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Mechanistic Studies of Mouse Polyamine Oxidase with N1,N12-Bisethylspermine as a Substrate†

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Abstract

In mammalian cells, the flavoprotein polyamine oxidase catalyzes a key step in the catabolism of polyamines, the oxidation of N1-acetylspermine and N1-acetylspermidine to spermidine and putrescine, respectively. The mechanism of the mouse enzyme has been studied with N1,N12-bisethylspermine (BESPM) as a substrate. At pH 10, the pH optimum, the limiting rate of reduction of the flavin in the absence of oxygen is comparable to the k_{cat} value for turnover, establishing reduction as rate-limiting. Oxidation of the reduced enzyme is a simple second-order reaction. No intermediates are seen in the reductive or oxidative half-reactions. The k_{cat} value decreases below a pK_a of 9.0. The k_{cat}/K_m value for BESPM exhibits a bell-shaped pH profile, with pK_a values of 9.8 and 10.8. These pK_a values are assigned to the substrate nitrogens. The rate constant for the reaction of the reduced enzyme with oxygen is not affected by a pH between 7.5 and 10. Active site residue Tyr430 is conserved in the homologous protein monoamine oxidase. Mutation of this residue to phenylalanine results in a 6-fold decrease in the k_{cat} value and the k_{cat}/K_m value for oxygen due to a comparable decrease in the rate constant for flavin reduction. This moderate change is not consistent with this residue forming a tyrosyl radical during catalysis.

The polyamines spermine, spermidine, and putrescine are ubiquitous in cells. Higher concentrations are found in rapidly growing cells (1-3), and compounds which deplete polyamines from cells inhibit cell growth (2). These observations have led to the general conclusion that polyamines are essential for cell growth, although their specific role in the cell is still a matter of discussion. Consequently, a variety of polyamine analogues have been examined as anticancer drugs (4-7); a number of clinical trials are underway, and analogues with cytotoxic potential have been developed (8,9). The metabolic pathways for synthesis and degradation of polyamines are generally conserved (1). In mammals, the biosynthetic pathway involves decarboxylation of ornithine to putrescine by ornithine decarboxylase, extension of putrescine to spermidine by spermidine synthase using decarboxylated *S*-adenosylmethionine as the propylamine donor, and a subsequent extension of spermidine with another propylamine moiety to form spermine catalyzed by the enzyme spermine synthase. Depletion of spermine and spermidine from the cell involves the action of two enzymes: spermidine/spermine N1-acetyltransferase converts spermine and spermidine to the respective N1-acetylated compound, and polyamine oxidase oxidizes N1-acetylspermidine and N1-acetylspermine to putrescine and spermidine, respectively, and 3-acetamidopropanal (Scheme 1). While polyamine oxidase can also oxidize spermine, the enzyme strongly prefers N1-acetylated polyamines as substrates (10). Instead, the related enzyme spermine oxidase is responsible for direct oxidation of

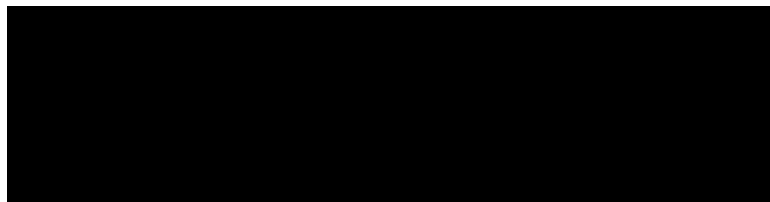
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spermine and spermidine (11,12). The mammalian polyamine oxidases have undergone little mechanistic study. The structure of a maize polyamine oxidase has been described with a number of inhibitors (13,14), establishing the enzyme as a homologue of mitochondrial monoamine oxidase, and preliminary kinetic studies with spermine as a substrate have been reported for that enzyme (15). However, the plant enzymes prefer spermine and spermidine as substrates instead of acetylated polyamines (16), so they are better described as spermine oxidases than polyamine oxidases.

Because of the role of monoamine oxidase in the metabolism of neurotransmitters, the mechanism of that enzyme has been extensively studied, yet the mechanism of amine oxidation by monoamine oxidase remains controversial. Edmondson and co-workers have proposed a mechanism involving transfer of two electrons from the amine of the substrate to the flavin concerted with abstraction of the α -hydrogen as a proton by N5 of the flavin (17,18). Alternatively, Silverman and co-workers have proposed a mechanism involving a substrate aminium radical cation as a discrete intermediate (19). In support of this latter proposal, Rigby et al. (20) recently reported that monoamine oxidase which has been partially reduced with dithionite contains a radical species identified as a tyrosyl radical. In light of the homology of polyamine oxidase to monoamine oxidase, these mechanistic proposals must be considered as possibilities for the former enzyme also.

