pH Dependence of Deuterium Isotope Effects and Tritium Exchange in the Bovine Plasma Amine Oxidase Reaction: A Role for Single-Base Catalysis in Amine Oxidation and Imine Exchange†

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ABSTRACT: The pH dependence of steady-state parameters for [1,1-$^1H_2$]- and [1,1-$^2H_2$]benzylamine oxidation and of tritium exchange from [2-^{3}H]dopamine has been measured in the bovine plasma amine oxidase reaction. Deuterium isotope effects on k_{cat}/K_m for benzylamine are observed to be constant, near the intrinsic value of 13.5, over the experimental pH range, indicating that C–H bond cleavage is fully rate limiting for this parameter. As a consequence, pK_a values derived from k_{cat}/K_m profiles, 8.0 ± 0.1 (pK_1) and 9.0 ± 0.16 (pK_s), can be ascribed to *microscopic* pK_a values for the ionization of an essential active site residue (EB_1) and substrate, respectively. Profiles for k_{cat} and $^Dk_{cat}$ show that EB_1 undergoes a pK_a perturbation from 8.0 to 5.6 ± 0.3 (pK_1') in the presence of substrate; additionally, a second ionization, pK_2 = 7.25 ± 0.25, is observed to mediate but not be essential for enzyme reoxidation. The pH dependence of the ratio of tritium exchange to product formation for dopamine also indicates base catalysis with a pK_{exch} = 5.5 ± 0.01, which is within experimental error of pK_1'. We conclude that the data presented herein support a single residue catalyzing both substrate oxidation and exchange, consistent with recent stereochemical results that implicate a syn relationship between these processes [Farnum, M., & Klinman, J. P. (1985) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 44*, 1055]. This conclusion contrasts with earlier kinetic data in support of a large rate differential for the exchange of hydrogen from C-1 vs. C-2 of phenethylamine derivatives [Palcic, M. M., & Klinman, J. P. (1983) *Biochemistry 22*, 5957–5966]. We have attempted to integrate the available kinetic and stereochemical data for plasma amine oxidase, using the structure of the recently proposed cofactor pyrroloquinoline quinone.

Bovine plasma amine oxidase is a copper-containing enzyme catalyzing the oxidative deamination of a wide variety of primary amines. Relatively little has been known about the active site of this enzyme: specifically, the role of copper, the structure of the organic cofactor, and the nature of the active site residues participating in catalysis. Recent studies by Lobensteen-Verbeek et al. (1984) and Ameyama et al. (1984) suggest that the organic cofactor may be pyrroloquinoline quinone, previously shown to be present in oxidative enzymes isolated solely from bacteria. If correct, the structure of this cofactor implies the formation of a covalent adduct between enzyme and substrate, consistent with recent findings by Rius et al. (1984) that enzyme reoxidation is a prerequisite for ammonia release during the catalytic cycle of amine oxidase from pig plasma. Measurements of deuterium isotope effects under pre-steady-state and steady-state conditions for the bovine plasma enzyme have implicated three kinetic steps involving enzyme reduction, imine hydrolysis, and enzyme reoxidation (Palcic & Klinman, 1983). Combination of the existing structural and kinetic data leads to the minimal mechanism in eq 1–3, where X and X′ represent oxidized and reduced enzyme-bound cofactor, respectively:

$$E_X + RCH_2NH_3^+ \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E_X{\cdot}RCH_2NH_3^+ \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E_{X'\text{-}N^+H=CHR} \quad (1)$$

$$E_{X'\text{-}N^+H=CHR} \xrightarrow[H_2O]{k_5} E_{X'\text{-}NH_2}(RCHO) \quad (2)$$

$$E_{X'\text{-}NH_2}(RCHO) \xrightarrow[O_2]{k_7} E_X + RCHO + H_2O_2 + NH_4^+ \quad (3)$$

In addition to the above reactions, bovine plasma amine oxidase has been shown to catalyze an exchange of tritium from the β-carbon of [2-^{3}H]phenethylamine derivatives (Lovenberg & Beaven, 1971). This exchange process, which appears rapid relative to other steps in the mechanism, is postulated to occur from the imine intermediate in eq 2, prior to its hydrolysis (Summers et al., 1979).

The relative rates of eq 1–3 can be inferred from a comparison of primary deuterium isotope effects on steady-state and pre-steady-state parameters. From a previous study at pH 7.2, k_{cat} is concluded to be partially rate limited by k_7 and k_{cat}/K_m to be fully rate limited by k_3 for benzylamine and a number of phenethylamines investigated. Significantly, both dopamine and *m*-tyramine show smaller steady-state isotope effects on k_{cat}/K_m than pre-steady-state effects on k_3/K_s. This important observation requires that eq 1 be reversible, i.e., that imine hydrolysis be the first irreversible step and, hence, that the hydrogen or deuteron derived from C-1 of substrate be sequestered in the EX′—N$^+$H=CHR complex. Thus, a minimum of two active site residues have been proposed to participate in catalysis, such that the residue catalyzing substrate oxidation undergoes a slow exchange with solvent, in contrast to a second solvent-accessible residue catalyzing a rapid exchange of hydrogen from C-2 of phenethylamines (Palcic & Klinman, 1983). It should be noted, however, that the conclusion of separate residues catalyzing oxidation and exchange leads to the expectation of an anti stereochemical relationship for C–H bond cleavage at C-1 and C-2 of phenethylamine substrates, whereas recent experiments using chirally deuterated and tritiated dopamine samples indicate a syn relationship (Farnum & Klinman, 1985).

