Magnitude of Intrinsic Isotope Effects in the Dopamine β-Monooxygenase Reaction†

Susan M. Miller and Judith P. Klinman*

ABSTRACT: Intrinsic primary hydrogen isotope effects (k_H/k_D) have been obtained for the carbon–hydrogen bond cleavage step catalyzed by dopamine β-monooxygenase. Irreversibility of this step is inferred from the failure to observe back-exchange of tritium from TOH into substrate under conditions of dopamine turnover; this result cannot be due to solvent inaccessibility at the enzyme active site, since we will demonstrate [Ahn, N., & Klinman, J. P. (1983) *Biochemistry* (following paper in this issue)] that a solvent-derived proton or triton must be at the enzyme active site prior to substrate activation. As shown by Northrop [Northrop, D. B. (1975) *Biochemistry 14*, 2644], for enzymatic reactions in which the carbon–hydrogen bond cleavage step is irreversible, comparison of $^D(V/K)$ to $^T(V/K)$ allows an explicit solution for k_H/k_D. Employing a double-label tracer method, we have been able to measure deuterium isotope effects on V_{max}/K_m with high precision, $^D(V/K) = 2.756 \pm 0.054$ at pH 6.0. The magnitude of the tritium isotope effect under comparable experimental conditions is $^T(V/K) = 6.079 \pm 0.220$, yielding $k_H/k_D = 9.4 \pm 1.3$. This result was obtained in the presence of saturating concentrations of the anion activator fumarate. Elimination of fumarate from the reaction mixture leads to high observed values for isotope effects on V_{max}/K_m, together with an essentially invariant value for $k_H/k_D = 10.9 \pm 1.9$. Thus, the large disparity between isotope effects, plus or minus fumarate, cannot be accounted for by a change in k_H/k_D, and we conclude a role for fumarate in the modulation of the partitioning of enzyme–substrate complex between catalysis and substrate dissociation. On the basis of literature correlations of primary hydrogen isotope effects and the thermodynamic properties of hydrogen transfer reactions, the very large magnitude of $k_H/k_D = 9.4$–10.9 for dopamine β-monooxygenase suggests an equilibrium constant not very far from unity for the carbon–hydrogen bond cleavage step. This feature, together with the failure to observe re-formation of dopamine from enzyme-bound intermediate or product and overall rate limitation of enzyme turnover by product release, leads us to propose a stepwise mechanism for norepinephrine formation from dopamine in which carbon–hydrogen bond cleavage is uncoupled from the oxygen insertion step.

Kinetic isotope effects, which have been used as probes of reaction mechanisms in solution for many years, recently have emerged as powerful tools for the investigation of enzymatic reactions. While the magnitude of isotope effects can be quite large, in kinetically complex enzyme-catalyzed reactions observed values frequently are diminished due to the kinetic dominance of isotope-insensitive steps. Consequently, measurement of the intrinsic isotope effect on the bond cleavage step is essential for accurate interpretation of the observed isotope effects in enzyme-catalyzed reactions. Comparison of observed effects to the intrinsic isotope effect can then provide detailed information regarding both kinetic and chemical mechanisms.

Existing experimental approaches to the evaluation of intrinsic effects include, for the general case, (1) variation of the enzymatic assay conditions in an effort to render the isotope-sensitive step rate limiting [e.g., Klinman (1972)], (2) the simultaneous measurement of more than one isotope effect (Hermes et al., 1982), and specifically for hydrogen abstraction reactions, (3) a comparison of the deuterium and tritium isotope effects on V_{max}/K_m (Northrop, 1975). The first method can afford ambiguous results since it is frequently unclear when the intrinsic effect is fully expressed. While there are limitations, the second method has the potential for providing unique values for intrinsic isotope effects and should be most useful when heavy-atom isotope effects are desired. As described by Northrop (1975), the third method provides a range of values for the intrinsic hydrogen isotope effect when the interconversion of enzyme-bound substrate(s) and products is rapid and reversible. By contrast, for enzymatic reactions characterized by an essentially irreversible chemical step, this method is capable of yielding a unique solution for the intrinsic isotope effect.

Monooxygenases catalyze a transfer of hydrogen from substrate to water, coupled to a splitting of dioxygen to hydroxylated product and water (eq 1). The expectation of large

$$RH + O_2 \xrightarrow[2H^+]{2e} ROH + H_2O \qquad (1)$$

primary isotope effects for the hydrogen abstraction step targets these enzymes for mechanistic isotope-effect investigations. Furthermore, the overall irreversibility of these reactions in solution suggests that intrinsic isotope effects may be readily obtained. In this paper, evidence is given for an irreversible hydrogen abstraction step in the reaction catalyzed by dopamine β-monooxygenase. This observation, together with the development of a double-label technique for the precise measurement of deuterium isotope effects on V_{max}/K_m, has allowed us to estimate the intrinsic isotope effect with a high degree of accuracy.

