Mechanism of Inhibition of Dopamine β-Monooxygenase by Quinol and Phenol Derivatives, As Determined by Solvent and Substrate Deuterium Isotope Effects†

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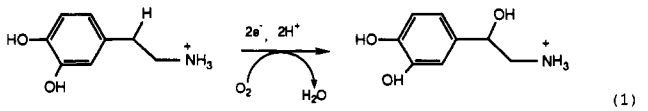
Received March 18, 1991; Revised Manuscript Received May 22, 1991

ABSTRACT: The mechanism of interaction of quinols and phenols with dopamine β-monooxygenase (DβM) has been investigated. The ratio of quinone formation (from catechol) to oxygen consumption rises from a value of 1 in the presence of phenethylamine substrate to 2 in the absence of substrate. These results implicate quinol oxidation at both the reductant- and substrate-binding sites of DβM. In the presence of saturating ascorbate, catechol and *p*-hydroquinol behave as mechanism-based inhibitors of DβM, with partitioning ratios of turnover to inactivation of 21:1 and 41:1, respectively. Phenol is found to inactivate the enzyme in a manner similar to *p*-cresol, suggesting that the methyl group of *p*-cresol is not an essential component of enzyme inhibition. Solvent isotope effects on inactivation and turnover have been measured for various inactivators. Although the majority of these inhibitors, including catechol, *p*-hydroquinol, aniline, phenethylenediamine, and benzylhydrazine, are characterized by relatively small solvent isotope effects (1.5–2.5) on the inactivation rate constant (k_i), solvent isotope effects on k_i for phenol and *p*-cresol are 5.7 and 7.4, respectively. By contrast, solvent isotope effects on the turnover of *p*-cresol are almost unity. Using *p*-cresol-d_7 as substrate, we observe $^D(k_{cat}) = 5.2$ and $^D(k_{cat}/K_m) = 3.1$, while isotope effects on inactivation are $^D(k_i) = 0.95$ and $^D(k_i/K_i) = 0.59$. These results lead us to propose that inhibitors fall into two mechanistic classes, involving either one-electron oxidation to form radical cation intermediates (quinols) or hydrogen atom abstraction (phenols). In the case of cresol inactivation, we conclude that turnover involves C–H cleavage, whereas inactivation involves O–H bond cleavage. The inverse isotope effects on inactivation with *p*-cresol-d_7 are shown to be the result of an isotope-dependent change in the partitioning between turnover and inactivation.

Dopamine β-monooxygenase (DβM)[1] is a copper-containing enzyme catalyzing a hydroxylation reaction at the β-carbon of dopamine to form the neurotransmitter and hormone norepinephrine (Skotland & Ljones, 1970; Rosenberg & Lovenberg, 1980; Villafranca, 1981; Stewart & Klinman, 1988).



(1)

As illustrated, the net reaction catalyzed by DβM requires two electrons and two protons. Ascorbate is generally ascribed the role of electron donor, given its efficiency in electron transfer to the enzyme-bound copper atoms and its presence in the DβM-containing storage vesicles of the adrenal gland and the sympathetic nervous system (Skotland & Ljones, 1980; Diliberto & Kaufman, 1981). Although cleavage at the β-carbon of the substrate has been shown to occur by hydrogen atom abstraction, generating a benzyl radical as the key intermediate (Miller & Klinman, 1983; Miller & Klinman, 1985), many details of the enzymatic reaction mechanism remain unclear.

Given its central role in the biosynthesis of norepinephrine and epinephrine, DβM has been a target for the rational design of inhibitors (Kruse et al., 1986; Bargar et al., 1986). During the last decade, a number of mechanism-based inhibitors have been developed with the dual goals of elucidating the enzymatic mechanism and of developing antihypertensive drugs (Fitzpatrick & Villafranca, 1987). Analogous to the proposed mechanism for substrate turnover, all mechanism-based inhibition can be rationalized by the generation of an unstable radical intermediate via either hydrogen atom abstraction from a benzylic carbon or electron abstraction from electron-donating groups by an unidentified copper-bound oxygen species (Stewart & Klinman, 1988). It is still not possible, however, to predict the precise structural features that will lead to enzyme inactivation vs turnover.

Goodhart et al. (1987) have reported that *p*-cresol is a mechanism-based inhibitor of DβM, proposing C–H cleavage to form a (4-hydroxyphenyl)methyl radical as the intermediate in both turnover and inactivation. These results suggested that the aminomethyl side chain, which is attached to the reactive benzyl radical position in phenethylamine substrates, may prevent inactivation under normal turnover conditions (Stewart & Klinman, 1988). In an effort to explore further the nature of the reactive intermediate(s) in *p*-cresol inactivation and turnover, we have examined structures similar to *p*-cresol but lacking the methyl group. As we describe, catechol, *p*-hydroquinol, and phenol inactivate DβM in pseudo-first-order processes; oxygen consumption is also detected when DβM is incubated with catechol or *p*-hydroquinol. Investigation of solvent isotope effects on inactivation with these new inactivators, as well as solvent and substrate isotope effects with *p*-cresol, leads to the conclusion that quinols inactivate DβM by a one-electron oxidation process, whereas phenols undergo O–H cleavage. These studies provide the first evidence for an O–H bond cleavage process, catalyzed by dopamine β-monooxygenase.

Materials and Methods

All chemicals were reagent grade, unless otherwise noted. Phenol, catechol, *p*-hydroquinol, *p*-cresol, aniline, phenethylenediamine (PEDA), L-ascorbate, and benzylhydrazine

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[1] Abbreviations: DβM, dopamine β-monooxygenase; PEDA, phenethylenediamine; MES, 2-(*N*-morpholino)ethanesulfonic acid.

