Kinetic Mechanism of Monoamine Oxidase A†

Rona R. Ramsay

Department of Biochemistry and Biophysics, University of California at San Francisco, San Francisco, California 94143, and Molecular Biology Division, Department of Veterans Affairs Medical Center, 151-S, 4150 Clement Street, San Francisco, California 94121

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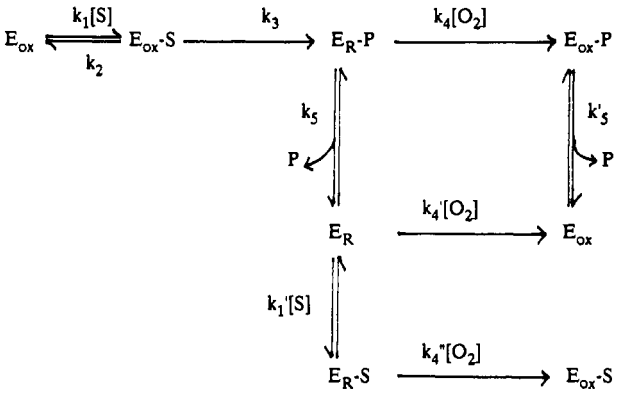
ABSTRACT: Steady-state kinetic data for monoamine oxidase A in crude extracts suggest an exclusively ping-pong mechanism, in contrast to those for monoamine oxidase B, which indicate alternate mechanisms involving either a binary or ternary complex. In this study, with use of purified monoamine oxidase A, steady-state data for the inhibition by D-amphetamine of the oxidation of primary amines indicate the possibility of a ternary complex mechanism for monoamine oxidase A also. Stopped-flow studies demonstrate that the rate of reoxidation of reduced enzyme is enhanced by substrates but not by the product, 1-methyl-4-phenylpyridinium. Thus, for the A enzyme, the ternary complex with substrate, but not product, is reoxidized at a faster rate than the free, reduced enzyme. For both the A and B forms of monoamine oxidase, the mechanism is determined by competition between alternate pathways on the basis of the relative rate constants and dissociation constants.

Monoamine oxidases are important in deamination of neurotransmitters and amines in peripheral tissues. The differences between the A and B forms of monoamine oxidase have been reviewed (Singer, 1985, 1991; Weyler et al., 1990). They have overlapping substrate specificity and sensitivity to inhibitors. The flavin sites have been shown to be identical (Nagy & Salach, 1981), so the specificity must lie in the substrate site. 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP),[1] a neurotoxic amine that causes experimental Parkinsonism in susceptible species (Burns et al., 1983; Langston et al., 1983), is oxidized by both forms of monoamine oxidase (Chiba et al., 1984; Singer et al., 1986) to $MPDP^+$, the dihydropyridinium. Unlike the imines formed after oxidation of primary amines, $MPDP^+$ is sufficiently stable to be used in product inhibition experiments. It is also itself a substrate and is slowly oxidized to the very stable product MPP^+ (Singer et al., 1986).

Steady-state kinetic patterns suggested that oxidation catalyzed by monoamine oxidase B followed a binary mechanism with some substrates (e.g., phenylethylamine) and a ternary complex mechanism with others (e.g., benzylamine) (Houslay & Tipton, 1973; Husain et al., 1982; Pearce & Roth, 1985). Stopped-flow experiments confirmed these results (Husain et al., 1982; Ramsay et al., 1987) and further provided direct evidence (Ramsay et al., 1987) for ternary complexes not only of reduced enzyme, oxygen, and product but also of reduced enzyme, oxygen, and substrate both for benzylamine and the quaternary amine MPTP. The rate of reoxidation of the reduced enzyme in a ternary complex was much faster than that in a binary complex (Ramsay et al., 1987). Comparison of the rate constants and dissociation constants suggested that the mechanism was not exclusive and determined by the substrate but rather was determined by competition between the alternate pathways shown in Scheme I as a result of the different rates of reoxidation of the binary and ternary complexes and their dissociation constants.

Steady-state data for monoamine oxidase A in crude preparations show only ping-pong kinetics (Tipton, 1968; Oi et al., 1971; Roth, 1979) except for inhibition of tyramine, tryptamine, and 5-hydroxytryptamine oxidation by D-amphetamine (J. A. Roth, personal communication). The inhibition patterns are competitive, as is seen for monoamine oxidase B oxidizing benzylamine (Pearce & Roth, 1985), suggesting that a ternary complex is involved. The present paper describes kinetic studies on purified monoamine oxidase A, including initial rate and inhibition patterns in the steady state and measurement of the rates of the half-reactions by stopped-flow techniques. As for monoamine oxidase B (Ramsay et al., 1987), ternary complexes of reduced monoamine oxidase A are more rapidly oxidized than the binary complex and the same spectrum of competing pathways may be used to account for the kinetic observations.

