit reasonably well within a pocket defined by ascorbate and ferrocyanide (Chart I-C), a number of key differences emerge. First, in contrast to ascorbate and ferrocyanide which are expected to bind as negatively charged species, the high pK_a values for both the protonated amino group and ring hydroxyl (Kappe & Armstrong, 1965) suggest that dopamine will bind either as a positively charged or ring-ionized, neutral species. Additionally, there is no possibility for the three hydrogen-bonding interactions proposed for ferrocyanide and ascorbate. Superposition of the five- and six-membered rings of ascorbate

Scheme I: Mechanistic Model of Dopamine β-Monooxygenase Active Site



and dopamine leads to different orientations for the ethylene glycol and ethylamine side chains, respectively, which may explain the very poor ability of (*R*)-norepinephrine to serve as reductant. Overall, a relative paucity of binding interactions between dopamine and enzyme, together with the charge repulsion generated upon oxidation of dopamine to a radical cation, is expected to lead to a greatly reduced rate for enzyme reduction.

The above considerations provide a simple and consistent rationalization for the relative efficiencies of ascorbate, ferrocyanide, and dopamine, as well as a working model for the properties of the reductant binding site of DβM. A surprising result is a pattern of specificity at the reductant site that differs markedly from that proposed for the active site copper. Recent investigations of structure reactivity correlations and isotope effects in the DβM reaction have led to a working model for the chemical details surrounding dopamine hydroxylation (Miller & Klinman, 1985). Incorporation of these results with previous studies demonstrating the requirement of a proton in catalysis prior to substrate activation (Ahn & Klinman, 1983) has led to the mechanism in Scheme I. Briefly, several points are noted. First, both electrons enter the catalytic cycle prior to substrate binding, leading to formation of a Cu(I-I)–OOH activated species. Homolysis of the peroxy bond is then predicted to proceed concomitant with an effective oxidation of the copper atom to yield a Cu(III)–O^{2-} as a short-lived intermediate. Although this type of copper redox chemistry is not expected to be thermodynamically favorable a priori (Miller & Klinman, 1985; Kust, 1979), the generation of Cu(III)–O^{2-} is postulated to be linked to the large energy release upon transfer of hydrogen from the carbon center of substrate to the oxygen of product water. Overall, the features of dopamine hydroxylation appear to differ markedly from those of reductant oxidation. Given the available data, we propose separate binding sites for reductants vs. substrates in DβM. This important conclusion is consistent with freeze–quench EPR studies, which indicate a very low level of E–Cu(II) in the steady state (Skotland et al., 1980; Brenner & Klinman, 1987), in contrast to pre-steady-state and steady-state studies of product formation, which indicate that the dominant enzyme form in the steady state is the E·P complex (Ahn & Klinman, 1983; Miller & Klinman, 1985; Murray & Klinman, 1985). It appears that reductants are capable of binding to E·P, reducing E–Cu(II) to E–Cu(I) on a time scale that is rapid relative to product release (Brenner & Klinman, 1987). Thus, while further studies are needed to establish the precise distance between copper centers, the available data on DβM argue against a *single binuclear copper binding center* performing the dual functions of electron transfer and substrate activation.