In this study the pH dependences of deuterium isotope effects in the oxidation of benzylamine and of tritium exchange

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from C-2 of dopamine have been measured, in an effort to estimate the pK_a values of ionizable groups involved in substrate oxidation and exchange and to assess the number of unique catalytic residues participating in each process. We have made the unusual observation that deuterium substitution does not alter the observed pK_a values in k_{cat}/K_m vs. pH profiles for benzylamine, while reducing the rate of enzyme reduction by a factor of 12–13 across the experimental pH range. This result indicates that pK_a values derived from k_{cat}/K_m profiles can be directly ascribed to microscopic ionization constants, implicating a basic residue, EB_1 ($pK_1 = 8.0$), in substrate oxidation. Examination of k_{cat} and $^Dk_{cat}$ plots reveals that EB_1 undergoes a marked perturbation to $pK_1' = 5.6 \pm 0.3$ in the enzyme–substrate complex. Significantly, an almost identical $pK_{exch} = 5.5 \pm 0.01$ is observed for the base catalysis of tritium exchange from C-2 of dopamine. Thus, the data presented herein are concluded to support a single basic residue catalyzing both substrate oxidation at C-1 and exchange at C-2, consistent with recent stereochemical results that implicate a syn relationship between these processes (Farnum & Klinman, 1985). As we describe, the structure of the newly proposed cofactor for bovine plasma amine oxidase may provide a mechanistic rationale for the large differential in hydrogen exchange rates from C-1 vs. C-2 of phenethylamine substrates and hence a successful integration of kinetic and stereochemical data.

Experimental Procedures

Materials

Benzylamine and [1,1-$^2H_2$]benzylamine were prepared in parallel by the reduction of benzonitrile in anhydrous tetrahydrofuran as described by Bardsley et al. (1973) using $LiAlH_4$ or $LiAl^2H_4$ (isotopic purity > 98%); they were converted to HCl salts by bubbling HCl (g) through the basic ethereal extracts and were recrystallized twice from methanol. Plasma amine oxidase was isolated as previously described (Summers et al., 1979). Horse liver alcohol dehydrogenase was obtained from Sigma and catalase from Boehringer. [2-^{3}H]Dopamine, sp act. 34.4 Ci/mmol, was from New England Nuclear. Other chemicals were obtained commercially and were reagent-grade.

Methods

pH Dependence of Benzylamine Oxidation. Initial velocity studies for the oxidation of deuterated and protonated benzylamine were carried out at 25 °C by monitoring either benzaldehyde production at 250 nm ($\Delta\epsilon = 12.8$ mM^{-1} cm^{-1}) on a Cary 118 spectrophotometer or O_2 consumption on a Yellow-Springs oxygen monitor. All measurements were carried out in air-saturated solutions ensuring saturating levels of oxygen for measurements. The K_m for O_2 has been determined to be 0.7% for 1.7 mM benzylamine (Oi et al.). Benzylamine concentrations varied from a low of 10 μM to a high of 130 mM across the pH range. Buffers of 0.10 ionic strength were prepared (Long et al., 1968) and were chosen to overlap wherever possible to test for specific buffer effects. The following buffers and pH ranges were used: sodium acetate, pH 5.03–5.40; sodium phosphate–potassium phosphate, pH 5.62–8.01; potassium pyrophosphate, pH 8.00–9.01; sodium carbonate, pH 8.98–9.74. The pH stability of plasma amine oxidase was checked by incubating enzyme for 20–30 min in the appropriate buffer and then measuring activity by the standard assay. The enzyme is stable from pH 5.0 to pH 9.0 under these conditions. Enzyme activities vs. substrate concentrations were fit to the expression

$$v = \frac{V_{max}(app)[S]}{K_m(app) + [S]} \quad (4)$$

with the program HYPER (Cleland, 1979), which had been translated to BASIC (Ahn & Klinman, 1983). In these experiments, enzyme concentrations were normalized to 0.36 unit/mg. First-order (k_{cat}) and second-order (k_{cat}/K_m) rate constants were then calculated from apparent V_{max} and V_{max}/K_m values assuming a subunit M_r of 85 000. In analyzing pH profiles, k_{cat}/K_m was fit to the expression

$$\log v = \log \frac{C}{1 + K_A/[H^+] + [H^+]/K_B} \quad (5)$$

with the program BELL (Cleland, 1979), which had been translated to BASIC. The pH profiles for k_{cat} and $^Dk_{cat}$ were found to be complex and poorly fit by nonlinear least-squares analysis. Consequently, these data were fit numerically, as described under Results.

C-2 Exchange of Dopamine. Employing randomly labeled [2-^{3}H]dopamine, the ratio of tritium exchange with solvent to product formation was measured as a function of pH. The following buffers and pH ranges were used: sodium acetate, pH 5.0–5.75; sodium phosphate–potassium phosphate, pH 5.75–8.1. To minimize spontaneous loss of tritium from the aldehyde product of the plasma amine oxidase reaction, the liver alcohol dehydrogenase–NADH system was used to convert aldehyde to the corresponding alcohol. Control experiments were conducted at the pH extremes to ensure complete coupling of the two reactions. A typical reaction mixture contained 1 mM [2-^{3}H]dopamine, sp act. = 10^7 cpm/μmol, 2 mM NADH, 0.03 unit of plasma amine oxidase (0.15 unit/mg), and 0.3 unit of liver alcohol dehydrogenase (1.6 units/mg), in air-saturated solutions with buffer concentrations to achieve a final ionic strength of 0.1. At appropriate time points, aliquots from the reaction were quenched with an equal volume of 2.0 N perchloric acid. Volatile tritium was separated from the residue by bulb-to-bulb distillation in vacuo. Both portions were counted, and the percentage of volatile counts in the total was determined. Volatile counts liberated nonenzymatically were measured in simultaneous control experiments and subtracted from the observed enzymatic exchange. Product formation was determined from the decrease in absorbance at 340 nm due to the oxidation of NADH to NAD^+. Infinity points for complete product formation were calculated from initial dopamine concentrations ($\epsilon = 2.7$ mM^{-1} cm^{-1}). These absorbance changes were corrected for nonenzymatic loss of NADH by applying the observed pseudo-first-order rate constant for the acid-catalyzed hydration of NADH determined under conditions identical with the enzymatic reactions except for the absence of plasma amine oxidase. At pH 7.0, $k_{obsd} = 3.0 \times 10^{-4}$ min^{-1}, which is comparable to the value of 4.0×10^{-4} min^{-1} reported by Wong and Whitesides (1981). The reported ratios of tritium exchange to product formation at each pH are the average of five time points, which varied from 20 to 100% reaction. As expected, observed ratios were found to be independent of percent reaction and substrate concentration. It should be noted that the fraction of tritium at C-2 for commercially obtained [2-^{3}H]dopamine is frequently less than 100%. Dopamine β-monooxygenase assays indicated a specific activity at C-2 of ca. 70% of the quoted value for the samples of [2-^{3}H]dopamine employed in these studies. The pH dependence of the ratio of exchange to product formation was fit to the expression