Recently, a number of true polyamine oxidases have been described which prefer acetylated polyamines as substrates. These include enzymes from yeast (21), humans (11), and mice (10); both the mouse and yeast enzymes have been expressed in *Escherichia coli*, although at low levels (10,21). These studies have made available for the first time sufficient amounts of a mammalian polyamine oxidase for mechanistic studies. We describe here mechanistic studies of mouse polyamine oxidase.



Scheme 1.

EXPERIMENTAL PROCEDURES

Materials

Spermine and N1,N12-bisethylspermine (BESPM)¹ were from Tocris Bioscience Inc. (Ellisville, MO). N1-Acetylspermine was from Fluka Chemical Co. (Milwaukee, WI). pET28b (+) was from Novagen (Madison, WI). All other reagents were of the highest purity commercially available.

Expression and Purification of Recombinant Proteins

The cDNA encoding murine polyamine oxidase (GenBank accession number AF226656) in a pDrive vector with codons optimized for expression in *E. coli* was obtained from DNA 2.0 (Menlo Park, CA). It was subcloned into pET28b(+) using the NdeI and EcoRI sites at the 5' and 3' ends, respectively, for expression of the His-tagged enzyme. The Y430F mutation was generated using the Stratagene QuikChange site-directed mutagenesis method, with the

¹Abbreviation: BESPM, N1,N12-bisethylspermine.

mutagenic primer 5'-CCATACACGCGTGGTTCT**TT**CAGCTATGTTGCAGTTGG-3'. (The mutated triplet is bold.) For all constructs, DNA sequencing of the entire coding region was performed to ensure that no adventitious mutations were present.

For protein expression, plasmids were transformed into BL21(DE3) *E. coli*. After induction with 0.15 mM isopropyl β -D-thioglycanopyranoside, the cells were incubated overnight at 20 °C. The cells were collected by centrifugation at 9000g for 30 min. The cell paste was resuspended in 50 mM potassium phosphate (pH 7.5), 10% glycerol, 150 μ M FAD, 2 μ M leupeptin, 2 μ M pepstatin, and 100 μ g/mL phenylmethanesulfonyl fluoride and lysed by sonication. After the lysate was centrifuged at 22400g, the supernatant was loaded onto a 5 mL Ni column (HiTrap Chelating HP, Amersham Biosciences). The column was washed with 10 column volumes of 50 mM potassium phosphate (pH 7.5), 10% glycerol, 0.4 M NaCl, 10 mM imidazole, 2 μ M leupeptin, and 2 μ M pepstatin, and the protein was eluted with a linear gradient from 0 to 200 mM imidazole in 20 column volumes of the same buffer. Fractions containing polyamine oxidase were pooled and concentrated by the addition of solid ammonium sulfate to 65% saturation. The pellet resulting upon centrifugation was resuspended in 50 mM potassium phosphate (pH 7.5), 10% glycerol, 1 μ M leupeptin, and 1 μ M pepstatin, loaded onto a 120 mL HiPrep Sephacryl S-200 column (Pharmacia Biotech), and eluted with the same buffer. The fractions containing the protein were pooled. The purified enzyme was stored at -80 °C, in 50 mM potassium phosphate (pH 7.5), 10% glycerol, 1 μ M leupeptin, and 1 μ M pepstatin. The concentration of polyamine oxidase was determined using an ϵ_{458} value of 10 400 M⁻¹ cm⁻¹ and a molecular mass of 56 101 Da (10).

Assays

Polyamine oxidase activity was typically determined in air-saturated buffer containing 10% glycerol by monitoring oxygen consumption with a computer-interfaced Hansatech Clark oxygen electrode (Hansatech Instruments, Pentney King's Lynn, U.K.). All assays were conducted at 30 °C. The buffers were 50 mM Tris-HCl from pH 7.5 to 9, 50 mM CHES from pH 9 to 10, and 50 mM CAPS from pH 10 to 11.5.