0006-2960/91/0430-8138$02.50/0

were purchased from Aldrich Chemical Co. and used without further purification. Tyramine hydrochloride and disodium fumarate were purchased from Sigma Chemical Co. *p*-Cresol-d_7 and deuterium oxide (99.9% purity) were purchased from Cambridge Isotope Laboratories. Catalase (sp act. = 5×10^4 units/mg) was purchased from Boehringer-Mannheim. Soluble dopamine β-monooxygenase was isolated from bovine adrenal glands as previously described (Miller & Klinman, 1983; Miller & Klinman, 1985). Oxygen consumption assays were performed on a Yellow Springs Instrument model 53 biological oxygen monitor. Absorbance spectroscopy determinations were carried out on a Cary 118 UV–Vis spectrometer.

Enzyme Turnover. Rates of oxygen consumption were determined in 1-mL reaction solutions containing 100 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer, pH 5.5 or pD 5.9, 10 mM ascorbate, 10 mM disodium fumarate, 100 μg/mL catalase, 200 mM NaCl, 2 μM $CuSO_4$, and various concentrations of substrate at 37 °C. Reactions were initiated by adding DβM. MES, disodium fumarate, NaCl, and $CuSO_4$ were dissolved in D_2O, rotary evaporated twice, and dissolved again in D_2O for complete removal of H_2O. Since 0.005 mL of catalase and at most 0.005 mL of DβM were used for each 1-mL assay solution, the content of D_2O in solution was 98.9% or greater.

Inhibition Studies. Dopamine β-monooxygenase was incubated at 37 °C in 0.4-mL solutions containing 100 mM MES, pH 5.5 or pD 5.9, 10 mM ascorbate, 10 mM disodium fumarate, 100 μg/mL catalase, 2 μM copper sulfate, and various concentrations of inhibitor. Inhibition reactions were started by adding 14.8 μg (2 μM) of DβM. At various time intervals, 0.02-mL aliquots were taken and assayed for residual activity. Assay solutions contained 100 mM MES, pH 5.5, 10 mM ascorbate, 10 mM fumarate, 100 μg/mL catalase, 200 mM NaCl, 12 μM copper, and 5 mM tyramine and were measured from the rate of oxygen consumption. Values for k_i at each inhibitor concentration were determined from linear least-squares fitting of time vs the natural log values of initial velocities.

Turnover with Alternate Reductants. Quinols can substitute for ascorbate in the DβM reaction. 10 mM quinol was incubated with 100 mM MES, pH 6.6, 10 mM fumarate, 100 μg/mL catalase, 200 mM NaCl, 2.0 μM $CuSO_4$, 10 mM tyramine, and 0.37 mg/mL DβM. The consumption of oxygen was followed by an oxygen electrode. In the case of catechol, *o*-benzoquinone formation was assayed at 380 or 340 nm by coupling with 0.2 mM NADH (Levin & Kaufman, 1961). Experiments were carried out both in the presence and absence of tyramine.

Analysis of Initial Velocity Data. All initial velocity data were analyzed by fitting to the computer program HYPER, which was written by Cleland (1979) and translated into BASIC for use on a Northstar Horizon computer. Enzyme activities vs substrate or inhibitor concentrations were fit to

$$v = k_{cat}(k_i)[E_T][S]/[K_m(K_i) + [S]] \quad (2)$$

Deuterium isotope effects and solvent isotope effects were calculated from the ratios of the kinetic parameters k_{cat}, k_{cat}/K_m, k_i, and k_i/K_i.

Results and Discussion

Quinols Function as Both Reductant and Substrate. Levin and Kaufman (1961) reported that catechol was a good substitute for ascorbate in the DβM reaction. The rate with catechol was approximately 10% of the rate with ascorbate in the absence of fumarate at pH 6.5 and was almost equal in the presence of fumarate. The product after two-electron oxidation was identified as *o*-benzoquinone, from its ability to oxidize NADH and its characteristic absorbance maximum at 380 nm. We have examined *p*-hydroquinol, as well as reinvestigated the role of catechol, as a replacement for ascorbate, with the relative rates for enzyme turnover summarized in Table I. An apparent ability of phenol to act as reductant, albeit at a very low rate, was unexpected. Although this was not pursued, it is consistent with an earlier result from this laboratory in which high concentrations of DβM were shown to catalyze a low level hydroxylation of tyramine in the absence of added reductant (Brenner and Klinman, unpublished results).

Table I: Turnover of Dopamine β-Monooxygenase with Alternate Reductants[a]

reductant	turnover rate (μmol/mg·min)
ascorbate	5.64 (1.0)
catechol	4.14 (0.73)
p-hydroquinol	2.38 (0.42)
phenol	0.194 (0.034)

[a] All kinetic parameters were obtained at a single oxygen concentration (0.2 mM), 37 °C, and pH 6.6. Assay mixtures contained 100 mM MES, 10 mM fumarate, 200 mM NaCl, 100 μg/mL catalase, 2 mM $CuSO_4$, 10 mM tyramine, and reductant at 10 mM. The values in parentheses are rates relative to ascorbate.