$$\log v = \log \frac{C}{1 + [H^+]/K} \quad (6)$$

with the program HABELL (Cleland, 1979), which had been translated to BASIC.

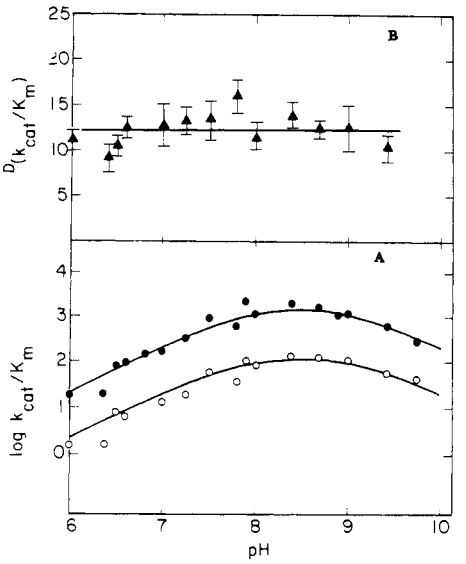


FIGURE 1: (A) pH dependence of k_{cat}/K_m for benzylamine (●) and [1,1-$^2H_2$]benzylamine (○) oxidation, catalyzed by bovine plasma amine oxidase. Errors on k_{cat}/K_m all lie within 5% of measured values with the exception of pH 6.0, 6.4, 7.0, and 7.8 for [1,1-$^2H_2$]benzylamine where errors were 6.5, 24, 12, and 6%, respectively. Data were fit to eq 5 as described under Methods. (B) pH dependence of the observed isotope effect on k_{cat}/K_m.

Table I: Absence of an Isotope Effect on pK_a Values from k_{cat}/K_m vs. pH Profiles for Benzylamine[a]

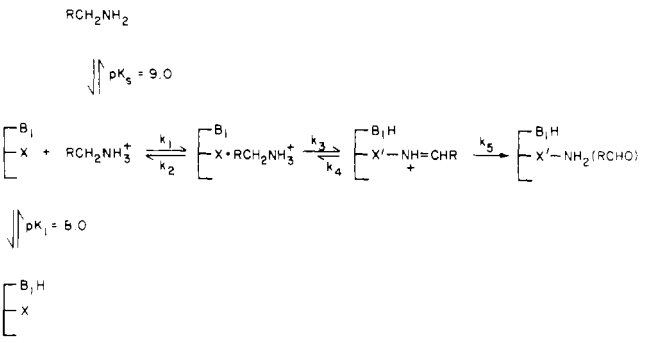
constant	benzylamine	[1,1-$^2H_2$]benzylamine
pK_1	8.0 ± 0.10	7.9 ± 0.08
pK_s	9.0 ± 0.16	9.1 ± 0.14
C^b	2440 ± 260	205 ± 17

[a] Date fitted to eq 5, pK_1 = pK_B and pK_s = pK_A. [b] pH-independent value of k_{cat}/K_m (M^{-1} s^{-1}).

RESULTS

pH Dependence of k_{cat}/K_m and $^D(k_{cat}/K_m)$. As shown in Figure 1A, benzylamine oxidation, catalyzed by bovine plasma amine oxidase, reveals a bell-shaped curve with pK_a values of 8.0 ± 0.10 and 9.0 ± 0.16, Table I. Since substrate ionizes with a pK_a = 9.3 (Lindstrom et al., 1976), we assign the alkaline pK_a to amine ionization and conclude that substrate binds to enzyme in a protonated form. The low pK is assigned to an active site residue that must be unprotonated for enzyme catalysis. Kinetic parameters for [1,1-$^2H_2$]benzylamine are also shown in Figure 1A for comparison with the protonated substrates. Significantly, deuteration reduces k_{cat}/K_m 12–13-fold across the pH rate profile, Figure 1B. Concomitant with this large reduction in rate, we fail to see a significant alteration in observed pK_a values: pK_1 = 7.9 ± 0.08 and pK_s = 9.1 ± 0.14, Table I. Thus, we conclude (i) that the CH bond abstraction step fully limits k_{cat}/K_m across the experimental pH range and (ii) that kinetically observed pK_a values reflect microscopic ionization constants. Scheme I summarizes the ionizations controlling the conversion of free amine through the first irreversible step, which is attributed to protonated imine hydrolysis (Palcic & Klinman, 1983). As previously discussed, the similarity of the isotope effect observed on k_{cat}/K_m for benzylamine at pH 7.2 to the pre-steady-state isotope effect of 13.5 indicates that $k_2 > k_3$ and $k_5 > k_4$. The invariance of $^D(k_{cat}/K_m)$ with pH indicates that this condition pertains across the experimental pH range, allowing us to formulate k_{cat}/K_m and $^D(k_{cat}/K_m)$ independent of k_4 and k_5 (Table II). Surprisingly, we have no evidence for even partial rate limitation by imine hydrolysis at high pH, suggesting that imine may remain protonated to pH 10.