Scheme I: Alternate Pathways of Reoxidation of Reduced Monoamine Oxidases via Binary or Ternary Complexes



Methods

Reagents. Kynuramine, tyramine, D-amphetamine and *n*-octyl glucoside were purchased from Sigma Chemical Co., and MPTP was from Aldrich. $MPDP^+$ and MPP^+ were ob-

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[1] Abbreviations: Hepes, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; $MPDP^+$, 1-methyl-4-phenyl-2,3-dihydropyridinium; MPP^+, 1-methyl-4-phenylpyridinium.

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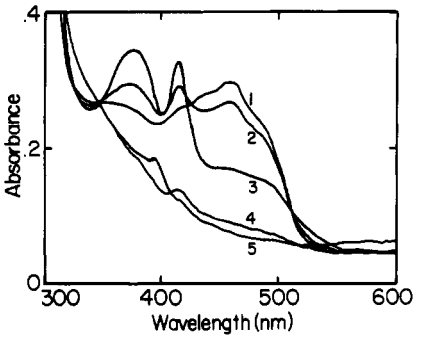


FIGURE 1: Reduction of monoamine oxidase A by xanthine and xanthine oxidase in the presence of methyl viologen. Monoamine oxidase A (15 μM) was incubated in 50 mM Hepes, pH 7.5, at room temperature, with 30 mM glucose/1 unit/mL glucose oxidase/24 units/mL catalase/200 μM xanthine/10 μM methyl viologen. Reduction was initiated by the addition of 50 nM xanthine oxidase and terminated after spectrum 5 by the addition of 100 μM allopurinol. This addition resulted in reoxidation of most of the excess reduced methyl viologen, which caused the increase in absorbance at 600 nm (spectrum not shown). The spectra were recorded at (1) 30 s after the xanthine oxidase addition (identical with oxidized spectrum), 5 min, (3) 16 min, (4) 26 min, and (5) 30 min.

tained as before (Ramsay et al., 1987). Stock solutions of $MPDP^+$ (20 mM) were prepared just before use in water adjusted to pH 2 to prevent disproportionation. Because of the neurotoxicity of these compounds, isolation procedures were followed whenever MPTP or its derivatives were used.

Enzyme. Monoamine oxidase A was purified from human placental mitochondria (Weyler & Salach, 1985, 1987). Its activity was assayed spectrophotometrically at 314 nm in a 1-mL system containing 50 mM NaP_i, pH 7.2, 0.2% Triton X-100, 1 mM kynuramine, and 0.236 mM O_2 (air-equilibrated) at 30 °C (Weyler & Salach, 1987). The specific activity was 1 unit/mg protein. The enzyme was stored at −20 °C in a solution of 50 mM NaP_i, pH 7.2, 0.8% *n*-octyl glucoside, 1.5 mM β-mercaptoethanol, 0.5 mM D-amphetamine, and 50% glycerol at a concentration of about 4 mg/mL (32 μM flavin). Before use, glycerol, mercaptoethanol, and D-amphetamine were removed by dialysis for 2.3 h with two changes of buffer.

Stopped-Flow Experiments. The apparatus and procedure for anaerobic stopped-flow spectrophotometry were as described previously (Ramsay et al., 1987). For the reductive half-reaction at 30 °C, the anaerobic enzyme solution was prepared in a tonometer and contained enzyme (6–20 μM flavin) in 50 mM Hepes, pH 7.5/30 mM glucose/glucose oxidase (1 unit/mL)/catalase (24 units/mL). For studies of the oxidative half-reaction, monoamine oxidase A was reduced by a novel application of a method for measuring the redox potentials of flavoproteins (Massey, 1991). Xanthine oxidase catalyzes the oxidation of xanthine to urate (redox potential −350 mV at pH 7), with methyl viologen as an electron acceptor. Reduced methyl viologen reacts rapidly with flavoproteins, such as monoamine oxidase, producing reduced flavoprotein with no substrate or product present. Monoamine oxidase A (15 μM) was reduced in 30 min by incubation in 50 mM Hepes, pH 7.5/30 mM glucose/1 unit/mL glucose oxidase/24 units/mL catalase/200 μM xanthine/10 μM methyl viologen/50 nM xanthine oxidase (Figure 1). In order to prevent continuing reduction of the methyl viologen after the reduction of the enzyme is complete, 100 μM allopurinol was added. The oxygen concentration was varied by mixing different volumes of anaerobic and oxygen-bubbled buffer (50 mM Hepes, pH 7.5). The resulting oxygen concentration was measured polarographically with a Clark oxygen electrode. The temperature in the oxidative experiments was 25 °C.