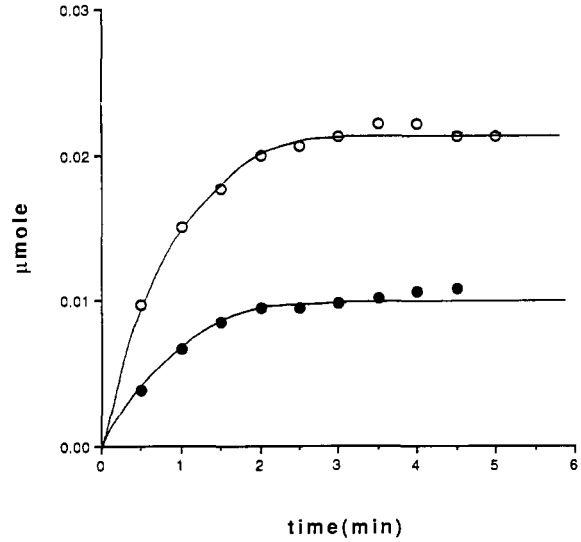


FIGURE 1: Time course of oxygen consumption and formation of *o*-benzoquinone when catechol was incubated with DβH in the absence of ascorbate and phenethylamine substrate: (●) oxygen consumption, (○) formation of *o*-benzoquinone. Experimental details are described under Materials and Methods.

Analogous to earlier studies (Levin & Kaufman, 1961), we observed a 1:1 stoichiometry between oxygen consumption and *o*-benzoquinone formation for the oxidation of catechol in the presence of tyramine. When tyramine is omitted from incubations, oxygen consumption is also observed. Although formation of benzoquinone and uptake of oxygen plateau fairly rapidly under these conditions due to enzyme inactivation (see below), *the stoichiometry between these processes is now 2:1* (Figure 1). This suggests that catechol can function to recycle the reduced enzyme by undergoing oxidation to *o*-benzoquinone at the substrate hydroxylation site. Subsequent studies focused on quinol oxidation in the presence of excess ascorbate to saturate the reductant site. As predicted, significant rates were observed for both quinol and *p*-hydroquinol turnover, 0.34 s^{-1} and 0.26 s^{-1}, respectively (Table II).

Quinols and Phenols as Enzyme Inhibitors. Catechol was found to be a very effective mechanism-based inhibitor. Figure

Table II: Comparison of Turnover to Inactivation of Dopamine β-Monooxygenase with Quinols and Phenols[a]

compound	k_{cat} (s^{-1})	K_m (mM)	k_i (s^{-1})	K_i (mM)	k_{cat}/k_i
catechol	0.336	3.04	0.0159	3.46	21.1
p-hydroquinol	0.225	1.65	0.00550	2.74	40.9
phenol	NR[b]	NR[b]	0.00173	4.28	
p-cresol[c]	3.33	1.50	0.00312	2.53	1070

[a] All kinetic parameters were obtained at a single oxygen concentration (0.2 mM), 37 °C, and pH 5.5 in the presence of 10 mM ascorbate as reductant. (See Materials and Methods for details of enzyme turnover and inhibition studies.) [b] No reaction. [c] According to DeWolf et al. (1988) at pH 5, k_{cat} = 8.8 s^{-1}, K_m = 2.4 mM, k_i = 0.0050 s^{-1}, and K_i = 2.0 mM.

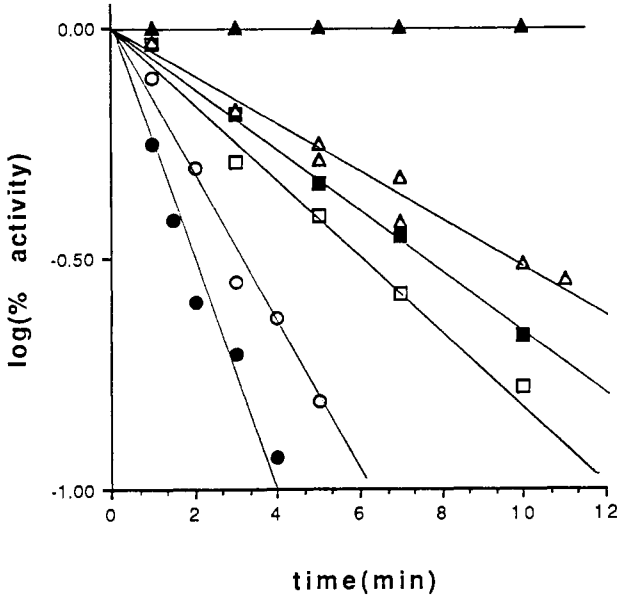


FIGURE 2: Kinetics of inactivation of DβH by catechol. The time course of inactivation of DβH is shown with various concentration of catechol: (●) 5.0 mM, (○) 2.0 mM, (□) 1.0 mM, (■) 0.7 mM, (△) 0.5 mM, and (▲) 0 mM. Experimental details are described under Materials and Methods.

2 shows the time- and concentration-dependent inactivation of DβM by catechol, producing k_i = 0.0159 s^{-1} and K_i = 3.46 mM (Table II). The partitioning ratio between turnover and inactivation is 21:1. Inhibition was also observed with *p*-hydroquinol and phenol, and the kinetic parameters are listed in Table II. Extensive dialysis of enzyme inactivated by catechol, *p*-hydroquinol, or phenol did not lead to a recovery of activity.

Goodhart et al. (1987) have reported that *p*-cresol is both a substrate and a mechanism-based inhibitor of DβM. We observe parameters for the interaction of DβM with *p*-cresol (Table II) that are similar to those previously reported. In the earlier work of Kruse and co-workers, amino acid analysis of inactivated DβM indicated two chemically distinct covalent adducts of cresol with an active-site tyrosine residue, attributed to (4-hydroxyphenyl)methyl-Tyr and (4-methylphenyl)oxy-Tyr. A hypothesis was put forth involving the initial generation of (4-hydroxyphenyl)methyl radical, followed by rearrangement to a (4-methylphenyl)oxy radical (DeWolf et al., 1988). Our present results, indicating that catechol, *p*-hydroquinol, and phenol are DβM inhibitors, suggest that the methyl group of *p*-cresol may not be a prerequisite for inactivation. This point was pursued further through the use of solvent and substrate isotope effects.