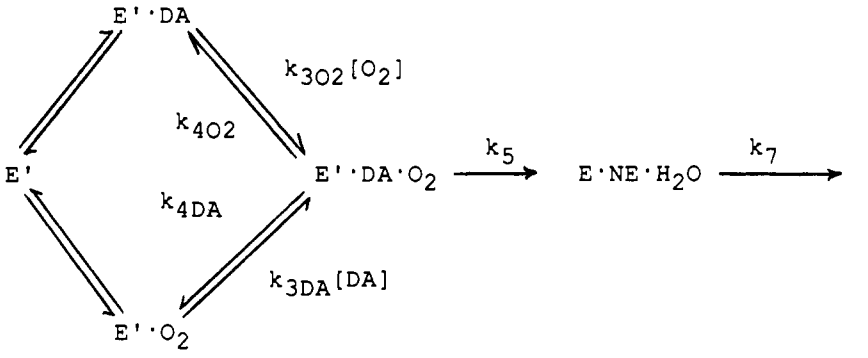
Active Site Electron Requirements. The use of alternate electron donors in the DβM reaction can provide insight into the electron requirements of the hydroxylation reaction itself. Previous investigations (Klinman et al., 1980) of the dependence of tritium isotope effects on oxygen concentration have demonstrated a kinetic scheme involving random substrate binding to DβM (Scheme II-A). Formation of the ternary reduced enzyme–substrate complex is followed by an irreversible chemical step (k_5), leading to a ternary oxidized enzyme–product complex, which undergoes reduction and product release to reinitiate the catalytic cycle. A notable feature of Schemes I and II-A is that enzyme is fully reduced prior to substrate binding, i.e., no further steps in the mechanism involve electron uptake. While this view is supported by steady-state kinetics, which indicate parallel lines in reciprocal plots of velocity vs. substrate concentration at varying ascorbate levels (Goldstein et al., 1968), it is extremely difficult to rule out quaternary complexes of substrate, oxygen, and reductant from such steady-state patterns (Segel, 1975). Certainly, the proposed existence of multiple binding sites for reductant and substrate/product raises the question of electron flux from ascorbate in the presence of substrate and oxygen. For example, as an alternative to the Cu(III)–O^{2-} intermediate in Scheme I, a further one-electron reduction of the E·S′ complex would lead to the formation of Cu(I)–OOH as the hydroxylating species. By analogy to Fenton's type chemistry (Groves, 1980), Cu(I)–OOH should be a *much* more active hydroxylating species than the analogous Cu(II) complex, generating Cu(II)–OH and OH• as the hydroxylating agent.[3] Although such a reaction has been considered unlikely on the basis of the magnitude of primary and secondary isotope effects (Miller & Klinman, 1985), direct information on this step would be of great value.

Attempts to discern whether an electron may be entering the catalytic cycle at the hydroxylation step (k_5) have involved the determination of limiting kinetic parameters with alternate reductants. As summarized in Tables I and II and discussed above, ferrocyanide and ascorbate appear similar in overall properties and will be satisfied by Scheme II-A. By contrast, utilizing dopamine as both reductant and substrate leads to an alteration in mechanism, such that enzyme reduction contributes to V_{max} and V/K_{DA} (Scheme II-B). As shown, the minimal mechanism of Scheme II-A has been expanded to include the reductant binding and oxidation step with the result that kinetic expressions for V_{max} and V/K_{DA} are altered while V/K_{O_2} is unchanged. From previous work by Ahn and Klinman (1983) showing large, invariant deuterium isotope effects on V/K_{O_2} and hence a sizable contribution of k_5 to this parameter, it is possible to use V/K_{O_2} as a monitor for changes in k_5. Specifically, a contribution of electron transfer to k_5 is predicted to generate a measureable decrease in V/K_{O_2} with kinetically poor reductants vs. ascorbate. Although a comparison of V/K_{O_2} values for ascorbate vs. dopamine as reductant has not been possible with [2,2-$^1H_2$]dopamine (Figure

[3] In principle, such a mechanism could be detected by a comparison of electron equivalents required under conditions of the approach to the steady state vs. the steady state. Starting with fully oxidized enzyme, three electron equivalents would be required to achieve steady-state conditions [involving reduction of both Cu(II) centers, followed by Cu(II)–OOH reduction]. Once in the steady state, however, the correct stoichiometry of two electrons per mole of product would be achieved, equvalent to a reduction of one copper center at the level of Cu(II)–OOH followed by a second (one-electron) reduction subsequent to product formation.