Previous investigations with dopamine β-monooxygenase have shown variations in observed isotope effects with changes in reaction conditions; in particular, significant reductions in both deuterium (Bachan et al., 1974) and tritium (Klinman et al., 1977) isotope effects have been reported in the presence of the anion effector fumarate. As reported herein, the magnitude of the intrinsic isotope effect in the absence vs. presence of fumarate is essentially unchanged. This result provides a key reference point for further consideration of the mechanism of fumarate activation.

Experimental Procedures

All chemicals were reagent grade unless otherwise noted. Disodium fumarate, catalase, α-chymotrypsin, dopamine hy-

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drochloride, norepinephrine hydrochloride, and 2-mercaptoethanol were from Sigma. Ascorbic acid was from BDH Chemicals, Ltd. DE-52 ion-exchange resin was from Whatman. Concanavalin A–Sepharose and Sephadex G-25 were from Pharmacia. Methyl mannoside was purchased from Calbiochem. Ag 50W-X8 ion-exchange resin was from Bio-Rad. Tritiated water (sp act. 18 mCi/mmol), [2-^{3}H]dopamine (sp act. 40–60 mCi/mmol), and Liquiflor PPO/POPOP[1] toluene concentrate were from New England Nuclear. Radioactivity was determined on a Beckman LS-8000 Liquid scintillation spectrometer with a toluene-diluted Liquiflor/95% ethanol mixture. A radiometer pH meter, type PHM 26, was used for pH determinations. Absorbance measurements were made on a Cary 118B, fluorescence assays on a Perkin-Elmer MPF-44A fluorescence spectrometer, and oxygen uptake assays on a Yellow Springs Instrument polarographic oxygen electrode, Model 53. High-pressure liquid chromatography (HPLC) was performed on a Beckman Model 332 gradient liquid chromatographic system equipped with a Model 155 variable-wavelength detector. Purification of dopamine and norepinephrine by HPLC was accomplished by using either an Altex C-18 Ultrasphere column with a mobile phase of 15% methanol/85–1% acetic acid, v/v (condition A), or a Waters C-18 reverse-phase column with a mobile phase of 0.1% acetic acid (condition B). Mass spectral analyses were obtained on an A.E.I. Model 12 mass spectrometer. Dopamine β-monooxygenase was prepared from bovine chromaffin granules through a series of concanavalin A, Sephadex G-25, and DE-52 anion-exchange columns as previously described (Klinman & Krueger, 1982). Enzyme activity was assayed by measuring the rate of oxygen uptake or norepinephrine production as previously described (Klinman et al., 1980).

Synthesis of [2(R)-^{2}H]Dopamine. 2-(3,4-Dimethoxyphenyl)-1,3-[2-^{2}H]dithiane was synthesized by the method of Seebach et al. (1966), followed by oxidation with ceric ammonium nitrate (Ho et al., 1972) to yield 3,4-dimethoxy-[*formyl*-^{2}H]benzaldehyde. Condensation of the aldehyde with *N*-acetylglycine (Niederl & Ziering, 1942) yielded 2-methyl-4-(3,4-dimethoxydeuteriobenzal)-5-oxazolone, which, when treated with sodium methoxide in dry methanol (distilled from magnesium), yielded methyl α-acetamido-3,4-dimethoxy-*trans*-[β-^{2}H]cinnamate. Hydrogenation of the cinnamate with 10% Pd/C in dry methanol yielded the racemic mixture of (2*S*,3*R*)- and (2*R*,3*S*)-*N*-acetyl-3-(3,4-dimethoxyphenyl)[3-^{2}H]alanine methyl ester. Resolution of the esters was afforded by α-chymotrypsin hydrolysis of the desired 2*S*,3*R* enantiomer to the acid (Clement & Potter, 1971). After removal of protein on a Sephadex G-25 column, the ester was extracted into chloroform and the aqueous layer evaporated, leaving pure *N*-acetyl-3-(3,4-dimethoxyphenyl)alanine. The free amino acid was obtained by refluxing in 2 N HCl for 10–12 h. Decarboxylation, catalyzed by acetophenone (Chatelus, 1964; Al-Sayyab & Lawson, 1968) afforded 2-(3,4-dimethoxyphenyl)[2(*R*)-^{2}H]ethylamine, which was then demethylated by refluxing with constant-boiling HBr. Final recrystallization, first from concentrated HCl and second from methanol/ether, yielded [2(*R*)-^{2}H]dopamine hydrochloride. The stereochemistry of the deuterated dopamine was checked by 100% conversion of an aliquot with dopamine β-monooxygenase to give norepinephrine. After purification by HPLC (condition A), norepinephrine was derivatized with pentafluoropropionic anhydride (Karoum et al., 1972) and subjected to mass spectral analysis in which the molecular ions M^+, $M^+ + 1$, and $M^+ + 2$ were monitored. A 5.7% excess above natural abundance detected in $M^+ + 1$ and $M^+ + 2$ corresponds to [2(*S*)-^{2}H]dopamine in [2(*R*)-^{2}H]dopamine.