Solvent Isotope Effects. Earlier studies of Wimalasena and May (1987) suggested that aniline and PEDA inactivate DβM by the generation of radical cation intermediates at nitrogen.

Table III: Solvent Isotope Effects on Kinetic Parameters[a]

compound	$^{D}k_i$	$^{D}(k_i/K_i)$	$^{D}k_{cat}$	$^{D}(k_{cat}/K_m)$
p-cresol	7.32 ± 0.83	3.83 ± 0.94	1.10 ± 0.06	0.96 ± 0.14
phenol	5.66 ± 0.78	2.49 ± 0.87		
catechol	2.54 ± 0.28	1.44 ± 0.20	1.69 ± 0.21	2.59 ± 0.58
p-hydroquinol	1.84[b]			
aniline	1.70[b]			
PEDA	1.50[b]			
benzyl-hydrazine	2.15[b]			

[a] All kinetic parameters were obtained at a single oxygen concentration (0.2 mM), 37 °C, and pH 5.5 or pD 5.9 in the presence of 10 mM ascorbate as reductant. (See Materials and Methods for details of enzyme turnover and inhibition studies.) [b] Measured at a single concentration of inhibitor (10 mM).

Table IV: Substrate and Solvent Isotope Effects with *p*-Cresol[a]

	$^{D}k_i$	$^{D}(k_i/K_i)$	$^{D}k_{cat}$	$^{D}(k_{cat}/K_m)$
substrate	0.95 ± 0.34	0.59 ± 0.20	5.19 ± 0.45	3.05 ± 0.31
solvent	7.32 ± 0.83	3.80 ± 0.94	1.10 ± 0.06	0.96 ± 0.14

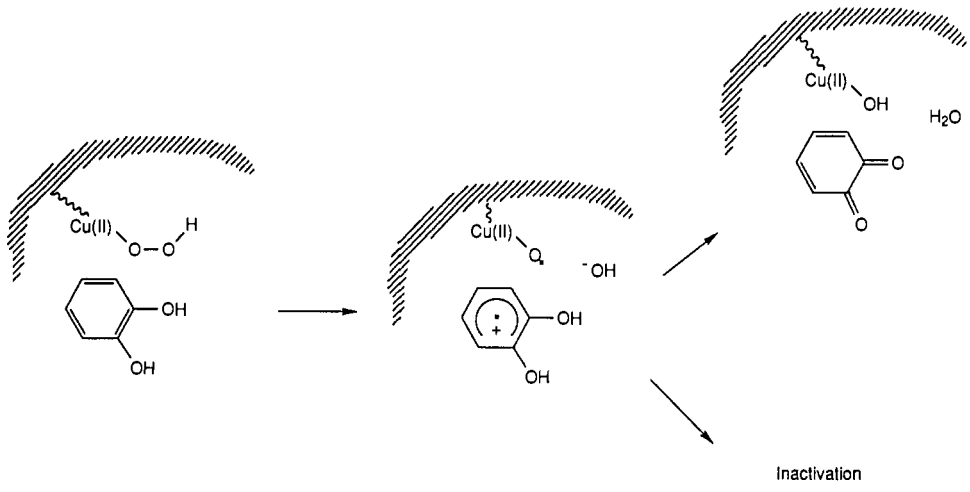
[a] Kinetic parameters obtained at a single oxygen concentration (0.2 mM), 37 °C, and pH 5.5 or pD 5.9 in the presence of 10 mM ascorbate as reductant. (See Materials and Methods for details of enzyme turnover and inhibition studies.)

We therefore used this class of inhibitors as a frame of reference in evaluating solvent isotope effects. As shown in Table III, measured solvent isotope effects fall between 1.5 and 2. Modest effects in this range can be ascribed to a cumulative effect of D_2O on pK_a perturbations and protein structure and are consistent with a radical abstraction mechanism in the course of enzyme inhibition. Analogous to amine inhibitors, catechol and *p*-hydroquinol show relatively small effects of D_2O (Table III). Solvent isotope effects on turnover of catechol are not very different from those on inactivation, which suggests that the character of the intermediates involved in inactivation and turnover is similar. The inactivation data for phenol and cresol are in marked contrast to quinols, with solvent isotope effects for k_i of 5.7 and 7.3, respectively, implicating cleavage of a solvent-exchangeable hydrogen as a key step in the inactivation process. As seen in Table III, solvent isotope effects on turnover of *p*-cresol are almost unity for both k_{cat} and k_{cat}/K_m, within the error range. These differences between $^{D}(k_{cat})$ and $^{D}(k_i)$ implicate divergent pathways for enzyme turnover and inactivation with *p*-cresol.

Substrate Isotope Effects. The mechanism of DβM with *p*-cresol was pursued further through the use of *p*-cresol-d_7. As shown in Table IV, substrate isotope effects on turnover are 5.19 and 3.05 for k_{cat} and k_{cat}/K_m, respectively. Although these isotopes effects reflect perdeuterated substrate, the effect of phenyl ring deuteration is expected to be quite small, on the basis of an earlier study of secondary tritium isotope effects by Miller and Klinman (1983). We therefore attribute these results to a kinetically significant hydrogen atom abstraction from the benzylic position. This view is supported by the nature of the products formed on cresol oxidation (Goodhart et al., 1987).