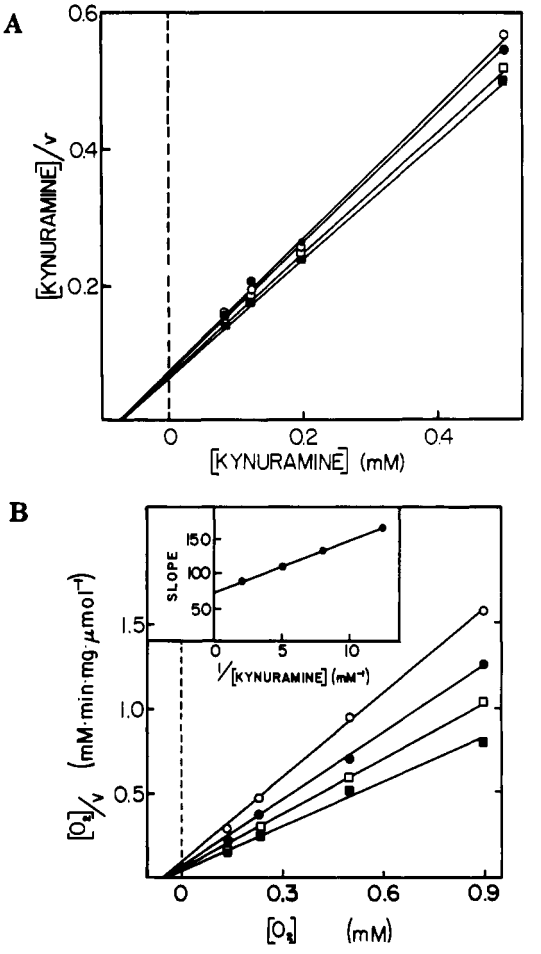


FIGURE 2: Steady-state kinetics for the oxidation of kynuramine by monoamine oxidase A. Assays were performed at 30 °C in stoppered cuvettes containing 50 mM sodium phosphate, pH 7.2, and 0.2% Triton X-100, preequilibrated with N_2/O_2 gas mixtures. The reaction was initiated by the addition of kynuramine and monitored spectrophotometrically at 316 nm. Panel A shows the effect of the oxygen concentration on the Hanes–Woolf plots for varying kynuramine. The oxygen concentrations (and gas mixture used) were (○) 0.14 mM (10% O_2/90% N_2), (●) 0.236 mM (air), (□) 0.5 nM (50% O_2/50% N_2); and (■) 0.9 mM (100% O_2). Panel B shows the effect of kynuramine concentration on the slopes of the Hanes–Woolf plots for varying oxygen. The same data as in (A) were replotted versus O_2 concentration. The kynuramine concentrations were (○) 0.08 mM, (●) 0.125 mM, (□) 0.2 mM, and (■) 0.5 mM. The inset is a secondary plot of the slopes from (B) against the reciprocals of kynuramine concentration.

Steady-State Kinetics. Assays at 30 °C in 50 mM NaP_i, pH 7.0, containing 0.2% Triton X-100 were followed either spectrophotometrically (kynuramine, MPTP) with enzyme at about 5 μg/mL or polarographically (tyramine, kynuramine) with enzyme at about 25 μg/mL. Oxygen concentration was varied by equilibration of the buffer with 10, 50, or 100% oxygen/nitrogen mixtures in stoppered cuvettes.

RESULTS

Steady-State Experiments

(*a*) *Varying Substrate Concentrations.* The activity of monoamine oxidase A was measured at different concentrations of both amine and oxygen with kynuramine (Figure 2) or tyramine (data not shown) as the amine substrate. The Hanes–Woolf plots with oxygen as the variable substrate (Figure 2B) intersect either on or very close to the *y* axis, so that the data could be said to indicate a ping-pong mechanism, with the rate equation

$$v = \frac{VAB}{K_m^B A + K_m^A B + AB} \quad (1)$$

where v is observed velocity, V is maximum velocity A is the concentration of amine B is the concentration of oxygen, and K_m^A and K_m^B are the Michaelis constants for A and B, respectively. However, for an ordered mechanism, the equation takes the form

$$v = \frac{VAB}{K_m^B A + K_m^A B + AB + K_i^A K_m^B} \quad (2)$$

where K_i^A is the dissociation constant for A. If K_i^A is very small, 2 approximates eq 1. Discrimination of the two mechanisms is made additionally difficult because of the extremely low K_m for oxygen, so that terms containing K_m^B are very small. The K_m for oxygen calculated from the experiments with kynuramine (Figure 2) or tyramine was 6 μM, well below the concentration present at air saturation (236 μM at 30 °C). Other kinetic constants obtained from these experiments: kynuramine V = 1.43 μmol/min·mg, K_m = 0.11 mM; tyramine V = 3.40 μmol/min·mg, K_m = 0.02 mM.