Ring Tritiation of Dopamine. Separate samples of [2-^{1}H]dopamine and [2(*R*)-^{2}H]dopamine were tritiated in the aryl ring by heating at 60 °C for 12–24 h with a mixture of 1 N HCl and 1 N HOAc in 1 Ci/mL of tritiated water (generated by addition of acetyl chloride to TOH). Similar treatment in D_2O showed that all three ring protons are exchangeable while the side-chain protons remain intact.

Isotope-Effect Measurements. The precise reaction conditions for each isotope-effect measurement are contained in the appropriate table legend. The measurement and calculation of tritium isotope effects were carried out as previously described (Klinman et al., 1980). For the measurement of deuterium isotope effects, [*aryl*-^{3}H,2(*R*)-^{2}H]dopamine hydrochloride (sp act. 6.3×10^6 cpm/μmol) and [1-^{14}C]dopamine hydrochloride (sp act. 4.4×10^5 cpm/μmol) were purified separately by HPLC and then mixed in a 1:1 mole ratio to give a final dopamine concentration of 1 mM. At the appropriate time points, 0.15-mL aliquots of reaction mixtures were quenched by addition to 0.09 mL of 0.1 N $HClO_4$ for reactions with fumarate or to 0.09 mL of 16.7 mM fumarate in 0.1 N $HClO_4$ for reactions without fumarate. Quenched samples from reaction mixtures were filtered on Amicon centriflo membrane cones 224-CF-50 to remove protein and were stored frozen. Samples were removed individually for purification of norepinephrine by HPLC (condition B). After solvent evaporation, the purified norepinephrine peak was redissolved in 1 mM HCl and its ^{14}C and ^{3}H content determined. ^{14}C/^{3}H ratios were calculated following correction of the tritium counts for the presence of 5.7% [*aryl*-^{3}H,2(*S*)-^{2}H]dopamine in [*aryl*-^{3}H,2(*R*)-^{2}H]dopamine (cf. Synthesis of [2(*R*)-^{2}H]Dopamine). Since under the reported experimental conditions the observed primary isotope effect is low relative to k_H/k_D, the contribution of secondary isotope effects to this correction is negligible; thus tritiated (*S*)-[^{2}H]dopamine and norepinephrine can be assumed to appear with ^{14}C-labeled substrate and product in a constant ratio, $(^{14}C/^3H)_H$, described by

$$(^{14}C/^3H)_H = \frac{n_H SA_H}{n_D f_s SA_D} \quad (2)$$

where n_H and n_D are the initial micromoles of protonated and deuterated substrate, f_s is the fraction of (*S*)-[^{2}H]dopamine in the deuterated dopamine sample, and SA_H and SA_D are the specific activities of original ^{14}C protonated substrate and tritiated deuterated substrate expressed in counts per minute per micromole. The corrected ^{14}C/^{3}H ratios for each sample are easily calculated according to eq 3. Isotope effects are

$$(^{14}C/^3H)_{corr} = \frac{\text{cpm } ^{14}C}{\text{cpm } ^3H - [\text{cpm } ^{14}C/(^{14}C/^3H)_H]} \quad (3)$$

calculated by comparison of the corrected ^{14}C/^{3}H ratios for product to starting material.

A control for the presence of a secondary isotope effect arising from ring tritiation of dopamine was performed by incubating a mixture of [*aryl*-^{3}H]dopamine and [1-^{14}C]dopamine (initial ^{14}C/^{3}H = 0.09522) under conditions identical with those of Table II. In this case, the isotope effect was calculated from comparison of the ^{14}C/^{3}H ratios for purified norepinephrine at fractional vs. complete conversion.

Results and Discussion

Assay for Reversibility. The expression describing the relationship of deuterium and tritium isotope effects on

[1] Abbreviations: PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis(5-phenyloxazol-2-yl)benzene.

Table I: Assay for Reversibility of Enzyme-Bound Norepinephrine to Dopamine in the Dopamine β-Monooxygenase Reaction

assay mixtures[a]	sp act. of dopamine (cpm/μmol)[b]	% exchange
complete	5.0×10^3	0.05
−fumarate	5.1×10^3[c]	0.05
−dopamine β-monooxygenase	4.6×10^3	0.05

[a] Assay mixtures contained in 0.5 mL of 1 Ci/mL TOH 100 mM potassium phosphate, pH 6.0, 10 mM ascorbate, 10 μg/mL catalase, 10 mM dopamine, 17 mM KCl, and ±12.5 μg of dopamine β-monooxygenase, ± 10 mM fumarate. Incubations were at 35 °C for 2.5 h. [b] Dopamine was purified by cation-exchange chromatography on Ag 50W-X8 (Bio-Rad) with a linear gradient of 0.3–2 N HCl. The peak fractions were combined, the solvent was evaporated, and the residue was dissolved in a known volume of 1 M potassium phosphate buffer, pH 6.0. Specific activity is based on the absorbance at 280 nm with $\epsilon_{DM} = 2.7\ mM^{-1}\ cm^{-1}$. [c] The isolated [^{3}H]dopamine was incubated with fresh dopamine β-monooxygenase in a mixture containing 100 mM potassium phosphate, pH 6.0, 10 mM fumarate, 10 mM ascorbate, 17 mM KCl, 10 μg/mL catalase, 12.5 μg of dopamine β-monooxygenase, and 0.2 mM [^{3}H]-dopamine (700 cpm) in 0.5-mL total volume. After 100% conversion, 0.2 mL of reaction was lyophilized, and both the water and residues were counted. Of 265 cpm expected, 250 cpm remained in the residue with only ca. 5 ppm in the water.