Scheme I: Kinetically Significant Ionizations, Accounting for Observed pH Dependence of k_{cat}/K_m, in Bovine Plasma Amine Oxidase Catalyzed Oxidation of Benzylamine[a]



[a] Scheme includes ionizations controlling benzylamine binding and catalysis up to and including the first irreversible step, imine hydrolysis.

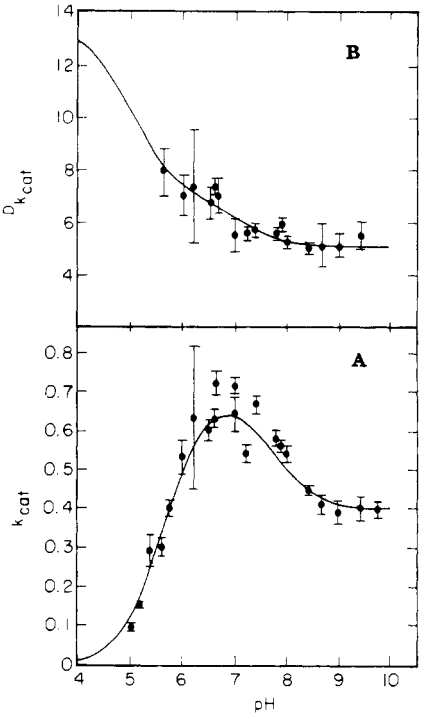


FIGURE 2: (A) pH dependence of k_{cat} for benzylamine oxidation. The solid line was generated from eq b in Table II where $k_3/k_7' = 2.13$, $k_3/k_7 = 0.80$, pK_1' = 5.3, and pK_2 = 7.0. (B) pH dependence of the observed isotope effect on k_{cat}. The solid line was generated from eq d in Table II, where $k_3/k_7' = 2.13$, $k_3/k_7 = 1.1$, pK_1' = 5.9, and pK_2 = 7.5.

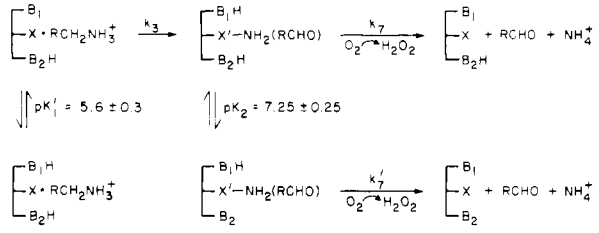
pH Dependence of k_{cat} and $^Dk_{cat}$. The behavior of k_{cat} for benzylamine oxidation is shown in Figure 2A. There is a small

Table II: Kinetic Expression for pH Dependencies in the Plasma Amine Oxidase Catalyzed Oxidation of Benzylamine[a]

parameter	eq	isotope effect	eq
$k_{cat}/K_m = k_1k_3/[k_2(1 + [H^+]/K_1 + K_s/[\ ^+])]$	a	$^D(k_{cat}/K_m) = {}^Dk_3$	c
$k_{cat} = k_3/[(1 + [H^+]/K_1') + (1 + K_2/[H\ ])[k_3/k_7 + k_7'(K_7/[H^+])]]$	b	$^Dk_{cat} = (^Dk_3 + c)/(1 + c)^b$	d

[a] The rate constants in eq a and b refer to Schemes I and II, respectively. [b] $c = (k_3/k_7)(1 + K_2/[H^+])/[(1 + [H^+]/K_1')[1 + k_7'/k_7(K_2/[H^+])]]$.

Scheme II: Mechanism That Will Account for Observed pH Dependencies of k_{cat} and $^{D}k_{cat}$ for Benzylamine Oxidation, Showing Ionizations Contributing to Substrate Oxidation in the E·S Complex and Enzyme Reoxidation



increase in rate between pH 10.0 and pH 6.0, followed by a marked decrease below pH 6.0. We attribute the low pH behavior of k_{cat} to protonation of the group that must be ionized for substrate oxidation, B_1 in Scheme I. A comparison of the pH dependencies for k_{cat}/K_m and k_{cat} indicates that this group undergoes a pK perturbation in the presence of substrate from pH 8.0 to below pH 6.0. Although substrate binding to EB_1H has not been included in Scheme I, this perturbation implies that substrate binds to EB_1H, but with more than a 100-fold weaker affinity than to EB_1.

The study of $^{D}k_{cat}$, Figure 2B, is consistent with the presence of multiple ionizing groups, such that protonation leads initially to a discrete increase in the observed isotope effect from 5.0 to ca. 7.4, followed by a rise in isotope effect toward the intrinsic value of $^{D}k_3 = 13.5$ (Palcic & Klinman, 1983) at low pH.[1] We attribute the modest changes in k_{cat} and $^{D}k_{cat}$ between pH 10.0 and pH 6.0 to a residue that mediates but is not essential for enzyme oxidation by O_2, EB_2 in Scheme II, in contrast to the pH behavior below pH 6, which implicates an essential basic residue, EB_1 in Scheme II. The kinetic expressions describing the pH behavior of k_{cat} and $^{D}k_{cat}$ are given in Table II. The relationship of rate constants for enzyme reoxidation below pK_2 (k_7) and above pK_2 (k_7') can be estimated from intrinsic isotope effect on k_3 and observed limiting isotope effects of 5.0 and 7.0–7.9. Utilizing the expression for $^{D}k_{cat}$ in Table II, the term c simplifies to $k_3/k_7' = 2.1$ in the alkaline range and to $k_3/k_7 = 0.80$–1.1 at intermediate pH values from which we estimate $k_7/k_7' = 2$–3. These values of k_3/k_7 and k_3/k_7' have been used in generating the solid lines in Figure 2, which correspond to p$K_1' = 5.6 \pm 0.3$ and p$K_2 = 7.25 \pm 0.25$.[2]