(*b*) *Inhibition by* D-*Amphetamine.* The classical monoamine oxidase inhibitor D-amphetamine inhibits preferentially the A form but also inhibits the B form of monoamine oxidase in a manner competitive with the amine substrate. It was used to confirm the alternative binary and ternary complex pathways for monoamine oxidase B (Pearce & Roth, 1985). With benzylamine (ternary complex mechanism), competitive inhibition was observed in double-reciprocal plots when the amine concentration was varied and mixed inhibition was observed when the oxygen concentration was varied. With phenylethylamine (binary complex mechanism), the action of amphetamine on monoamine oxidase B was mixed inhibition with either substrate varied (Pearce & Roth, 1985). With monoamine oxidase A, the effect of D-amphetamine on the oxidation of kynuramine was purely competitive. The double-reciprocal plots with the amine as the varied substrate intersected on the y-axis but were parallel with oxygen as the varied substrate. This pattern suggests that the reduced enzyme is not available for amphetamine binding, i.e., that a ternary complex mechanism analogous to monoamine oxidase B oxidizing benzylamine must be considered. The same competitive pattern was observed for inhibition by D-amphetamine of monoamine oxidase A in brain mitochondria with tyramine, tryptamine, or 5-hydroxytryptamine as the varied substrates (J. A. Roth, personal communication), so this is not an anomaly introduced by the purification procedure.

Stopped-Flow Experiments

(*a*) *Stopped-Flow Monitored Turnover.* The extent of reduction of the flavin during turnover depends on the relative rates of the reductive and oxidative half-reactions and can be monitored by stopped-flow spectrophotometry. For monoamine oxidase B, the steady-state level of oxidized enzyme decreased as the rate constants for different substrates in the reductive half-reaction increased (Husain et al., 1982). For phenylethylamine, k_3 is very high (572 s^{-1}), so that the enzyme is almost completely reduced on entering the steady state (Husain et al., 1982). In contrast, for benzylamine where the rates of oxidation and reduction are comparable (Ramsay et al., 1987), the flavin is only 40% reduced and for the slower substrate, dideuteriobenzylamine (k_3 = 1.3 s^{-1}), the flavin remains 90% oxidized (Husain et al., 1982).

Figure 4 shows that no reduction of flavin occurs for a considerable time after the enzyme and substrate are mixed in the presence of oxygen. Thus, the steady-state concentration

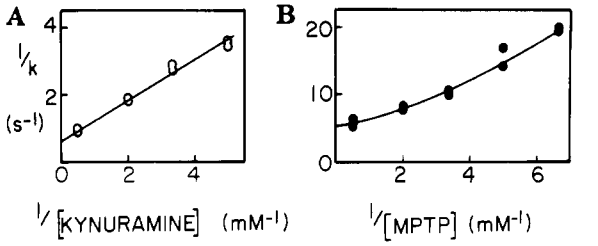


FIGURE 3: Dependence of the observed rate constant for reduction of monoamine oxidase A on substrate concentration. The rates of the reductive half-reaction were measured at 30 °C as described in Methods. The reaction mixture contained 9 μM flavin, 50 mM Hepes, pH 7.5, 30 mM glucose, 1 unit/mL glucose oxidase, 24 units/mL catalase, and substrate as indicated in the double-reciprocal plots. The substrates were kynuramine (A) or MPTP (B).