V_{max}/K_m to the intrinsic isotope effect k_H/k_D (Northrop, 1977) is given in eq 4. This equation is a general one, incorporating

$$\frac{^D(V/K) - 1}{^T(V/K) - 1} = \frac{k_H/k_D - 1 + C_r(^DK_{eq} - 1)}{k_H/k_T - 1 + C_r(^TK_{eq} - 1)} \quad (4)$$

a partitioning ratio, C_r, for steps that occur after the isotope-sensitive step. In the case where $C_r \simeq 0$ or $^DK_{eq} = 1$, eq 4 simplifies to eq 5, where $(k_H/k_D)^{1.442}$ has been substituted

$$\frac{^D(V/K) - 1}{^T(V/K) - 1} = \frac{k_H/k_D - 1}{(k_H/k_D)^{1.442} - 1} \quad (5)$$

for k_H/k_T (Swain et al., 1958).

As indicated in eq 5, a unique value for the intrinsic isotope effect should be accessible for enzymatic reactions characterized by essentially irreversible C–H bond cleavage steps. While the reactions catalyzed by monooxygenases are generally believed to be irreversible, it is known that numerous enzymes significantly alter the equilibrium constants for the interconversion of bound vs. free substrates and products [e.g., Nageswara Rao et al. (1979)]. Hence, an assessment of the ability of enzyme-bound norepinephrine to revert to dopamine in the dopamine β-monooxygenase reaction was undertaken.

Partial conversion of dopamine to norepinephrine was carried out in the presence of 1 Ci/mL of tritiated water, followed by isolation of unreacted starting material. As can be seen from the data in Table I, an ca. 0.05% exchange of tritium was observed independent of reaction conditions, indicating a nonenzymatic exchange process. In an effort to confirm this conclusion, the [^{3}H]dopamine was reincubated with dopamine β-monooxygenase with the expectation that enzymatically incorporated counts would be released to water. As summarized in Table I, incubation of the purified [^{3}H]-dopamine labilized <2% of the initial tritium under conditions of complete conversion of dopamine to norepinephrine. These results indicate a low-level, nonenzymatic incorporation of tritium into dopamine under the reaction conditions. Importantly, no enzyme-catalyzed reversal of norepinephrine to dopamine was detected.

In principle, the absence of back-exchange from solvent to substrate could arise from an inaccessibility of solvent to the enzyme active site. However, as shown in the following paper (Ahn & Klinman, 1983), dopamine β-monooxygenase must be protonated for substrate activation to occur. Since protons are consumed in the overall reaction, the protonated residue will be regenerated from solvent such that in the presence of tritiated water tritium will be available in the active site. Considering the chemistry of this reaction, it seems most likely that the requisite proton is necessary for protonation of the partially reduced oxygen during its activation. Furthermore, the stereochemical outcome of retention of configuration implies that the bound amine substrate is oriented with the hydrogen undergoing abstraction projecting toward the activated oxygen and, hence, also toward the basic residue. Both the activated oxygen and base are considered possible catalysts for C–H bond cleavage via hydrogen atom and proton abstraction, respectively. In either case, the substrate-derived hydrogen would be in close proximity to the solvent-derived tritium, and scrambling is expected. We therefore conclude that the *complete absence* of specific tritium exchange from solvent into dopamine is evidence of an irreversible C–H bond cleavage step.

Measurement of Isotope Effects. As originally noted by Albery & Knowles (1977), the application of eq 5 to obtain a statistically significant value for k_H/k_D requires careful experimental design to minimize sources of error in measured isotope effects. Noncompetitive methods are frequently employed for measuring deuterium isotope effects; while straightforward, these measurements may suffer large errors. For example, in the case of dopamine β-monooxygenase, independent measurement of $(V_m/K_m)_H$ (±4.8%) and $(V_m/K_m)_D$ (±7.1%) yielded $^D(V/K)$ (±8.7%). Combination of this value with $^T(V/K)$ characterized by an error of only 1.9% resulted in a range for k_H/k_D of $5.6 < 9.1 < 15.9$. Although smaller errors in the data are attainable, a 3% error in each V_{max}/K_m value would still generate limits of 7.1–12.1 for k_H/k_D.