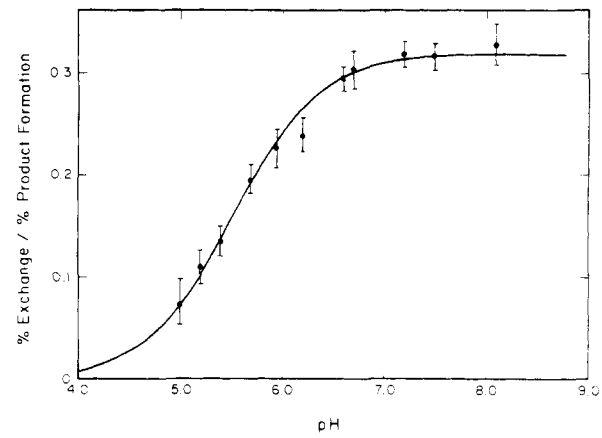
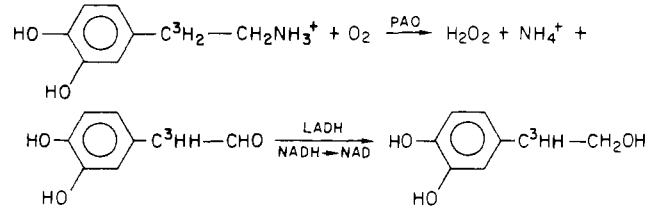


FIGURE 3: pH dependence of the ratio of tritium exchange to product formation for [2-^{3}H]dopamine. Data were fit to eq 6 as described under Methods.

An important feature of k_{cat}, distinct from k_{cat}/K_m, is the inclusion of steps for oxygen reduction to hydrogen peroxide, k_7 and k_7' in Scheme II, which are concluded to be partially rate limiting from the reduced magnitudes of $^{D}k_{cat}$ relative to $^{D}(k_{cat}/K_m)$. As noted previously in the dopamine β-monooxygenase reaction, general-acid catalysis appears to play a key role in the activation of oxygen to a reduced form capable of substrate hydroxylation (Ahn & Klinman, 1983). Yet, whereas Figure 2 supports a role for base catalysis in substrate oxidation, no obligatory role for general-acid catalysis has been detected.[3] One possible explanation is that enzyme reoxidation is limited by product desorption step(s) rather than chemistry, such that the anticipated pH dependencies are not expressed in k_{cat} across the experimental pH range. Alternatively, large perturbations in pK_a may occur such that enzyme remains protonated (either on an active site residue, EB_1, or cofactor) in its reduced form. Although we currently have no information regarding the rate-limiting step(s) controlling k_7 and k_7', partial support for the latter is provided by the failure of substrate-derived deuterium to exchange with solvent in the reduced enzyme–imine complex formed with phenethylamine substrates.[4]

pH Dependence of Tritium Exchange from C-2 of Dopamine. Exchange experiments were carried out by incubating dopamine, randomly tritiated at C-2, with plasma amine oxidase in the pH range of 5.0–8.0. Tritium release to solvent and loss of NADH due to reduction of product aldehyde to alcohol were monitored as a function of time:



(7)

where PAO and LADH are plasma amine oxidase and liver alcohol dehydrogenase, respectively. Ratios of tritium exchange to product formation were observed to be essentially

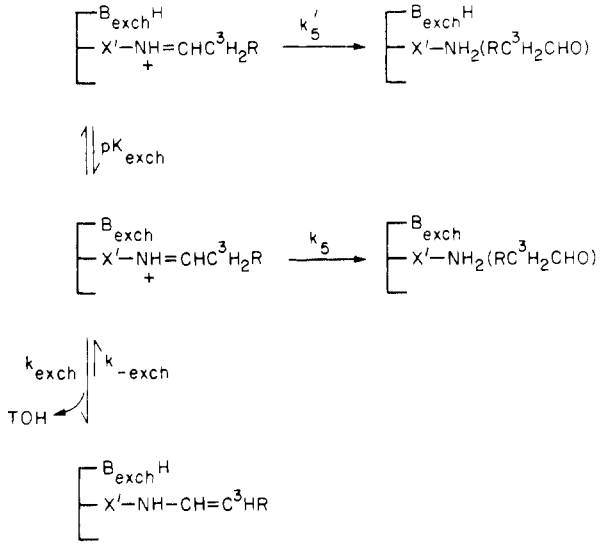
[1] The solid line in Figure 2B indicates that $^{D}k_{cat}$ rises to the intrinsic value at low pH. Ideally, isotope effects would have been measured below pH 5.5, in an effort to confirm this point. However, the combination of high K_m values and isotope effects at low pH leads to large experimental error in rates measured with deuterated substrate. A referee has pointed out that in the absence of evidence that $^{D}k_{cat}$ approaches an intrinsic value this parameter may reflect a single pK due to the ionization of EB_1 and it may plateau at a value of 8. If such a mechanism were correct, we would have to postulate that the alkaline ionization in k_{cat} does not alter $^{D}k_{cat}$, i.e., that enzyme can undergo an isomerization or oligomerization that affects rate without altering partitioning ratios. This seems unlikely, since a nonspecific change in protein structure would be expected to alter k_{cat}/K_m analogous to k_{cat}, whereas we have no evidence for such an effect, Figure 1A. Alternatively, a mechanism in which EB_1 must be ionized for all steps in both the reductive and oxidative half-reactions is conceivable. In this instance, a single ionization would be observed on $^{D}k_{cat}$ reflecting an average of pK_1' and pK_2. We consider this mechanism unlikely, since an absolute dependence on EB_1 for all kinetically significant steps in the oxidative half-reaction is contrary to our expectation of acid catalysis in dioxygen reduction. Clearly, however, detailed characterization of enzyme reoxidation is needed.

[2] These pK_a values are the average of the best fit to Figure 2A, pK_1' = 5.9 and pK_2 = 7.5, and to Figure 2B, pK_1' = 5.3 and pK_2 = 7.0. As noted in footnote 1, a more accurate determination of pK_1' is precluded by the very slow enzymatic rates below pH 5.5.