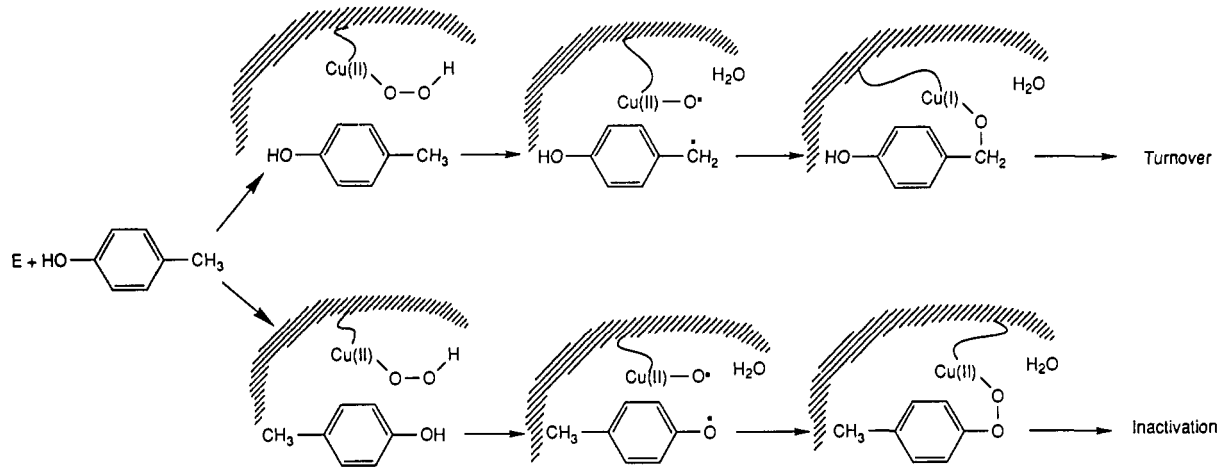
When substrate deuterium isotope effects on inactivation were measured, they were found to be almost unity (0.95) on k_i but inverse (0.59) on k_i/K_i. This important result argues against hydrogen abstraction from the benzylic position during the inactivation process and is consistent with the conclusion of involvement of a solvent exchangeable position (from solvent isotope effects; Table III). Inverse isotope effects on inactivation have been reported with dideuterated benzylhydrazine (Fitzpatrick & Villafranca, 1986) and dideuterated PEDA (Wimalasena & May, 1987). Kinetic isotope effects on

Scheme I[a]



[a] Proposed mechanism for enzyme turnover (upper pathway) and inactivation (lower pathway) with quinols.

Scheme II[a]



[a] *p*-Cresol is proposed to partition between binding modes that place either the methyl or hydroxyl group in position for hydrogen abstraction, leading to turnover (upper pathway) vs inactivation (lower pathway).

turnover with benzylhydrazine were 13, but 0.25 on k_i; in the case of PEDA, the k_{cat} isotope effect was 2.8 and that on k_i was unity. In both instances, these observations have been attributed to partitioning between hydrogen abstraction for turnover and electron transfer for inactivation. In a similar manner, the observed inverse isotope effects for *p*-cresol inactivation can be ascribed to partitioning between hydrogen atom abstraction from the benzylic carbon in turnover vs hydrogen abstraction from a solvent-exchangeable position during inactivation.

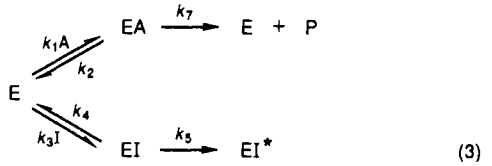
Mechanistic Implications. A major finding from the present study is that simple quinols undergo oxidation within the substrate-binding site of DβM. As summarized in Table II, catechol and *p*-hydroquinol show partitioning ratios for turnover vs inactivation of 21:1 and 41:1, respectively. Solvent isotope effects on both inactivation and turnover are relatively small and in the range seen with amine-containing inactivators, which are thought to act via one-electron transfer processes (Table III). Taken together, the available data support electron transfer processes in both turnover and inactivation. Extensive studies of DβM have implicated an active-site Cu(II)–OOH as the hydroxylating agent (Brenner & Klinman, 1989). As shown in Scheme I, placement of quinol into this active site is expected to lead to a rapid one-electron reductive cleavage of Cu(II)–OOH to produce either OH^- and Cu(II)–$O^•$ or $OH^•$ and Cu(II)–O^- (plus the radical cation derived from quinol). Either of these intermediates should lead to facile oxidation/labeling of active-site residues (inhibition) as well as further oxidation to quinone (turnover).

The behavior of phenol and cresol is markedly different from quinols, consistent with the lower reducing potential of these compounds. As summarized in Table III, solvent isotope effects on enzyme inactivation are very large [$^D(k_i)$ = 6–7], providing evidence for O–H cleavage as the key inactivation event. Whereas phenol and cresol show similar rate constants and isotope effects on inactivation, only cresol produces enzyme turnover. The resulting implication, that C–H cleavage is essential for cresol turnover, has been confirmed through studies of solvent and substrate isotope effects. As seen in Table IV, cresol turnover yields a substrate isotope effect on k_{cat} of 5, in contrast to a solvent isotope on k_{cat} of unity.

A plausible mechanism to explain the behavior of *p*-cresol is presented in Scheme II. As illustrated, cresol can bind to DβM in two unique orientations with regard to active-site Cu(II)–OOH. Placement of the methyl group near the postulated hydroxylating species [Cu(II)–OOH] leads to a (4-hydroxyphenyl)methyl radical, which upon recombination with a reactive Cu(II)–$O^•$ intermediate, yields product. An analogous mechanism has been proposed for the hydroxylation of phenethylamine substrates (Miller & Klinman, 1985). By contrast, when cresol binds with its hydroxyl group closer to copper-bound peroxide, a hydrogen atom will be lost from the

hydroxyl group to produce a (4-methylphenyl)oxy radical. In this case, the rebound reaction generates 4-peroxytoluene, an unstable intermediate that is expected to react further with one (or more) active-site residues. We note that the postulate that cresol undergoes hydrogen atom abstraction from either its methyl or hydroxyl group is compatible with the similarity of bond dissociation energies at these positions [88 and 87 kcal/mol, respectively (Weast, 1971)].