In light of the above limitations, a more accurate, competitive design was sought. Typically, competitive isotope effects are obtained by comparing the isotopic abundance of product at fractional vs. complete reaction as shown in eq 6 where f

$$^T(V/K) = \frac{\ln(1-f)}{\ln[1 - f(SA_f/SA_{f\infty})]} \quad (6)$$

is the fraction of unlabeled substrate converted to product and $SA_f/SA_{f\infty}$ is the ratio of the specific activities of product at fractional vs. complete reaction. With monooxygenases, the design of competitive experiments is complicated by the fact that C–H bond cleavage releases hydrogen, or its isotopes, to water. While $^T(V/K)$ is easily determined by analysis of tritium in water, an analogous experiment for $^D(V/K)$ would require a measurement of the deuterium enrichment in water.[2] In the present studies, a competitive double-label method

[2] Geochemists routinely measure such enrichments as D/H ratios by isotope ratio mass spectrometry (Friedman, 1963). Reported accuracies for D/H ratios referenced to a natural abundance standard are ca 0.2% (Craig, 1961; Friedman, et al., 1970; Kishima & Sakai, 1980). Since calculation of $^D(V/K)$ from eq 6 would require comparison of D/H for fractional vs. 100% conversion, propagation of this error must be considered. In the case of dopamine β-monooxygenase, under conditions of an isotopic discrimination of 2.5 and high substrate concentration (50 mM dopamine), a 10% conversion of dopamine to norepinephrine would contribute only ~15 ppm above background to the D/H ratio. Assuming a 0.2% error in D/H ratios at 10% and 100% conversion, a propagated error of ≥3–4% is calculated for $^D(V/K)$. Thus, unless uncertainties of <0.2% in each D/H ratio are attainable (Hartley, 1980; Hartley reports accuracies of 0.1% for D/H ratios in water using a modified spectrometer), this method does not appear sufficiently accurate for determination of the intrinsic isotope effect.

Table II: Competitive Measurement of $^{D}(V/K)$ in the Dopamine β-Monooxygenase Reaction[a]

% conversion of dopamine to norepinephrine	$(^{14}C/^{3}H)_{corr}$[b]	$^{D}(V/K)$[c]
0	0.07329	
reaction 1		
2.0	0.2041	2.784
reaction 2		
0.75	0.1956	2.668
2.0	0.2003	2.733
reaction 3		
2.0	0.2026	2.765
0	0.08253	
reaction 4		
1.5	0.2052	2.829
		av: 2.756 ± 0.054

[a] Reaction mixtures contained 100 mM potassium phosphate, pH 6.0, 10 mM ascorbate, 10 mM fumarate, 17 mM KCl, 40 μg/mL catalase, 1 μM $CuCl_2$, 1 mM total dopamine (H:D, 1:1) and 1.25 μg of dopamine β-monooxygenase in a total volume of 0.5 mL, 35 °C, 20% O_2. For the time points, 0.15 mL of reaction was quenched with 0.09 mL of 0.1 N $HClO_4$. [b] Counting channels were adjusted so that ^{3}H spillover to the ^{14}C channel was 0.2% of ^{3}H channel counts and ^{14}C spillover to the ^{3}H channel was 23% of the ^{14}C channel counts. [c] $^{D}(V/K)$ was determined by comparison of $^{14}C/^{3}H$ ratios for product to initial substrate as opposed to product at 100% conversion. Substrate was purified by HPLC immediately preceding isotope effect determinations.

similar to that described by Dahlquist et al. (1969) was employed for the measurement of $^{D}(V/K)$. This technique utilizes the radioisotopes carbon-14 and tritium to trace the course of reaction of protonated and deuterated substrate, respectively. As summarized in Table II, comparison of corrected $^{14}C/^{3}H$ ratios for product at early percent conversion to initial dopamine provides a direct measure of $^{D}(V/K)$ (±2%).

For the design of experiments involving measurement of $^{D}(V/K)$ by radiolabeling, consideration of the following points is important: (i) The stereochemical purity of the deuterated substrate must be accurately determined, and, if <100%, a correction applied to the $^{14}C/^{3}H$ ratios. As described under Experimental Procedures, a correction was made for the presence of 5.7% [*aryl*-^{3}H,2(*S*)-^{2}H]dopamine in [*aryl*-^{3}H,2(*R*)-^{2}H]dopamine samples. (ii) Calculation of $^{D}(V/K)$ by direct comparison of $^{14}C/^{3}H$ ratios in product to substrate is valid only if the fractional conversion of protonated substrate is ≤0.05. These studies employed deuterated substrate of relatively low specific activity, requiring the use of deuterated dopamine at a concentration equal to protonated dopamine. Under the condition of ≤0.02 total fractional conversion (corresponding to a ≤0.029 fractional conversion for protonated substrate), the magnitudes of isotope effects derived from direct comparison of $^{14}C/^{3}H$ ratios are within experimental error of values calculated according to eq 6.[3] (iii) Incorporation of tritium into substrate could give rise to a nonnegligible secondary hydrogen isotope effect. In these studies, analysis of $^{14}C/^{3}H$ ratios in product following incubation of a mixture of [*aryl*-^{3}H]dopamine and [1-^{14}C]dopamine yielded an isotope effect of 0.99 ± 0.012; hence, we are able to rule out a secondary effect arising from ring tritiation in the dopamine β-monooxygenase reaction.