[3] Despite the requirement for 2 equiv of protons in the reduction of dioxygen to hydrogen peroxide.

[4] As observed previously, $^{D}(k_{cat}/K_m) < {}^{D}(k_3/K_s)$ for dopamine and *m*-tyramine, implicating sequestration of a substrate-derived deuteron in the reduced enzyme complex (Palcic & Klinman, 1983).

Scheme III: Mechanism To Explain Observed pH Dependence of Ratio of Tritium Exchange to Product Formation for [2-^{3}H]Dopamine, Illustrating Absolute Dependence of Exchange on Ionized Form of an Active Site Residue



invariant with time, after correction for a pH-dependent, spontaneous loss of NADH.

As shown in Figure 3, the ratio of tritium exchange to product formation indicates a $pK_{exch}(app) = 5.5 \pm 0.01$, with the ratio plateauing at a value of 0.32, rather than 0.50 as previously reported (Summers et al., 1979). This is a consequence of multisite labeling in commercial [2-^{3}H]dopamine, which after assay with dopamine β-monooxygenase was concluded to contain 66% of the label at the β-carbon. Since the measurements in Figure 3 reflect the partitioning of a common intermediate between exchange and product formation, the marked fall-off in the ratio of these processes with decreasing pH requires different pH dependencies for exchange and product formation. In principle, the observed pH pattern could result from acid catalysis of imine hydrolysis. From a chemical perspective, such a mechanism is reasonable, since protonation of imine is expected to lead to much faster hydrolysis than that from the neutral species. However, in order to observe the profile in Figure 3, it would also be necessary to postulate an uncatalyzed exchange process that could occur from either neutral or protonated imine. We consider this possibility improbable, especially in light of the much faster rate of exchange than of hydrolysis at pH 7.0 and above (Summers et al., 1979). Thus, we attribute the data in Figure 3 to a preferential increase in exchange with increasing pH, i.e., general-base catalysis of tritium exchange from protonated imine, Scheme III.

Properties of the plasma amine oxidase reaction, which have been incorporated into Scheme III, are as follows: (i) the presence of imine hydrolysis, k_5, as a kinetically significant step for phenethylamine derivatives, in contrast to benzylamine oxidation where imine hydrolysis is fast relative to C–H bond cleavage (Palcic & Klinman, 1983); (ii) the availability of more than one pathway for imine hydrolysis, i.e., via EB_1, k_5, and EB_1H, k_5', since, as noted, we would not observe an effect of pH on the ratio of exchange to product formation if both processes required the unprotonated form of the same active site residue; (iii) the formation of an enamine upon proton removal from C-2, where enamine is formed off the reaction path; i.e., protonated imine must re-form before any significant hydrolysis occurs. The latter point takes into account an anticipated far greater reactivity of protonated imine toward addition of water [cf. Jencks (1969)]. The expression relating the relative velocities for imine exchange vs. hydrolysis to pH is given by

$$\frac{V_{exch}}{V_{hydrol}} = \frac{k_{exch}/k_5}{1 + (k_5'/k_5)([H^+]/K_{exch})} \quad (8)$$

where $pK_{exch}(app) = pK_{exch} - \log (k_5/k_5')$.

Discussion

Relationship of Observed to Microscopic pK_a Values. Several aspects of pH-dependent isotope effect data are highlighted by the studies presented herein. First, the use of alternate substrates can lead to simplified kinetic schemes in which a single step dominates k_{cat} and k_{cat}/K_m (Klinman, 1972). Measurement of kinetic isotope effects, in conjunction with initial rate parameters, provides a probe for the nature of rate-determining steps. In the special instance where the magnitude of isotope effects supports the dominance of a single step across the experimental pH range, microscopic pK_a values are obtained directly from steady-state data. As shown in Figure 1A, the pH dependence of k_{cat}/K_m for the plasma amine oxidase catalyzed oxidation of both [1,1-$^1H_2$]- and [1,1-$^2H_2$]benzylamine leads to identical pK_a values, despite a 12–13-fold reduction in rate. Thus, we conclude that C–H bond cleavage limits k_{cat}/K_m at all pH values and that measured pK_a's are microscopic values, providing $pK_1 = 7.9$–8.0 for an active site residue that must be unprotonated for catalysis and $pK_s = 9.0$–9.1 for a substrate that interacts with enzyme in a protonated form. The key aspect of the data in Figure 1 is the large reduction in rate upon isotopic substitution, coupled to no observable alterations in pH dependencies.

A more general situation arises when isotope effects are measureable but reduced from maximal values. In those instances where the magnitude of the intrinsic isotope effect is known, microscopic pK_a values can be obtained from steady-state data (Cook & Cleland, 1981a,b; Ahn & Klinman, 1983). As shown in Figure 2B, the pH dependence of $^Dk_{cat}$ indicates a transition between enzyme forms characterized by different partitioning ratios, for the formation (k_3) and reoxidation (k_7) of reduced enzyme. From the magnitude of $^Dk_3$ previously described (Palcic & Klinman, 1983), eq d in Table II leads to estimates for k_3/k_7' (above pH 7.5) and k_3/k_7 (below pH 6.5). Utilizing these partitioning ratios, the data in Figure 2 can be fit by microscopic pK_a values, $pK_1' = 5.6 \pm 0.3$ and $pK_2 = 7.25 \pm 0.25$. We note that whereas pK_1' can be attributed to an active site residue that must be unprotonated for catalysis, pK_2 reflects a very small change in k_7/k_7' (2–3-fold), making its mechanistic significance unclear at this time.