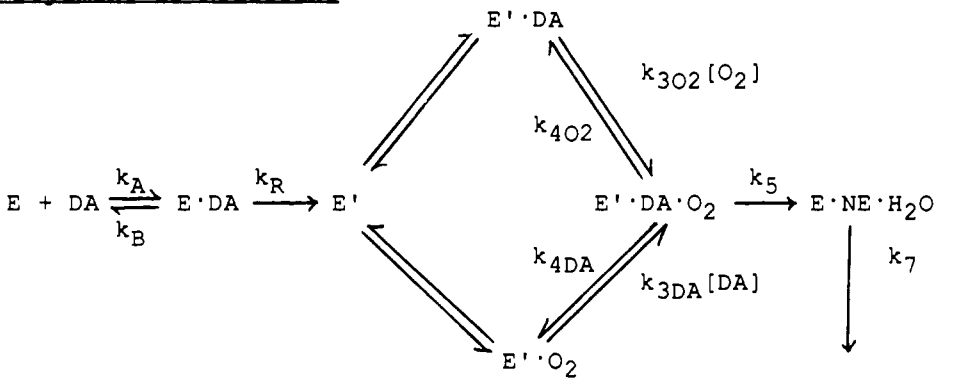
Scheme II: Kinetic Mechanisms and Parameters for DβM Turnover in the Presence of Ascorbate (A) or Dopamine (B) as Reductants[a]

A. Ascorbate as Reductant



$$(1)\ V_{max} = \frac{k_5k_7}{k_5+k_7};\ (2)\ V/K_{DA} = \frac{k_{3DA}k_5}{k_{4DA}+k_5};\ (3)\ V/K_{O2} = \frac{k_{3O2}k_5}{k_{4O2}+k_5}$$

B. Dopamine as Reductant



$$(4)\ V_{max} = \frac{k_Rk_5k_7}{k_Rk_7+k_Rk_5+k_5k_7};$$

$$(5)\ V/K_{O2} = \frac{k_{3O2}k_5}{k_{4O2}+k_5};\qquad (6)\ V/K_{DA} = \frac{k_Ak_Rk_{3DA}k_5}{k_Ak_R(k_{4DA}+k_5)+k_{3DA}k_5(k_R+k_B)}$$

[a] According to this scheme, E and E′ represent oxidized and reduced enzyme forms, respectively. V_{max} and V/K_m expressions reflect rate constants with units of s^{-1} and $M^{-1}\ s^{-1}$, respectively.

1A), a dependence of rate on oxygen concentration is observed with [2,2-$^2H_2$]dopamine, allowing direct comparison of parameters (Table II). As seen, the magnitude of V/K_{O_2} in the presence of [2,2-$^2H_2$]dopamine is observed to be *independent of reductant*. This result provides strong evidence against any requirement for electron transfer subsequent to formation of the reduced enzyme–dopamine binary complex. We conclude that the mechanism in Scheme I is correct, insofar as enzyme reduction is complete prior to oxygen binding and activation.

In conclusion, the data presented herein have expanded our understanding of the DβM mechanism in several areas. The finding that the one-electron reductant ferrocyanide can function nearly as well kinetically as the optimal reductant ascorbate is consistent with both its reduction potential and an unexpected, but striking, similarity to ascorbate with regard to postulated binding interactions. This similarity, together with the contrasting slow reduction of enzyme by dopamine, lends supports to a model in which the reductant binding site is physically distinct from the site leading to substrate activation. Despite the postulate of separate binding sites, examination of kinetic parameters with an efficient (ascorbate) vs. poor (dopamine) reductant permits us to rule out any further, reductive activation of the putative E–Cu(II)–OOH species, consistent with the direct insertion mechanism illustrated in Scheme I.

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Registry No. DβM, 9013-38-1; DA, 51-61-6; O_2, 7782-44-7; ascorbate, 50-81-7; ferrocyanide, 13408-63-4; (*R*)-norepinephrine, 51-41-2; (*R*)-epinephrine, 51-43-4; deuterium, 7782-39-0.

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