Intrinsic Isotope Effects. The data summarized in Tables II and III indicate values for $^{D}(V/K)$ (±2%) and $^{T}(V/K)$

[3] Since $f_H/f_D = (^{14}C/^{3}H)_{product}/(^{14}C/^{3}H)_{substrate}$, f_H and f_D may be calculated by combining this equation with $X_H/f_H + X_D/f_D = f_T$, where X_H and X_D are the initial mole fractions of protonated and deuterated substrate, respectively, and f_T is the total fractional conversion. f_H is then used in eq 6 to calculate isotope effects.

Table III: Measurement of $^{T}(V/K)$[a]

% conversion of dopamine to norepinephrine	sp act. (cpm/μmol × 10^{-5})[b]	$^{T}(V/K)$[c]
100	15.668	
reaction 1		
1.8	2.545	6.157
2.4	2.504	6.258
2.9	2.555	6.134
reaction 2		
1.5	2.532	6.189
2.0	2.475	6.330
2.3	2.686	5.835
reaction 3		
1.3	2.549	6.029
2.1	2.440	6.442
reaction 4		
2.2	2.709	5.783
reaction 5		
2.0	2.741	5.716
reaction 6		
0.9	2.600	6.016
		av: 6.079 ± 0.220

[a] Reaction mixtures contained 100 mM potassium phosphate, pH 6.0, 10 mM sodium ascorbate, 1 μM $CuCl_2$, 40 μg/mL catalase, 17 mM KCl, 10 mM fumarate, 1 mM [2-^{3}H]dopamine (sp act. 3.72 × 10^{6} cpm/μmol), and 1.25 μg of dopamine β-monooxygenase in a total volume of 0.5 mL and were run at 35 °C, 20% O_2. The 100% conversion reaction was identical except that 0.1 mM [2-^{3}H]-dopamine and 50 μg of dopamine β-monooxygenase were used. For time points, 0.15 mL of reaction was quenched with 0.09 mL of 0.1 N $HClO_4$. [b] Product specific activities are calculated from the ratio of tritium counts in water from lyophilized aliquots of quenched reaction mix to the micromoles of norepinephrine produced, as previously described (Klinman et al., 1980). [c] Isotope effects are calculated from eq 4 or when the fractional conversions are <0.05, from the ratio of specific activities at complete vs. fractional conversion.

Table IV: Comparison of the Magnitude of Intrinsic Isotope Effect, Minus Fumarate, for Two Enzyme Preparations[a]

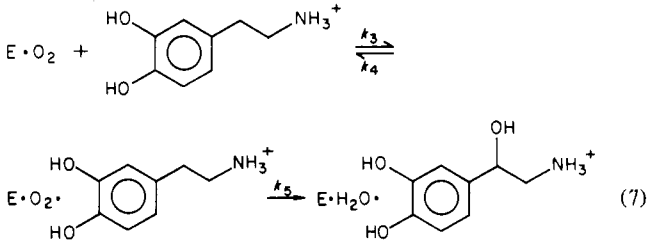
prepn[b]	$^{D}(V/K)$	$^{T}(V/K)$	k_H/k_D
I	5.931 ± 0.288	16.11 ± 0.52	9.2 < 10.9 < 13.0
II	3.024 ± 0.172	7.224 ± 0.355	8.95 < 11.0 < 13.7

[a] Reaction mixtures contained 100 mM potassium phosphate, pH 6.0, 10 mM sodium ascorbate, 1 μM $CuCl_2$, 40 μg/mL catalase, 1 mM [2-^{3}H]dopamine or 1 mM dopamine (H:D, 1:1), 27 mM KCl, and 5 μg of dopamine β-monooxygenase in a total volume of 0.5 mL and were run at 35 °C, 20% O_2. Time points were taken by quenching 0.15 mL of reaction with 0.09 mL of 16.7 mM fumarate in 0.1 N $HClO_4$. [b] Preparation I was the same enzyme used in the plus fumarate experiments. The two preparations were obtained by identical methods, approximately 8 months apart.

(±3.6%). By employment of eq 5, these data yield a value for k_H/k_D of 9.4 ± 1.3. Measurement of $^{T}(V/K)$ (±2%) would lower the uncertainty in k_H/k_D from 14% to 10%. With the methods described herein, further reduction of the experimental error in each V_{max}/K_m isotope effect to less than 2% appears unlikely, leading to the conclusion that 10% represents a realistic lower limit for the uncertainty in intrinsic isotope effects determined by this method.

The reactions of Tables II and III were performed in the presence of fumarate, an activator of dopamine β-monooxygenase. Similar experiments conducted in the absence of fumarate are summarized in Table IV for two separate enzyme preparations. Inspection of the data in Table IV shows that the observed V_{max}/K_m isotope effects are reduced approximately 2-fold for the second enzyme preparation; yet, the calculated values of k_H/k_D are identical for both preparations.