Table I: Constants for Reduction of Monoamine Oxidase A by Kynuramine and MPTP

$$E_{ox} + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E_{ox}S \xrightarrow{k_3} E_RP$$

	substrate	
constants[a]	kynuramine	MPTP
k_1 ($M^{-1}\cdot s^{-1}$)		(484)[b]
k_2 (s^{-1})		0.038
k_3 (s^{-1})	1.69 ± 0.15	0.185 ± 0.003[c]
K_a (mM)		(0.46)[b]
K_D (mM)	0.10	(0.08)[b]

[a] $K_a = (k_2 + k_3)/k_1$ and $K_D = k_2/k_1$. Experimental details are given in Figure 5. [b] Because the double-reciprocal plot is only slightly curved, the values of k_1 and constants derived from it are inherently inaccurate. [c] The value of k_3 from a linear fit to the data was 0.23 ± 0.10 s^{-1}.

of oxidized flavin for monoamine oxidase A is 100% both for kynuramine and for MPTP, which has a 10-fold lower turnover number (see Table III). This suggests that k_3 is much less than the rate of reoxidation, so that reduction of the flavin is seen only when the oxygen concentration becomes limiting.

(*b*) *Reductive Half-Reaction.* The rate constants for the reduction of monoamine oxidase A by kynuramine (Figure 3A) or MPTP (Figure 3B) were determined by anaerobic stopped-flow spectrophotometry. In Figure 3A, the double-reciprocal plot for reduction by kynuramine is virtually linear, so that only the rate constant for the reduction of the flavin in the enzyme–substrate complex (k_3) and the K_D for that complex can be calculated. For the reduction by MPTP (Figure 3B), the double-reciprocal plot is curved, so that the data could be analyzed as described previously for monoamine oxidase B (Ramsay et al., 1987) to obtain all three rate constants for eq 3. The rate constants obtained by this analysis are shown in Table I. As indicated by the curved double-reciprocal plot, k_3 is comparable to k_2 for MPTP. The values for k_3 for each substrate are similar to the turnover numbers obtained by steady-state methods, indicating that reduction must be rate-limiting. The ratios of the kynuramine to MPTP values are approximately 10 for both the k_3 values and the turnover numbers.

$$E_{ox} + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E_{ox}S \xrightarrow{k_3} E_RP \quad (3)$$

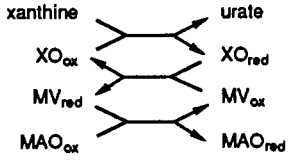
(*c*) *Oxidative Half-Reaction.* In the early phases of this work, the only way to obtain reduced enzyme was by titration with substrate because dithionite reduction yielded only the semiquinone. Only the fully reduced form is observed on titration with substrate. However, if substrate or product affected the reoxidation, the traces of kynuramine present would affect the apparent rate constants. This proved to be

Table II: Effect of Ligands on the Rate Constants for the Reoxidation of Reduced Monoamine Oxidase A[a]

	k_{app} (s^{-1})		
	substrate reduced	xanthine reduced	
ligand	ligand in enzyme	ligand in enzyme	ligand in O_2
none	1.86 ± 0.81 (5)	0.62	0.46 ± 0.05 (3)
kynuramine	58 ± 12 (4)	65	0.72 ± 0.20 (2)
MPTP	33.7 ● 4.3 (5)	26.4 ± 1.5 (3)	
$MPDP^+$	26.1 ± 5.5 (2)		
MPP^+	2.8	1.0	0.49 ± 0.04 (2)
D-amphetamine	0.9 ± 0.4 (3)		

[a] Monoamine oxidase A was reduced either by titration with kynuramine (substrate reduced) or by the xanthine–urate couple (xanthine reduced) as described in Methods. The final flavin concentration was 3–8 μM, the oxygen concentration was 0.129 mM, and the final ligand concentration was 2 mM. Column 3 (ligand in O_2) gives data from experiments where the ligand was present only in the oxygen buffer and was therefore mixed with enzyme at the same time as oxygen. For the other experiments (ligand in enzyme), the ligand was made anaerobic, premixed with the enzyme in the stopped-flow syringe, and incubated with the enzyme for 5 min before oxidation was initiated by mixing with the oxygen buffer containing the same concentration of ligand. The temperature was 25 °C. Where several experiments were done, data is given as the mean ±SD of (n) experiments.

a serious problem since kynuramine enhanced the rate of reoxidation by 2 orders of magnitude (Table II). In order to circumvent this problem, a method developed by Massey (1991) for determining the redox potential of flavoproteins was adapted to produce fully reduced monoamine oxidase A. Electrons from the xanthine oxidase catalyzed reduction of xanthine were transferred from xanthine oxidase to monoamine oxidase by the mediator, methyl viologen.



Since allopurinol could be added to inhibit xanthine oxidase, reduced monoamine oxidase A was obtained without excess reductant present and without interfering traces of substrate or product. The components of this system do not affect monoamine oxidase activity.