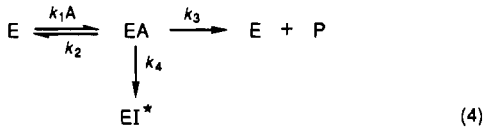
The equations for substrate and solvent deuterium isotope effects on turnover and inactivation have been derived in the Appendix for Scheme II. One feature, which was initially incorporated into Scheme II, involves discrimination between the orientations leading to turnover vs inactivation during the initial binding process



(3)

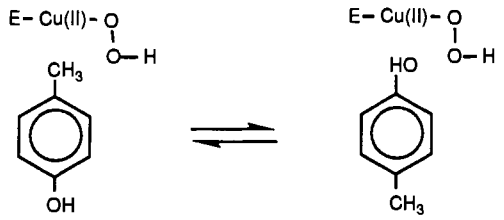
We find that this feature leads to substrate isotope effect equations for inactivation with the property $^{D}k_i > 1$ and $^{D}(k_i/K_i) = 1$. Inspection of Table IV, however, indicates that $^{D}k_i = 0.95 \pm 0.34$ and $^{D}(k_i/K_i) = 0.59 \pm 0.20$. Thus, either Scheme II is wrong or it must be modified to accommodate the available data.

We therefore examined the form of isotope effect equations for a mechanism in which the partitioning between turnover and inactivation occurs from a common enzyme intermediate

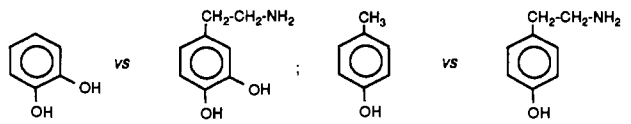


(4)

This minor modification, involving partitioning between inactivation and turnover *after* binding of cresol, leads to equations for isotope effects with the property $^{D}(k_i) = 1$ and $^{D}(k_i/K_i) < 1$, fully compatible with the experimental data (Table IV). We propose that the small size of *p*-cresol, relative to normal substrates, permits an active-site rotation that is fast on the time scale of either C–H or O–H cleavage:



The rather large partitioning toward turnover ($k_{cat}/k_i = 1100$; Table II) most likely reflects a preferential orientation of the hydroxyl group away from the Cu(II)–OOH species, since, as noted earlier, bond dissociation energies for cleavage of O–H and C–H from *p*-cresol are almost equivalent. One very interesting feature of the quinol and phenol inactivators described within this paper is that they represent subsets of the normal substrates, dopamine and tyramine:



quinol dopamine phenol tyramine

Yet, neither dopamine nor tyramine has been reported to lead to significant enzyme inhibition. It appears that the aminomethyl group of phenethylamine substrates is a critical structural feature in preventing an active site binding orientation, which we now know is capable of rapid and irreversible enzyme inhibition.

Appendix: Derivation of Substrate and Solvent Isotope Effects on Turnover and Inactivation Based on Eqs 3 and 4 in the Text

(I) Equation 3 in the Text

From the steady-state approximation for eq 3

$$d[EA]/dt = k_1[A][E] - (k_2 + k_7)[EA] = 0 \quad (A1)$$

$$d[EI]/dt = k_3[A][E] - (k_4 + k_5)[EI] = 0 \quad (A2)$$

$$[E_T] = [E] + [EA] + [EI] + [EI^*] \quad (A3)$$

From eqs A1 and A2

$$[EA] = \frac{k_1[A][E]}{k_2 + k_7} \quad (A4)$$

$$[EI] = \frac{k_3[A][E]}{k_4 + k_5} \quad (A5)$$

Equations A4 and A5 give

$$[EA]\frac{k_2 + k_7}{k_1} = [EI]\frac{k_4 + k_5}{k_3} \quad (A6)$$

After substitution of eq A3 with eqs A4, A5, and A6, eq A3 becomes

$$[E_T] = [EA]\left[\frac{k_2 + k_7}{k_1[A]} + 1 + \frac{(k_2 + k_7)k_3}{k_1(k_4 + k_5)}\right] + [EI^*] \quad (A7)$$

$$[E_T] = [EI]\left[\frac{k_4 + k_5}{k_3[A]} + \frac{k_1(k_4 + k_5)}{(k_2 + k_7)k_3} + 1\right] + [EI^*] \quad (A8)$$

(*A*) *Turnover.* Since there is no inactivated enzyme at the initial point of reaction, $[EI^*] = 0$ in eq A7 in initial velocity studies. Then, eq A7 becomes

$$[E_T] = [EA]\left[\frac{k_2 + k_7}{k_1[A]} + 1 + \frac{(k_2 + k_7)k_3}{k_1(k_4 + k_5)}\right] \quad (A9)$$