As indicated in eq 7 and 8, if k_H/k_D is constant, variable



(7)

$$^{D}(V/K) = \frac{k_H/k_D + k_5/k_4}{1 + k_5/k_4} \quad (8)$$

observed isotope effects must be due to changes in the partitioning of enzyme–dopamine–oxygen complexes between catalysis and substrate dissociation, k_5/k_4. We attribute these differences to inherent variations in enzyme preparations, which is a current problem of interest.

More interesting is a comparison of the observed and intrinsic isotope effects determined in the presence vs. the absence of fumarate, employing the same enzyme preparation (Tables II and III and preparation I of Table IV). The data indicate large reductions in the observed values for both $^{D}(V/K)$ and $^{T}(V/K)$ upon inclusion of fumarate in reaction mixtures, consistent with previous reports of the effect of fumarate on steady-state isotope effects in dopamine β-monooxygenase (Bachan et al., 1974; Klinman et al., 1977). By contrast, the intrinsic isotope effect shows only a minor decrease from 10.9 ± 1.9 in the absence of fumarate to 9.4 ± 1.3 in its presence, and the two values are within experimental error of one another. Since this small effect of fumarate on k_H/k_D cannot account for the large changes in the observed isotope effects, the role of the anion effector must be in the modulation of enzyme partitioning, k_5/k_4 in eq 8. Clearly, either an increase in k_5 or a decrease in k_4 would lead to diminished isotope effects on V_{max}/K_m. With the data at hand, distinction between these two is not possible; however, as shown in the following paper, direct comparison of deuterium isotope effects on V_{max} and V_{max}/K_m to the intrinsic isotope effect provides a quantitative measure of the effect of fumarate on both k_5 and k_4.

One further point of interest is the mechanistic implications of the magnitude of the intrinsic isotope effect. In this context, some insight may be gained by considering published correlations of the magnitude of primary hydrogen isotope effects with the overall free energy for the hydrogen-transfer process. In the case of both proton and hydrogen atom transfer reactions, highly exo- or endoenergetic processes have been shown to correlate with small primary isotope effects whereas isoenergetic processes correlate with large, maximal values (10–12) for k_H/k_D [Bell (1973) and references cited therein]. In this light, the values of 9.4–11 obtained for k_H/k_D herein suggest that the C–H bond cleavage step in the dopamine β-monooxygenase reaction is characterized by a ΔG value within several kilocalories per mole of zero. Under these conditions, it is expected that C–H bond cleavage would be freely reversible; on the contrary, the results presented within show *no detectable re-formation* of dopamine from enzyme-bound intermediate or product. To account for these results, together with a kinetically significant dissociation of dopamine from enzyme–ternary complex and the faster rate for norepinephrine formation than its dissociation from enzyme (cf. Ahn & Klinman, 1983), we propose a two-step mechanism for conversion of enzyme–substrate to enzyme–product complex in which removal of the substrate benzylic hydrogen occurs first, forming an intermediate E′ of approximately equal

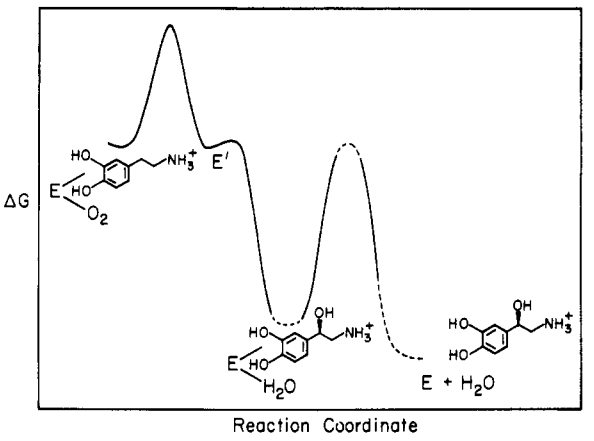


FIGURE 1: Postulated energy diagram for reaction from enzyme–substrate ternary complex to free enzyme and products for dopamine β-monooxygenase.

energy to the enzyme–substrate complex. As shown in the energy diagram for this mechanism, Figure 1, irreversibility of the C–H bond cleavage step is then due to the very rapid and essentially irreversible collapse of E′ to the enzyme–product complex.[4] Additional features of Figure 1 that should be noted are (1) the large energy of activation for product release, (2) the form of oxygen in the enzyme–substrate complex, and (3) the nature of the intermediate E′. The first two points have arisen from the data of the following paper and will be discussed more thoroughly there. Briefly, for the first point, low magnitudes of deuterium isotope effects on V_{max} were obtained, indicating that product release is much slower than the hydrogen-transfer reaction; for the second point, the necessity of a proton in the enzyme–substrate complex prior to C–H bond cleavage suggests that the oxygen is present in some activated form rather than as the molecular oxygen indicated in Figure 1. At present, insufficient data are available to determine the ionization state of the intermediate in E′ or of the hydrogen (H^+ vs. H·) that is abstracted; the nature of this process is currently under investigation.