Rapid Reaction Kinetics

Rapid reaction kinetics measurements were performed with an Applied Photophysics SX-18MV stopped-flow spectrophotometer. For anaerobic experiments, the instrument was flushed with anaerobic buffer immediately prior to use. Solutions were made anaerobic by placing them under vacuum and then flushing them with oxygen-scrubbed argon; glucose and glucose oxidase were then added at final concentrations of 5 mM and 36 nM, respectively, to maintain anaerobic conditions. To study the reaction of the reduced enzyme with oxygen, the enzyme (40 μ M) was reduced with 0.2 mM BESPM. The reduced enzyme was then mixed with buffer equilibrated with different concentrations of oxygen. Under these conditions, the subsequent re-reduction of the oxidized enzyme by the remaining substrate was sufficiently slow not to interfere with the oxidation kinetics.

Data Analysis

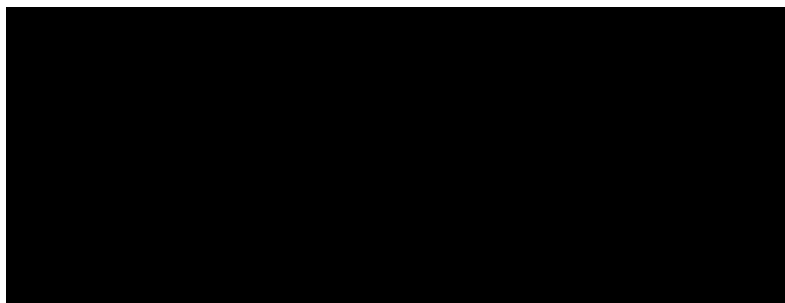
Data were analyzed using Kaleidagraph (Adelbeck Software, Reading, PA). The Michaelis-Menten equation was used to determine k_{cat} , k_{cat}/K_m , and K_m values when initial rates were measured as a function of the concentration of a single substrate. Equation 1 was used to analyze the pH dependence of kinetic parameters which decreased at low pH. Equation 2 was used to analyze the pH dependence of kinetic parameters which decreased at both low and high pH. For rapid reaction studies, rate constants were obtained from the change in absorbance of the flavin with time by fitting eq 3 to the data

$$\log Y = \log \left(\frac{C}{1 + \frac{H}{K_1}} \right) \quad (1)$$

$$\log Y = \log \left(\frac{C}{1 + \frac{H}{K_1} + \frac{K_2}{H}} \right) \quad (2)$$

$$A = A_{\infty} + A_1 e^{-\lambda_1 t} \quad (3)$$

where A is the measured absorbance, A_{∞} is the final absorbance, A_1 is the amplitude, λ_1 is the rate constant, and t is time.



Scheme 2.

RESULTS

Kinetic Mechanism

Initial kinetic characterization of mouse polyamine oxidase by Wu et al. (10) established that the k_{cat}/K_m value for N1-acetylspermine is independent of the concentration of oxygen and that the k_{cat}/K_m value for oxygen is the same for N1-acetylspermine and N1-acetylspermidine. This was consistent with a ping-pong mechanism in which the oxidized polyamine dissociates before the reduced enzyme reacts with oxygen. These data suggest that the kinetic mechanism of polyamine oxidase resembles the general mechanism of most flavoprotein oxidases shown in Scheme 2 (22,23). In the reductive half-reaction, transfer of a hydride equivalent from the substrate to the flavin reduces the flavin; the reduced flavin then reacts with oxygen in the oxidative half-reaction to regenerate the oxidized enzyme and form hydrogen peroxide. Prior to carrying out the mechanistic analyses described here, we carried out a preliminary analysis of the effects of pH. This showed that polyamine oxidase is much more active at pH 10 than at pH 7.6, the pH used in the studies of Wu et al. (10) (results not shown). While N1-acetylspermine is the best substrate for the enzyme, kinetics of slower substrates are much more likely to be limited by chemical steps, so intrinsic values of kinetic parameters such as pK_a values are more readily measured (24). BESPM is a slower substrate than N1-acetylspermine. It is also effective in preventing the growth of tumor cells (25), and its success at this has led to the development of more effective antineoplastic agents (26). Accordingly, more detailed kinetic studies were carried out with BESPM at pH 10.