In addition to the measurement of steady-state parameters, enzyme partial reactions can provide considerable insight into chemical mechanism. In this study, we have measured the *ratio* of exchange to imine hydrolysis for dopamine. The relationship of $pK_{exch}(app)$ for base catalysis of exchange to microscopic pK_a values, eq 5, indicates the dependence of this ionization constant on k_5/k_5'. Thus, it is important to consider the anticipated impact of the ionization of EB_{exch} on imine hydrolysis. For example, it is possible that EB_{exch} plays a role in both β-exchange and imine hydrolysis. However, as discussed under Results and shown in Scheme III, the data require that imine hydrolysis occurs from both the EB_{exch} and $EB_{exch}H$ complexes, in contrast to β-exchange which is absolutely dependent on the ionized form of EB_{exch}. Since imine hydrolysis is an obligatory step in product formation whereas exchange occurs off the reaction path, such a mechanism appears highly unlikely. We therefore propose that imine hydrolysis is catalyzed by a separate functional group titrating

outside the experimental pH range of Figure 3,[5] leading to $k_5' = k_5$ in eq 8 and $pK_{exch}(app) = pK_{exch}$. We further note the magnitude of $pK_{exch} = 5.5 \pm 0.01$ is within experimental error of $pK_1' = 5.6 \pm 0.3$.

Mechanistic Implications. These studies reveal two microscopic pK_a values in the plasma amine oxidase reaction: pK_2, which has a very modest effect on catalysis and will not be considered further; $pK_1' = pK_{exch}$, which indicates an obligatory role for basic catalysis in substrate oxidation and exchange. The magnitude of pK_1' has been perturbed 2.4 pH units in proceeding from free enzyme to the E·S complex. Such a pK_a perturbation may result from charge repulsion upon combination of protonated substrate with cofactor in proximity to a cationic active site residue (see below).

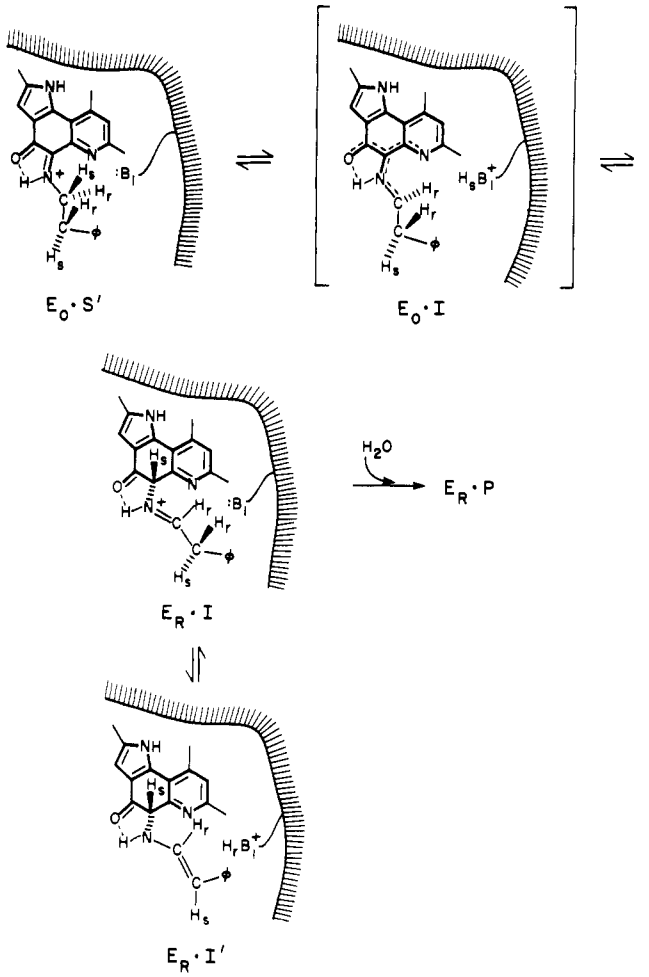
A major mechanistic dilemma that these studies have attempted to address is the large difference in exchange rates for hydrogens derived from C-1 and C-2 of phenethylamine derivatives, which led to the proposal of separate active site residues catalyzing oxidation and exchange (Palcic & Klinman, 1983) vs. the observation of a syn stereochemical relationship for hydrogen loss from C-1 and C-2, consistent with a single active site base (Farnum & Klinman, 1985). The current studies, which reveal almost identical microscopic pK_a values for substrate oxidation and exchange, provide support for a single catalytic activity. Thus, the origin of an apparent sequestration of hydrogen derived from C-1 of substrate at the active site remains to be explained.

As indicated in the introduction, two laboratories have recently presented evidence that pyrroloquinoline quinone functions as cofactor in the bovine plasma amine oxidase reaction. For example, Lobensteen-Verbeek et al. (1984) have prepared a dinitrophenylhydrazone derivative of the enzyme-bound cofactor and shown that limited proteolysis followed by HCl hydrolysis leads to an adduct that coelutes on HPLC with an authentic derivative of pyrroloquinoline quinone. However, it should be noted that the yield of enzyme-derived product was only 6%, and isolation and characterization of the native cofactor has not yet been achieved. In the case of studies by Ameyama et al. (1984), fluorescence spectra of native enzyme and pyrroloquinoline quinone were compared and shown to be identical. Clearly, more precise and quantitative data are needed regarding the nature of the active site cofactor of bovine plasma amine oxidase. Nonetheless, the available findings are quite suggestive and provide a working basis for the interpretation of kinetic data.

As shown in Scheme IV, and by analogy to model studies conducted by Eckert et al. (1983), the formation of a covalent adduct between substrate and the C-5 carbonyl of cofactor will precede catalysis. In light of the subsequent chemistry, product imine is expected to remain protonated. In fact, we have no evidence for imine ionization in these studies, and it is likely that hydrogen bonding to the neighboring carbonyl (or pyridine ring) would increase the imine pK_a above the experimental pH range. The retention of a positive charge on bound imine could also provide a simple charge-repulsion mechanism for the perturbation of EB_1, ensuring that this essential residue remains in the correct prototropic form for catalysis of substrate oxidation. The cofactor structure would be expected to facilitate a base-catalyzed proton abstraction from substrate, which is shown leading to a short-lived, delocalized carbanion intermediate. If we allow reprotonation of this intermediate to be kinetically fast relative to exchange,

[5] Given the rapid rate of imine hydrolysis in benzylamine oxidation, it appears likely that the group catalyzing imine hydrolysis is fully ionized across the experimental pH range, i.e., $pK_{hyd} < pK_{exch}$.