Registry No. [2(*R*)-^{2}H]Dopamine, 85479-75-0; 2-methyl-4-(3,4-dimethoxydeuteriobenzal)-5-oxazolone, 85479-76-1; methyl α-acetamido-3,4-dimethoxy-*trans*-[β-^{2}H]cinnamate, 85479-77-2; (2*S*,3*R*)-*N*-acetyl-3-(3,4-dimethoxyphenyl)[3-^{2}H]alanine methyl ester, 85479-78-3; (2*R*,3*S*)-*N*-acetyl-3-(3,4-dimethoxyphenyl)[3-^{2}H]alanine methyl ester, 85479-79-4; (2*S*,3*R*)-*N*-acetyl-3-(3,4-dimethoxyphenyl)[3-^{2}H]alanine, 85479-80-7; (2*S*,3*R*)-3-(3,4-dimethoxyphenyl)[3-^{2}H]alanine, 85479-81-8; dopamine, 51-61-6; dopamine β-monooxygenase, 9013-38-1; fumaric acid, 110-17-8; deuterium, 7782-39-0; tritium, 10028-17-8.

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[4] Maximal hydrogen isotope effects have been observed to occur over a range for ΔG of several kilocalories per mole for the hydrogen-transfer step. Hence, we have estimated the anticipated level of back-incorporation of tritium from solvent to dopamine in the event of a single-step mechanism characterized by $K_{eq} = 10^3$. Employing the rate constants in the accompanying paper (Ahn & Klinman, 1983), and intrinsic tritium isotope effects of 25–30, ±fumarate, the specific activity of unreacted dopamine is calculated as 10^3 cpm/μmol at 10% conversion, well within our levels of detection. Thus, $K_{eq} > 10^3$ for the formation of E·P from E·S.

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Mechanism of Modulation of Dopamine β-Monooxygenase by pH and Fumarate As Deduced from Initial Rate and Primary Deuterium Isotope Effect Studies†

Natalie Ahn and Judith P. Klinman*

ABSTRACT: Dopamine β-monooxygenase catalyzes a reaction in which 2 mol of protons are consumed for each turnover of substrate. Studies of the pH dependence of initial rate parameters (V_{max} and V_{max}/K_m) and their primary hydrogen isotope effects show that at least two ionizable residues are involved in catalysis. One residue (B1, pK = 5.6-5.8) must be protonated prior to the carbon-hydrogen bond cleavage step, implying a role for general-acid catalysis in substrate activation. A second protonated residue (B2, pK = 5.2-5.4) facilitates, but is not required for, product release. Recent measurement of the intrinsic isotope effect for dopamine β-monooxygenase [Miller, S. M., & Klinman, J. P. (1983) *Biochemistry* (preceding paper in this issue)] allows an analysis of the pH dependence of rate constant ratios and in selected instances individual rate constants. We demonstrate large changes in the rate-determining step as well as an unprecedented inversion in the kinetic order of substrate release from ternary complex over an interval of 2 pH units. Previously, fumarate has been used in dopamine β-monooxygenase assays because of its property of enzyme activation. Studies of the pH behavior in the presence of saturating concentrations of fumarate have shown two causes of the activation: (i) fumarate perturbs the pK of B1 to pK = 6.6-6.8 such that the residue remains protonated and the enzyme optimally active over a wider pH range; (ii) fumarate decreases the rate of dopamine release from the ternary enzyme–substrate complex, increasing the equilibrium association constant for dopamine binding. Both effects are consistent with a simple electrostatic stabilization of bound cationic charges by the dianionic form of fumarate.

Dopamine β-monooxygenase is a copper containing monooxygenase catalyzing the hydroxylation of a variety of substituted phenylethylamines at their benzylic positions. These reactions involve a two-electron reductive cleavage of dioxygen via a poorly understood interaction of dioxygen with enzyme-bound copper and an exogenous electron donor. Properties of this enzyme have been recently reviewed (Skotland & Ljones, 1979a; Rosenberg & Lovenberg, 1980; Villafranca, 1981). In vivo, dopamine β-monooxygenase is compartmentalized to storage vesicles that support both an ATP-dependent accumulation of dopamine and the hydroxylation of dopamine to norepinephrine. The detection of high levels of ascorbic acid in vesicles derived from bovine adrenal glands implicates ascorbic acid as the physiological electron donor (Terland & Flatmark, 1975).

As summarized in eq 1, the overall reaction catalyzed by dopamine β-monooxygenase involves a formal transfer of two electrons and two protons from ascorbic acid to the products norepinephrine and water. We have analyzed the pH dependence of initial rate parameters and primary hydrogen isotope effects on these parameters in an effort to identify steps in the kinetic mechanism that involve proton transfer. Previous measurements of pH dependencies in the dopamine β-monooxygenase reaction were carried out at a single oxygen concentration, precluding a clear-cut determination of V_{max} and V_{max}/K_m parameters (Miras-Portugal et al., 1973; Craine et

† From the Department of Chemistry, University of California, Berkeley, California 94720. *Received November 1, 1982.* Supported by a research grant from the National Institutes of Health.

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