The steady-state kinetic parameters with BESPM as a substrate for polyamine oxidase were determined by varying both oxygen and BESPM concentrations at pH 10; the resulting values are summarized in Table 1. The rate constants for the individual half-reactions were determined by stopped-flow methods. When polyamine oxidase and BESPM are mixed rapidly in the absence of oxygen, the flavin spectrum rapidly changes to that of the reduced enzyme (Figure 1A). The spectral changes can be described by a single exponential (Figure 1B), with no

evidence for intermediates between the oxidized and reduced enzyme, and there is no increase in absorbance at wavelengths greater than 530 nm. The rate constant for reduction is independent of the concentration of BESPM at concentrations of 50 μM to 6 mM (results not shown), indicating that the K_d value is less than 10 μM . The rate constant for reduction is close to the k_{cat} value from steady-state kinetic analyses, consistent with enzyme reduction being rate-limiting for turnover. In support of this conclusion, when 0.3 mM BESPM is added to 20 μM enzyme in air-saturated buffer, the absorbance spectrum of the enzyme resembles that of the oxidized enzyme for most of the reaction, only converting to that of the reduced enzyme when the oxygen is depleted (results not shown). The kinetics of the oxidative half-reaction were determined by mixing enzyme which had been reduced with BESPM with buffer solutions equilibrated with varying concentrations of oxygen. At all concentrations of oxygen, there was a monophasic change from the spectrum of the reduced enzyme to that of the oxidized enzyme (Figure 2A). The rate constant for oxidation varied directly with the concentration of oxygen (Figure 2B), consistent with a simple bimolecular reaction. The second order rate constant was the same at pH 10 and 7.5, 121 ± 2 and $141 \pm 3 \text{ mM}^{-1} \text{ s}^{-1}$, respectively, establishing that k_{ox} is pH-independent over that pH range.

pH Dependence

The effects of pH on steady-state kinetic parameters was determined with BESPM as a substrate. The $k_{\text{cat}}/K_{\text{BESPM}}$ pH profile is bell-shaped (Figure 3B), with the $k_{\text{cat}}/K_{\text{BESPM}}$ value decreasing when a group with an apparent $\text{p}K_a$ value of 9.8 ± 0.1 is protonated or when a group with an apparent $\text{p}K_a$ value of 10.8 ± 2 is deprotonated. The k_{cat} value is independent of pH at high pH, but decreases at low pH when a group with an apparent $\text{p}K_a$ value of 9.0 ± 0.1 is protonated (Figure 3A). The effect of pH on the k_{cat}/K_M value for oxygen was not determined directly with BESPM as a substrate. However, the k_{ox} value, which is equivalent to the $k_{\text{cat}}/K_{\text{O}_2}$ value for the mechanism of Scheme 2, was the same at pH 7.5 and 10 (vide supra), and the $k_{\text{cat}}/K_{\text{O}_2}$ is independent of pH between pH 6.5 and 11.5 with N1-acetylpermine as a substrate (results not shown.) The effect of pH on the visible absorbance spectrum of the enzyme-bound flavin was also determined; there was no significant change between pH 7.5 and 11 (results not shown).

Y430F Polyamine Oxidase

While the structure of mouse polyamine oxidase has not yet been reported, the structure of a homologous maize spermine oxidase has been determined (14). In addition, polyamine oxidase is a homologue of monoamine oxidase (18), and the structure of human monoamine oxidase B has been determined (27). Tyrosyl residues 60, 398, and 435 in monoamine oxidase B have been proposed as possibilities for a tyrosyl radical in that enzyme (20). Of these, the only corresponding residue in mouse polyamine oxidase which is a tyrosine is Tyr430, which corresponds to Tyr398 in monoamine oxidase B and Tyr407 in monoamine oxidase A. Accordingly, the Y430F mutant of mouse polyamine oxidase was characterized in an effort to evaluate the role of that residue in catalysis. The effects of this mutation on the steady-state and rapid reaction kinetic parameters of polyamine oxidase are summarized in Table 1. The k_{cat} value and the K_m value for oxygen are both altered in the mutant enzyme. The kinetics of the reductive half-reaction were studied by monitoring changes in the absorbance spectrum of the flavin when the enzyme was mixed with BESPM anaerobically in the stopped-flow spectrophotometer. The results were qualitatively identical to those with the wild-type enzyme, with a monophasic change from the oxidized to reduced enzyme with no indication of intermediates. There was a slight dependence of the rate constant for reduction on the concentration of BESPM, consistent with a K_d value of $\sim 8 \mu\text{M}$. The limiting rate of reduction, k_{red} , was ~ 6 -fold smaller than the wild-type value, and close to the k_{cat} value. In contrast, the k_{ox} value for the mutant, determined by measuring the rate constant for the reaction of the reduced enzyme with oxygen, was identical to the wild-type value.