Scheme IV: Postulated Mechanism for Bovine Plasma Amine Oxidase, in Which a Single Active Site Residue, EB_1, Catalyzes both Substrate Oxidation and Imine Exchange[a]



[a] The processing of (1*S*,2*R*)-phenylethylamine has been shown for illustrative purposes. The stereochemical relationship between C-1 and C-2 derives from recent findings (Farnum & Klinman, 1985) indicating a syn relationship in the oxidation of dopamine.

$E_0 \cdot I$ would be rapidly and quantitatively converted to either $E_0 \cdot S$ or $E_R \cdot I$, *thereby preventing an exchange of substrate-derived proton or deuteron with solvent.* This mechanism corresponds to a 1,3-prototropic shift, analogous to the better characterized transamination reactions catalyzed by pyridoxal phosphate dependent enzymes (Walsh, 1979). Such a hydrogen-transfer mechanism would lead to the regeneration of the basic form of EB_1, which is postulated to catalyze a subsequent exchange of the β-hydrogen of phenethylamines with solvent. As indicated in Scheme IV, proton abstraction from imine leads to the thermodynamically more stable enamine. We propose that the stability of this enamine, coupled to the absence of a proton acceptor in $E_R \cdot I'$, is the cause of the rapid and complete exchange of the C-2 hydrogen of phenethylamines with solvent. Since $E_R \cdot I'$ lies off the reaction path, reprotonation of enamine is a prerequisite for product formation. Hence, the pK_a of EBH in $E_R \cdot I'$ is expected to contribute to product formation in the turnover of phenethylamine derivatives. It will be of considerable interest to see if this pK_a can be detected from steady-state kinetic studies with dopamine and whether its magnitude is more similar to the (putatively) charge-perturbed pK_1' vs. the pK_1 of free enzyme.

As the above discussion indicates, the newly proposed cofactor provides a framework for the satisfactory integration of the kinetic and stereochemical properties of bovine plasma

amine oxidase. Further, the mechanism in Scheme IV makes a wide range of predictions that can be examined experimentally, such as the formation of covalent adducts between substrate and enzyme, the transfer of both hydrogen and nitrogen from substrate to cofactor, and the activation of substrate via a proton activation mechanism. Although the former two questions have yielded negative results when addressed by former investigators (Suva & Abeles, 1978; Berg & Abeles, 1980), they clearly require reinvestigation in light of the unusual chemical properties of pyrroloquinoline quinone. In support of a proton activation mechanism, Abeles et al. (1975) have previously demonstrated an enzyme-catalyzed elimination of HCl from β-chlorophenethylamine. Structure–reactivity correlations currently under way in our laboratory are expected to provide evidence concerning the mode of C–H bond cleavage in the course of substrate (benzylamine) oxidation.

Relationship between Monoamine and Plasma Amine Oxidases. There has been considerable speculation regarding the relationship of plasma amine oxidases from different sources to one another and the extent of similarity of amine oxidases from plasma to the FAD-containing monoamine oxidase (Hamilton, 1983). Lindstrom et al. (1976) and Olsson et al. (1976) have examined pre-steady-state pH dependencies for amine oxidase from pig plasma, observing a pH-independent rate for enzyme reduction under conditions of saturating benzylamine. By contrast, a bell-shaped curve was observed for the pH dependence of K_m, with maximal activity correlating with the basic form of a catalytic residue, pK = 8.8, and the protonated form of substrate, pK = 9.3. Deuterium isotope effects, determined at two pH values (7 and 9), were 2.8 and 2.7, respectively. Although these values are significantly reduced relative to that of the bovine plasma enzyme, they indicate partial rate limitation by the C–H bond cleavage step. Thus, in analogy with bovine plasma amine oxidase, the pig plasma enzyme appears to require the ionized form of a catalytic residue and the protonated form of substrate in the conversion of oxidized enzyme and substrate to the reduced enzyme–imine intermediate, with both enzyme and substrate pK_a values undergoing significant perturbation upon formation of the enzyme–substrate complex.

Despite considerable interest and activity in the pharmacology of monoamine oxidases, relatively little is known regarding the molecular mechanisms of these enzymes. Recent measurements of deuterium isotope effects for the bovine liver monoamine oxidase catalyzed oxidation of benzylamine reveals $^{D}k_{cat} = {}^{D}(k_{cat}/K_m) = 7$ at pH 7.5, indicating that both parameters are significantly limited by the rate of enzyme reduction (Husain et al., 1982). Reexamination of an early pH study by Oi et al. (1971) using the same enzyme source and substrate shows a dependence of *both* k_{cat} and k_{cat}/K_m on the ionized form of enzyme, pK = 7.1–7.3 (atmospheric O_2); these results support a role for a basic residue in catalysis but also rule out a significant perturbation in the pK_a of this residue upon forming the enzyme–substrate complex. Thus, bovine liver monoamine oxidase differs from plasma amine oxidase in several ways, which include (i) the absence of a pK perturbation for an essential catalytic residue in the E·S complex, (ii) the inability of enzyme to catalyze imine hydrolysis (Suva & Abeles, 1978), and (iii) the absence of hydrogen exchange from the β-carbon of phenethylamines (Lovenberg & Beaven, 1971). Clearly significant differences exist between these enzymes, which may derive in large part from the unique properties of the covalent complexes postulated in Scheme IV for plasma amine oxidase.

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