Therefore,

$$v_{cat} = \frac{dP}{dt} = k_7[EA] = \frac{k_1k_7(k_4 + k_5)[A][E_T]}{(k_2 + k_7)(k_4 + k_5) + [k_3(k_2 + k_7) + k_1(k_4 + k_5)][A]} \quad (A10)$$

$$k_{cat} = \frac{k_1k_7(k_4 + k_5)}{k_1(k_4 + k_5) + k_3(k_2 + k_7)} \quad (A11)$$

$$\frac{k_{cat}}{K_M} = \frac{k_1k_7}{k_2 + k_7} \quad (A12)$$

(1) Substrate isotope effects (when k_7 is isotope sensitive) are

$$^{D}k_{cat} = \frac{^{D}k_7 + [k_3k_7/[k_2k_3 + k_1(k_4 + k_5)]]}{1 + [k_3k_7/[k_2k_3 + k_1(k_4 + k_5)]]} \quad (A13)$$

$$^{D}(k_{cat}/K_M) = \frac{^{D}k_7 + (k_7/k_2)}{1 + (k_7/k_2)} \quad (A14)$$

(2) Solvent isotope effects (when k_5 is isotope sensitive) are

$$^{D}k_{cat} = \frac{1 + (k_5/k_4)}{^{D}k_5 + (k_5/k_4)} \frac{^{D}k_5 + [k_1k_5/[k_1k_4 + k_3(k_2 + k_7)]]}{1 + [k_1k_5/[k_1k_4 + k_3(k_2 + k_7)]]} \quad (A15)$$

$$^{D}(k_{cat}/K_M) = 1 \quad (A16)$$

Since the inactivation rate constant (k_5) is much smaller than the other rate constants, eq A15 for $^{D}k_{cat}$ becomes almost unity.

(*B*) *Inactivation.*

$$d[EI^*]/dt = k_5[EI] \quad (A17)$$

From eq A8

$$d[EI^*]/dt = \frac{k_3k_5(k_2 + k_7)[A]([E_T] - [EI^*])}{(k_2 + k_7)(k_4 + k_5) + [k_3(k_2 + k_7) + k_1(k_4 + k_5)][A]} \quad (A18)$$

$$\frac{-dE_T}{[E_T] - [EI^*]} = \frac{k_3k_5(k_2 + k_7)[A]dt}{(k_2 + k_7)(k_4 + k_5) + [k_3(k_2 + k_7) + k_1(k_4 + k_5)][A]} \quad (A19)$$

$$k_i = \frac{k_3k_5(k_2 + k_7)}{k_1(k_4 + k_5) + k_3(k_2 + k_7)} \quad (A20)$$

$$\frac{k_i}{K_I} = \frac{k_3k_5}{k + k_5} \quad (A21)$$

(1) Substrate isotope effects (when k_7 is isotope sensitive) are

$$^{D}k_i = \frac{1 + (k_7/k_2)}{^{D}k_7 + (k_7/k_2)} \frac{^{D}k_7 + [k_3k_7/[k_2k_3 + k_1(k_4 + k_5)]]}{1 + [k_3k_7/[k_2k_3 + k_1(k_4 + k_5)]]} \quad (A22)$$

$$^{D}(k_i/K_I) = 1 \quad (A23)$$

The magnitude of $^{D}k_i$ depends on the relationship between k_7/k_2 and $k_3k_7/[k_2k_3 + k_1(k_4 + k_5)]$. If $k_3 \simeq k_1$, then $k_7/(k_2 + k_4 + k_5) < k_7/k_2$, and the value of $^{D}k_7$ is predicted to be normal. As seen from eq A23, $^{D}(k_i/K_i) = 1$.

(2) Solvent isotope effects (when k_5 is isotope sensitive) are

$$^{D}k_i = \frac{^{D}k_5 + [k_1k_5/[k_1k_4 + k_3(k_2 + k_7)]]}{1 + [k_1k_5/[k_1k_4 + k_3(k_2 + k_7)]]} \quad (A24)$$

$$^{D}(k_i/K_I) = \frac{^{D}k_5 + (k_5/k_4)}{1 + (k_5/k_4)} \quad (A25)$$

(II) Equation 4 in the Text

From the steady-state approximation for eq 4

$$d[EA]/dt = -(k_2 + k_3 + k_4)[EA] + k_1[E][A] = 0 \quad (A26)$$

$$[E_T] = [E] + [EA] + [EI^*] \quad (A27)$$

by the same method employed above for eq 3.

(*A*) *Turnover.*

$$v_{cat} = \frac{dP}{dt} = k_3[EA] = \frac{k_1k_3[A][E_T]}{(k_2 + k_3 + k_4) + k_1[A]} \quad (A28)$$

$$k_{cat} = k_3 \quad (A29)$$

$$\frac{k_{cat}}{K_M} = \frac{k_1k_3}{k_2 + k_3 + k_4} \quad (A30)$$

(1) Substrate isotope effects (when k_3 is isotope sensitive) are

$$^{D}k_{cat} = ^{D}k_3 \quad (A31)$$

$$^{D}(k_{cat}/K_M) = \frac{^{D}k_3 + [k_3/(k_2 + k_4)]}{1 + [k_3/(k_2 + k_4)]} \quad (A32)$$

(2) Solvent isotope effects (when k_4 is isotope sensitive) are

$$^{D}k_{cat} = 1 \quad (A33)$$

$$^{D}(k_{cat}/K_M) = \frac{(1/^{D}k_4) + [(k_2 + k_3)/k_4]}{1 + [(k_2 + k_3)/k_4]} \quad (A34)$$

Since the inactivation rate (k_4) is smaller than the other rates (k_2 and k_3), $^{D}(k_{cat}/K_M)$ is equal to unity.