DISCUSSION

The results presented here establish the kinetic mechanism of mouse polyamine oxidase and provide insights into the catalytic mechanism of the enzyme. BESPM was chosen as a substrate for these studies because it is a slower substrate than N1-acetylspermine or N1-acetylspermidine (10); enzyme kinetics with slow substrates are more likely to reflect intrinsic kinetic parameters. In addition, BESPM is effective in preventing the growth of tumor cells (25) so that its metabolism is of interest, and polyamine oxidase is responsible for the catabolism of N-substituted spermine analogues (7,28). The results of steady-state and rapid reaction kinetic analyses are consistent with the kinetic mechanism of Scheme 2 for polyamine oxidase. In the case of the reductive half-reaction, in which the amine substrate is oxidized by transfer of a hydride equivalent to the flavin, the reaction occurs as a single first-order reaction with a rate constant that is independent of the concentration of BESPM. This is consistent with a low K_d value for this substrate; the estimate for the K_d value of $\leq 10 \mu\text{M}$ is in reasonable agreement with the K_m value from steady-state kinetic analyses. In addition, k_{red} , the rate constant for flavin reduction, agrees with the steady-state k_{cat} value. This establishes that this step is limiting for turnover, a conclusion further supported by the predominance of the oxidized enzyme during turnover. The immediate product of reduction is the Schiff base bound to the reduced enzyme. With a number of flavoproteins, the oxidized substrate does not dissociate from the reduced enzyme at a kinetically significant rate, instead dissociating after the flavin has been oxidized (23). The presence of the oxidized enzyme in the active site adjacent to the reduced flavin frequently can frequently be detected by a long-wavelength visible absorbance band due to a charge-transfer interaction between the product and enzyme (29). No such long-wavelength absorbance is detected upon reduction of polyamine oxidase by BESPM; a simple explanation is that the product dissociates from the reduced enzyme rapidly. Consistent with this interpretation, the structure of maize polyamine oxidase reduced by spermine shows that the product is not in the active site (13). The oxidative half-reaction of mouse polyamine oxidase can be described as a simple second-order reaction of reduced polyamine oxidase with oxygen with the second-order rate constant k_{ox} to complete the catalytic cycle. For the mechanism of Scheme 2, k_{ox} is equal to the k_{cat}/K_m for oxygen in steady-state kinetics; the values of these kinetic parameters given in Table 1 are comparable.

Both the k_{cat} and k_{cat}/K_m values for BESPM are pH-dependent, while the rate constant for the reaction of the reduced enzyme with oxygen is pH-independent. BESPM contains four nitrogens, making definitive interpretation of the pH dependence difficult; their $\text{p}K_a$ values range from ~ 8 to ~ 11 (30). Since both the acid and base limbs of the pH profile have a slope of unity, one can rule out the possibility that all four nitrogens must be in the same protonation state for catalysis. The $\text{p}K_a$ value of 10.8 in the $k_{\text{cat}}/K_{\text{BESPM}}$ profile is not seen in the k_{cat} profile, so it is only involved in binding. The simplest explanation for this $\text{p}K_a$ is that it is N12 of BESPM, the nitrogen with the highest $\text{p}K_a$, and it must be protonated for binding. In the case of monoamine oxidase, the substrate amino nitrogen must be unprotonated for catalysis, and it has been suggested that the reactive nitrogen in the substrate for polyamine oxidase must also be deprotonated for catalysis (18). This provides a reasonable assignment for the $\text{p}K_a$ of 9.8 seen in the $k_{\text{cat}}/K_{\text{BESPM}}$ profile as N4 of the substrate; this $\text{p}K_a$ is shifted to 9.0 when BESPM is bound. The pH independence of the flavin spectrum over the pH range covered in Figure 3 suggests that ionization of the flavin is not responsible for one of the $\text{p}K_a$ values. The pH independence of the rate constant for flavin oxidation establishes that the ionization state of the flavin is unchanged between pH 7.5 and 10. Reduced flavin reacts much more quickly with oxygen when the flavin is anionic (31,32), and the $\text{p}K_a$ for the deprotonation of N1 of free flavin is ~ 6.7 (33). The pH independence of the k_{cat}/K_m value for oxygen with polyamine oxidase can be explained by a moderate decrease in this $\text{p}K_a$ when FAD is bound to the enzyme.