Reoxidation by O_2 of this reduced monoamine oxidase A was a simple bimolecular process. The second-order rate constant was 4500 $M^{-1}\cdot s^{-1}$. In the initial experiments, ligand was added to the oxygen syringe before rapid mixing with anaerobic reduced enzyme. Table II (column 3) shows that mixing substrate (kynuramine, MPTP), product (MPP^+), or inhibitor (amphetamine) with the enzyme at the same time as oxygen did not alter the rate of oxidation.

In contrast, when the substrate is premixed with enzyme reduced by either method, a dramatic enhancement of the rate of reoxidation is observed (Table II, columns 1 and 2), suggesting that a ternary complex with substrate ($S–E_R–O_2$) is formed and that it reoxidizes faster than does the free enzyme, just like monoamine oxidase B (Ramsay et al., 1987). With 2 mM kynuramine, the enhancement was 100-fold and with MPTP (2 mM) was 42-fold. As the MPTP (0.1, 0.5, and 2 mM) and oxygen (0.09, 0.13, and 0.25 mM) concentrations were increased, the intercepts of the second-order rate plots increased only slightly. Thus, even at the lowest concentrations used, the enzyme was virtually saturated with both substrates. From this data, the second-order rate constant for the reoxidation of reduced monoamine oxidase A in the presence of MPTP was 138 000 $M^{-1}\cdot s^{-1}$.

The product, MPP^+, does not affect the rate of reoxidation significantly (Table II). Either oxygen cannot bind to the enzyme–product complex or the reduced enzyme–product–oxygen ternary complex is reoxidized at the same rate as free enzyme. Reoxidation of the reduced enzyme is completely inhibited when the reduced enzyme is premixed with amphetamine (Table II, column 1). Thus, amphetamine does indeed bind to the reduced enzyme, and further, it prevents reoxidation of the flavin by oxygen in addition to blocking the substrate site to prevent reduction by substrate.

Discussion

The steady-state experiments varying both substrates (e.g., kynuramine and O_2 in Figure 2) and in published experiments using mitochondria, brain slices, or homogenates (Tipton, 1968; Oi et al., 1971; Roth, 1979) are consistent with a ping-pong mechanism for all substrates with monoamine oxidase A. However, if the dissociation constant for the amine is small, i.e., the rate of dissociation is slower than the rate of binding, the equation for an ordered mechanism (eq 2) approximates that for a ping-pong mechanism (eq 3). In addition, the low K_m for oxygen (6 μM from Figure 2) makes it difficult to discriminate between the two mechanisms in steady-state experiments. The inhibition by amphetamine, on the other hand, clearly indicates that free reduced enzyme is not available for binding amphetamine in the steady state with any of the substrates tested. Thus, as for monoamine oxidase B with benzylamine (Pearce & Roth, 1985), a ternary mechanism must also be considered for monoamine oxidase A.

To test the possibility of ternary complex formation we examined the rates of oxidation of reduced monoamine oxidase A in the absence and presence of products (MPP^+, $MPDP^+$), substrates (MPTP, kynuramine), or inhibitor (amphetamine). While a ternary complex might be reoxidized at the same rate as that of the binary complex of reduced enzyme and oxygen, this was not the case for monoamine oxidase B, with the ternary complex being reoxidized approximately 5 times faster than the complex without substrate (Ramsay et al., 1987). Tables II and III show that the same is true for monoamine oxidase A. Free enzyme reduced by the novel xanthine oxidase

Table III: Kinetic Parameters for Monoamine Oxidase A from Steady-State and Stopped-Flow Half-Reaction Experiments[a]

	steady-state		reductive half-reaction		oxidative half-reaction	
substrate	turnover no. (s^{-1})	K_m (mM)	k_3 (s^{-1})	K_D (mM)	apparent second-order rate constant ($M^{-1}\cdot s^{-1}$)	apparent first-order rate constant at air saturation (s^{-1})
none					4500	1.2
kynuramine	1.9	0.12	1.7	0.10	nd	65
MPTP	0.18	0.14	0.19	0.08	138000	35.7

[a] The data for the steady state and for the reductive half-reaction are for 30 °C. The oxidative half-reaction was measured at 25 °C.