(*B*) *Inactivation.*

$$\frac{dEI^*}{dt} = \frac{k_1k_4[A]\{[E_T] - [EI^*]\}}{(k_2 + k_3 + k_4) + k_1[A]} \quad (A35)$$

$$k_i = k_4 \quad (A36)$$

$$\frac{k_i}{K_I} = \frac{k_1k_4}{k_2 + k_3 + k_4} \quad (A37)$$

(1) Substrate isotope effects (when k_3 is isotope sensitive) are

$$^{D}k_i = 1 \quad (A38)$$

$$^{D}(k_i/K_I) = \frac{(1/^{D}k_3) + [(k_2 + k_4)/k_3]}{1 + [(k_2 + k_4)/k_3]} \quad (A39)$$

The latter effect is due to a normal isotope effect on turnover, such that deuteration increases the flux through the inactivation pathway. As can be seen from eqs A38 and A39, the model in eq 4 in the text predicts isotope effects on inactivation that are unity for k_i and inverse for k_i/K_i.

(2) Solvent isotope effects (when k_4 is isotope sensitive) are

$$^{D}k_i = ^{D}k_4 \quad (A40)$$

$$^{D}(k_i/K_I) = \frac{^{D}k_4 + [k_4/(k_2 + k_3)]}{1 + [k_4/(k_2 + k_3)]} \quad (A41)$$

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Investigation of an Octapeptide Inhibitor of *Escherichia coli* Ribonucleotide Reductase by Transferred Nuclear Overhauser Effect Spectroscopy†

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Received December 11, 1990; Revised Manuscript Received April 25, 1991

ABSTRACT: Several peptides contained within the C-terminal sequence of the B2 subunit of *Escherichia coli* ribonucleotide reductase (RNR) were investigated for their ability to inhibit the enzyme, presumably by interfering with association of the B1 and B2 subunits. AcYLVGQIDSE, corresponding by sequence homology to a nonapeptide that inhibits herpes simplex RNR [Gaudreau et al. (1987) *J. Biol. Chem. 262*, 12413] shows no inhibition of the *E. coli* enzyme ($IC_{50} > 3$ mM), whereas AcDDLSNFQL, the C-terminal octapeptide of the *E. coli* B2 subunit, is a noncompetitive inhibitor (K_i = 160 μM). Neither bradykinin (RPPGFSPFR) nor the pentapeptide AcSNFQL inhibits the *E. coli* enzyme. Transferred nuclear Overhauser enhancement spectroscopy was used to probe the conformation of AcDDLSNFQL when it is bound to the B1 subunit. These experiments suggest that the peptide adopts a turn in the region of Asn_5 and Phe_6 and that a hydrophobic cluster of the phenylalanine and leucine side chains is involved in the interaction surface.

Ribonucleotide reductase (RNR, EC 1.17.4.1)[1] catalyzes the conversion of ribonucleotides to the corresponding deoxyribonucleotides. Since this enzyme is responsible for the first committed step in de novo DNA biosynthesis, considerable effort has been devoted to its mechanistic and structural elucidation (Reichard, 1988; Eriksson & Sjöberg, 1989; Stubbe, 1989, 1990a,b). The enzyme from *Escherichia coli* is composed of two readily dissociated nonequivalent subunits, designated B1 (171 kDa) and B2 (87 kDa) (Carlson et al., 1984), each of which is in turn homodimeric; the overall composition is therefore $\alpha_2\beta_2$. The substrate-binding sites are located on the B1 subunit; however, the B2 subunit is required for activity since it contains a tyrosine-based radical, stabilized by a binuclear μ-oxo-bridged iron center, that is thought to initiate the radical reaction (Thelander & Reichard, 1979). The electrons necessary for reduction of the ribosyl center are provided by redox-active cysteines on the B1 subunit, which are in turn reduced by thioredoxin or glutaredoxin to complete the catalytic cycle. The binding affinity of the B1 and B2 subunits is Mg^{2+} dependent and relatively weak (Thelander & Reichard, 1979).

A number of virally encoded RNRs have been identified, including those from the herpes simplex viruses (HSV) 1 and 2 (Cohen et al., 1974), equine herpes virus type 1 (Cohen et al., 1977), Epstein-Barr virus (Henry et al., 1978), pseudorabies virus (Lankinen et al., 1982), and vaccinia virus (Tengelsen et al., 1988). These enzymes are heterodimeric, like the RNR from *E. coli*, and significant sequence homology is observed between the *E. coli* enzyme and those from HSV, Epstein-Barr virus, and vaccinia virus (Sjöberg et al., 1985). The best characterized of the viral enzymes is that from HSV-1 (Preston et al., 1984). Two proteins of 136 and 36 kDa are associated with the activity, but, unlike the *E. coli* enzyme, these two subunits form a tight complex whose formation is not Mg^{2+} dependent (Ator et al., 1986).

It has been shown that the peptide YAGAVVNDL, corresponding to the C-terminus of the small subunit of HSV-1 RNR, inhibits the enzyme with a K_i value of 15 μM (Dutia et al., 1986; Cohen et al., 1986; Paradis et al., 1991). This peptide also shows high specificity for the viral over the mammalian RNR. The reversible, noncompetitive nature of the inhibition led Cohen et al. (1986) to suggest that the peptide inhibits association of the enzyme subunits. Affinity labeling (Paradis et al., 1988) and immunoprecipitation experiments (McClements et al., 1988) subsequently confirmed this interpretation.

† This research was supported by National Institutes of Health Grant GM 30759.
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[1] Abbrevations: Ac, acetyl; RNR, ribonucleotide reductase; HSV, herpes simplex virus; Fmoc, (9-fluorenyl)methoxycarbonyl; HOBT, hydroxybenzotriazole; BOP, benzotriazol-1-yloxytris(dimethylamino)-phosphonium hexafluorophosphate. For identification of NOE interactions, each hydrogen involved is identified by the amino acid, residue number, and position on the carbon framework; e.g., Q7α/L8NH represents a cross-peak between the α-hydrogen on Gln_7 and the NH of Leu_8.

0006-2960/91/0430-8144$02.50/0