While polyamine oxidase has seen little mechanistic study, the mechanism of the homologous monoamine oxidase has been extensively studied. At present, there are two dominant proposals for the mechanism of monoamine oxidase. In the mechanism of Edmondson and colleagues (17), the neutral amine adds to the flavin at the C4a position; collapse of this adduct to form reduced flavin is concerted with transfer of the substrate α -proton to the flavin N5. Since no intermediate is seen between oxidized and reduced flavin in the reduction of monoamine oxidase (17,34), initial formation of the initial flavin adduct must be unfavorable so that it does not accumulate. An alternative possibility is that the reaction involves concerted transfer of two electrons and a proton without formation of an intermediate adduct; however, on the basis of the ρ value of 2 for benzylamine oxidation by monoamine oxidase A, Miller and Edmondson (17) have argued against a mechanism involving a direct hydride transfer. A very different mechanism has been proposed by Silverman (19), based primarily upon inactivation of monoamine oxidase by a series of mechanism-based inhibitors predicted to act via radical intermediates. In this mechanism, a substrate aminium cation radical is generated by abstraction of a single electron from the substrate nitrogen. Subsequent transfer of a hydrogen atom to the flavin semiquinone would yield the products. Alternative versions of this mechanism involving an amino acid radical have also been proposed (19).

A flavin semiquinone, formed by one-electron oxidation of the amine substrate, should be readily detectable in the reductive half-reaction of polyamine oxidase. The spectral changes seen during the reductive half-reaction of the enzyme show no evidence for the presence of an intermediate between the oxidized enzyme and the fully reduced enzyme. This does not preclude the involvement of such an intermediate; it only establishes that it does not accumulate. This is similar to the situation with monoamine oxidase.

The Y430F enzyme provides a more direct test of the involvement of a tyrosyl radical in polyamine oxidase. Tyr430 is the only tyrosyl residue conserved in monoamine oxidase and mouse polyamine oxidase which is close to the flavin. It corresponds to the tyrosyl residue of monoamine oxidase which has been suggested by Rigby et al. (20) to be the most likely candidate for the putative tyrosyl radical. The data in Table 1 are consistent with the Y430F mutation affecting only the k_{red} value. The 6-fold decrease in the value of this parameter results in a comparable decrease in the k_{cat} value, since reduction is effectively rate-limiting with BESPM as a substrate. The decrease in the K_{m} value for oxygen can also be rationalized by the decrease in the k_{cat} value. For the mechanism of Scheme 2, the K_{m} value for oxygen equals $k_{\text{cat}}/k_{\text{ox}}$. Consequently, the K_{m} value for oxygen will decrease by the same amount as the k_{cat} value if the k_{ox} value is unaffected by the mutation. The effects of this mutation are consistent with a role for Tyr430 in the active site, but not with that role being formation of a tyrosyl radical. A decrease of only 6-fold in the rate constant for reduction is far less than would be expected for replacement of a tyrosyl moiety with phenylalanine in such a case. Mutagenesis of Tyr407 of monoamine oxidase A to phenylalanine similarly has only a small effect on the enzyme activity (35), providing further evidence against a mechanism involving obligatory oxidation of this residue to a radical.

These results provide several insights into the catalytic mechanism of mouse polyamine oxidase. With BESPM, flavin reduction is rate-limiting for turnover. No intermediates are seen during flavin reduction, and Tyr430 is unlikely to form a tyrosyl radical during catalysis; these results are contrary to the expectations of mechanistic proposals involving radical intermediates.