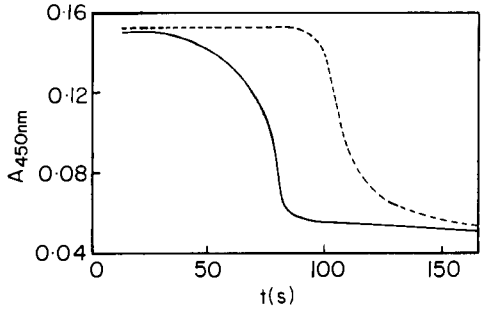


FIGURE 4: The steady-state concentration of oxidized flavin during stopped-flow-monitored turnover of monoamine oxidase A with either kynuramine of MPTP. The enzyme (final concentration 9 μM) and substrate (final concentration 10 mM) in equal volumes of aerobic 50 mM Hepes, pH 7.5, at 30 °C were mixed in the stopped-flow spectrophotometer, and the decrease in absorbance at 450 nm was recorded. The substrates were kynuramine (—) or MPTP (---).

catalyzed method is reoxidized only very slowly, with a second-order rate constant of 4500 $M^{-1}\cdot s^{-1}$ (Table III), similar to the rate of reoxidation of dithionite reduced monoamine oxidase B (6000 $M^{-1}\cdot s^{-1}$) (Husain et al., 1982). The equivalent apparent first-order rate constant at air saturation (1.2 s^{-1}) is barely adequate for the turnover number with kynuramine (1.9 s^{-1}, Table III) and is too low to explain the higher turnover numbers observed with tyramine (5.3 s^{-1}; from the steady state V reported above) and 2′-*n*-propyl-MPTP (4.2 s^{-1}; Youngster et al., 1989). However, preincubation of the reduced enzyme with substrate dramatically increases the rate of reoxidation, giving apparent first-order rate constants at air saturation (Table II) far in excess of even the fastest turnover number in steady-state experiments. With all substrates, the rate of reduction of rate-limiting, and thus, in the steady state virtually all the enzyme is in the oxidized form (see Figure 4). The steady-state turnover experiment (Figure 4) confirms that the ternary complex must form during turnover as well as during experimental measurement of the oxidative half-reaction, because the slow rate of reoxidation of the free enzyme (1.2 s^{-1}) would result in build-up of reduced flavin in the presence of excess kynuramine. That only oxidized flavin is observed initially in Figure 4 emphasizes that the rate of reoxidation must be considerably faster than the rate of reduction, which is the case only for the ternary complexes (Table II).

It is often assumed that MPTP is a poor substrate for monoamine oxidase A because it is oxidized at only one-tenth of the rate of kynuramine. However, it is clear that it is a good ligand for the enzyme, both from the K_D (0.08 mM) and from the fact that it can stimulate the rate of reoxidation of the flavin by 40-fold (Table II, column 2). The observed stimulation of the rate of reoxidation depends on the nature of the substrate ligand, not just on having the substrate site occupied, since product has no effect. The order of efficacy is kynuramine > MPTP > $MPDP^+$ (Table II), and the differences are significant.

In contrast to the enhancement of oxidation caused by substrate, the product MPP^+ has no significant effect on the rate of reoxidation of reduced monoamine oxidase A (Table II). Either oxygen cannot bind to the enzyme–product complex or the ternary complex with product cannot facilitate reoxidation in the same way as substrate. In experiments where the enzyme was reduced by a slight excess of substrate (1.5–2-fold excess of either kynuramine or MPTP) or a modest excess (up to 10-fold) kynuramine, MPP^+ so strongly inhibited the substrate-enhanced rate of reoxidation that it was suggested initially that the E_R–MPP^+ complex was not reoxidized. (Note that the difference in the rates of reoxidation of the free enzyme and the substrate–enzyme complex is 2 orders of magnitude). However, by use of xanthine oxidase catalyzed reduction, it was possible to measure the rates in the absence of interfering traces of substrate and show that the E_R–MPP^+ complex is reoxidized, but only at the same rate as is free enzyme. Thus, MPP^+ can interact with the same form of the enzyme as oxygen, namely, free reduced enzyme (E_R). In steady-state kinetics, mixed inhibition by MPP^+ would be expected if monoamine oxidase A followed a ping-pong mechanism. However, as in the amphetamine experiments, only strictly competitive inhibition is observed for MPP^+, reflecting the lack of free reduced enzyme in the steady state.

It can be inferred from these data that the preferred pathway for the reoxidation of monoamine oxidase A (see Scheme I) is via the ternary E_R–S–O_2 complex except at very low substrate concentration when $k_4''[S][O_2]$ becomes less than $k_4'[O_2]$. It is possible that the substrate must displace the product from the enzyme, as is the case for many NADH-dependent enzymes. For the normal monoamine substrates of monoamine oxidase A, it may be that only part of the initial imine product remains bound. However, chemical and spectral experiments suggest that the imine is released from the enzyme prior to hydrolysis (Patek at al., 1972; Walker & Edmondson, 1987).

Physiologically, the mechanism discussed means that at low amine concentrations regeneration of oxidized monoamine oxidase A will be slow, particularly if substrate displacement of the product is necessary. However, when a surge of amine is produced by synaptic transmission, the turnover capacity is immediately available. The very low K_m of monoamine oxidase A for oxygen (6 μM) in contrast to that of monoamine oxidase B (230 μM; Husain et al., 1982) means that the A enzyme is relatively insensitive to changes in the oxygen concentration. At O_2 concentrations where monoamine oxidase B activity becomes minimal, monoamine oxidase A can still be saturated by O_2. Consequently, the pattern of amine oxidation in regions with both forms of the enzyme could alter dramatically as the oxygen level of the region decreased.

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The AP-1 Sequence Is Necessary but Not Sufficient for Phorbol Induction of Collagenase in Fibroblasts†

David T. Auble‡ and Constance E. Brinckerhoff*
Departments of Biochemistry and Medicine, Dartmouth Medical School, Hanover, New Hampshire 03756
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ABSTRACT: Collagenase, the only enzyme active at neutral pH that initiates collagen degradation, is a major gene product of fibroblasts that have been stimulated with a variety of agents, including phorbol esters. To study mechanisms controlling collagenase gene expression, we transiently transfected rabbit synovial fibroblasts with chimeric constructs containing up to 1.2 kb of the rabbit collagenase 5′-flanking DNA linked to the chloramphenicol acetyltransferase gene (CAT). Our data indicate that the magnitude of the phorbol response is directly linked to the size of the promoter fragment and that the smallest piece of promoter DNA conferring phorbol inducibility is 127 bp. Deletional and mutational analysis of this fragment revealed that the AP-1 sequence alone is insufficient for phorbol inducibility and the presence of at least two additional sequences (a PEA3-like element and a sequence that includes 5′-TTCA-3′) is required. In addition, a substantial increase in responsiveness is seen when a fragment containing 182 bp of 5′-flanking DNA is transfected, implicating a 36 bp region located between −182 and −149 as an enhancer. We conclude (1) that the AP-1 sequence is necessary but insufficient for expression of collagenase in adult fibroblasts, (2) that phorbol inducibility depends on cooperation among several sequence elements within the collagenase promoter, and (3) that regulation of this promoter is more complex than previously described.

Collagen is the body's most abundant protein and is a major component of the extracellular matrix. Collagen degradation is initiated by the enzyme collagenase (Jeffrey, 1986) and occurs in a number of normal and disease processes including wound healing, uterine resorption, tumor invasion, and arthritis. The same collagenase gene product is expressed by a variety of cell types including endothelial cells (Herron et al., 1986), keratinocytes (Lin et al., 1987), macrophages (Campbell et al., 1987), chondrocytes (Lin et al., 1988; Stephenson et al., 1987), and fibroblasts (Goldberg et al., 1986; Whitham et al., 1986; Brinckerhoff et al., 1987). Collagenase is a major gene product of fibroblasts, and, indeed, increased production of collagenase by synovial fibroblasts lining the joint is largely responsible for the extensive destruction of connective tissue seen in rheumatoid arthritis (Harris, 1985).

Previous work by a number of investigators has implicated the AP-1 sequence (also known as the TRE) in the transcriptional regulation of the human collagenase gene (Angel et al., 1987a,b; Schonthal et al., 1988; Chiu et al., 1988; Brenner et al., 1989; Conca et al., 1989; Gutman & Wasylyk, 1990). AP-1 plays an important role in the induction of collagenase gene transcription in response to agents such as phorbol esters, interleukin-1, and tumor necrosis factor. As demonstrated recently, AP-1 also has been implicated in the repression of transcription by glucocorticoids (Lucibello et al., 1990; Diamond et al., 1990; Jonat et al., 1990; Yang-Yen et al., 1990; Schule et al., 1990). Many of the previous studies on the role of the AP-1 sequence in collagenase gene expression have utilized tandem repeats of this sequence linked to reporter genes and have studied expression of these chimeric genes in immortal or tumorigenic cell lines (Angel et al., 1987a,b; Schonthall et al., 1988; Chiu et al., 1988; Brenner et al., 1989; Conca et al., 1989; Gutman & Wasylyk, 1990).

Alternatively, we have investigated collagenase gene expression with chimeric constructs containing the natural configuration of the collagenase promoter transfected into primary cultures of adult fibroblasts that express this gene

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* Address correspondence to this author at the Department of Biochemistry, Dartmouth Medical School.
‡ Present address: Fred Hutchinson Cancer Center, Seattle, WA 98104.

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