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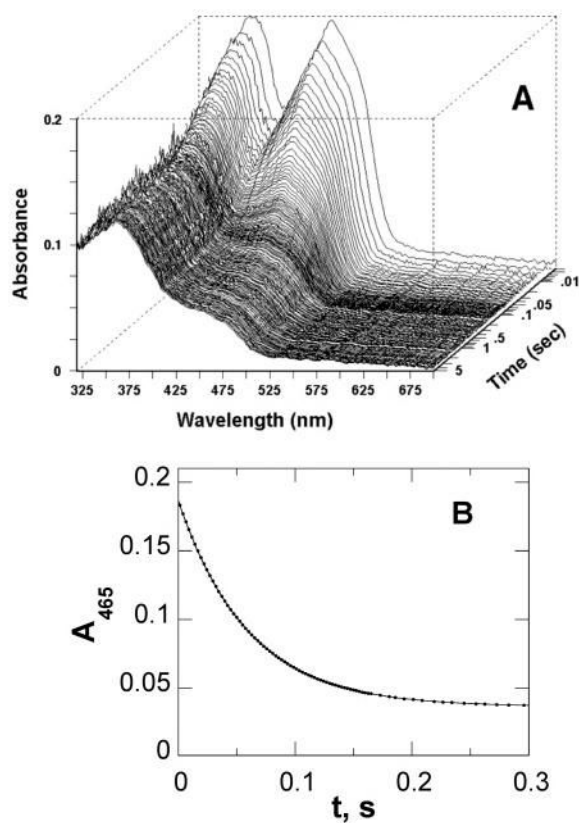


Figure 1. Reductive half-reaction of polyamine oxidase with BESPM as the substrate: (A) visible absorbance spectra after mixing polyamine oxidase with BESPM (1.2 mM final concentration) anaerobically at pH 10 and 30 °C and (B) time dependence of the absorbance change at 465 nm in panel A. The line in panel B is from a fit to eq 3.



Figure 2. Oxidative half-reaction of polyamine oxidase with BESPM as the substrate: (A) time dependence of the absorbance change at 465 nm after mixing reduced polyamine oxidase with oxygen (0.12 mM final concentration) at pH 10 and 30 °C and (B) dependence of the rate constant for flavin oxidation on oxygen concentration. The line in panel A is from a fit to eq 3.

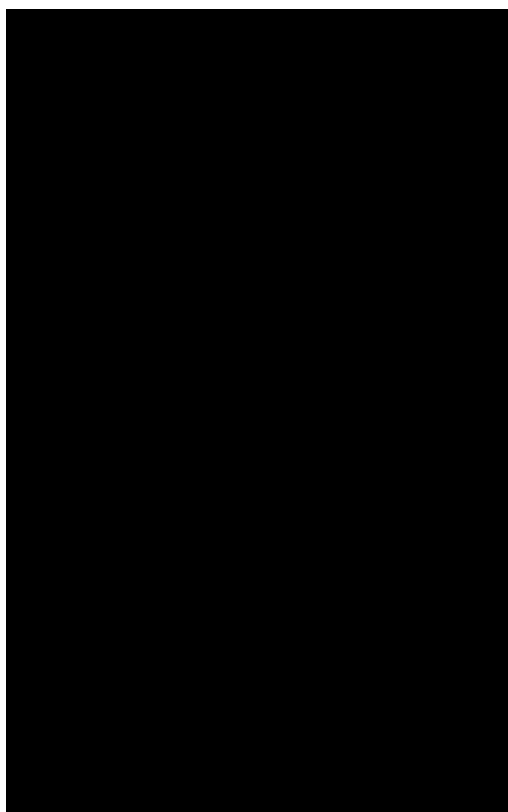


Figure 3. pH dependence of polyamine oxidase with BESPM as the substrate: (A) $\text{pH}-k_{\text{cat}}$ profile and (B) $\text{pH}-k_{\text{cat}}/K_{\text{BESPM}}$ profile. $T = 30\text{ }^{\circ}\text{C}$. The lines are from fits of the data to eq 1 (A) and eq 2 (B).

Table 1
Kinetic Parameters for Polyamine Oxidase with BESPM as the Substrate^a

kinetic parameter	wild-type enzyme	Y430F
k_{cat} (s ⁻¹)	12.3 ± 0.5	1.6 ± 0.04
K_{BESPM} (μM)	18 ± 1.6	11 ± 0.9
K_{O_2} (μM)	150 ± 20	26 ± 5
$k_{\text{cat}}/K_{\text{BESPM}}$ (mM ⁻¹ s ⁻¹)	690 ± 50	200 ± 20
$k_{\text{cat}}/K_{\text{O}_2}$ (mM ⁻¹ s ⁻¹)	81 ± 7	63 ± 11
k_{red} (s ⁻¹)	18.9 ± 0.8	2.9 ± 0.04
K_{d} (μM)	<10	8 ± 2
k_{ox} (mM ⁻¹ s ⁻¹)	121 ± 2	119 ± 2

^a At pH 10 and 30 °C.