On the Mechanism and Rate of Substrate Oxidation by Amine Oxidase from Lentil Seedlings*

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The kinetics of reaction between lentil seedlings amine oxidase and two amine substrates, namely putrescine and dimethylaminomethylbenzylamine, have been studied by rapid mixing with diode array detection. In this way several wavelengths can be monitored at once allowing the simultaneous measurement of enzyme bleaching and formation of a yellow radical intermediate. The two substrates are oxidized at rates that differ by one order of magnitude in favor of putrescine. Of the individual five rate constants measured and/or calculated from the experimental ones, $k_2$ alone, the monomolecular transformation of ES to EP accounts for this difference. The reoxidation step is instead not rate limiting and identical for the two substrates.

Amine oxidase (EC 1.4.3.6) from lentil seedlings is a soluble dimeric enzyme containing one copper atom and one carbonyl-like organic cofactor per monomer of 70 kDa (1). This enzyme catalyzes the oxidation of diamines, the physiological substrate being probably putrescine, using molecular oxygen as the oxidant.

Its absorption spectrum in the visible range makes it suitable for a detailed analysis of the reaction mechanism, since at least three distinct spectroscopic states of the enzyme are detectable and, using a chromogenic substrate, product formation can be followed.

The absorption in the visible range is associated with the organic cofactor. This cofactor was thought to be pyridoxal phosphate (2, 3) or pyrroloquinoline quinone (4). More recently, however, Klinnmann et al. (5) have reported that several amine oxidases contain 6-hydroxydopa as the redox organic moiety. Moreover Pedersen et al. (6) have shown that 6-hydroxydopamine, but not pyrroloquinoline quinone, gives a radical species whose spectroscopic (optical and ESR) features are quite similar to those of lentil seedlings amine oxidase. In particular, the radical species is associated with a yellow form of the enzyme showing two sharp absorption peaks at ~440 and ~460 nm. The presence of copper is absolutely required for the formation of this radical.

In a previous report (6) it was shown that a simple catalytic scheme with four intermediates is able to describe the reaction of this enzyme with the synthetic chromogenic substrate p-dimethylaminomethyl benzylamine (p-DABA). Now we have extended our preliminary study to the physiological substrate putrescine. In this paper we present a thorough spectroscopic characterization of the various states and a more complete analysis of the reaction mechanism, together with a scheme which takes into account the kinetic and chemical data altogether.

**MATERIALS AND METHODS**

Amine oxidase was purified from lentil (Lens culinaris) seedlings according to the procedure described by Floris et al. (1).

p-DABA was synthesized as described by Bardsley et al. (7); this substrate is colorless in the reduced state, but its aldehyde has an absorption peak at 250 nm with an extinction coefficient of 11,000 M$^{-1}$ cm$^{-1}$. All other chemicals were of analytical grade. Stopped flow experiments were carried out using a Tracor Northern TN6500 rapid scanning photodiode array spectrophotometer connected to a Gibson Durrum stopped flow apparatus (2-cm light path). This instrument acquires spectra of 1024 photodiode readings in 10 ms and stores up to 64 spectra recorded at chosen time intervals. Data analysis was carried out on Compaq desktop 286 and MicroVax 3500 computers.

Simulation of the time course was carried out by numerical integration, using the same kinetic scheme for both substrates (p-DABA and putrescine; for Scheme 1 see also Refs. 6, 8, and 9).

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R - \text{CHO} + \text{NH}_3 \xrightarrow{k_{r-1}} \text{R} + \text{NH}_3;
$$

$\text{ES} \xrightarrow{k_2} \text{EP}$

$\text{ER} + \text{H}_2\text{O} \xrightarrow{k_4} \text{ER} + \text{H}_2\text{O} + \text{NH}_3$

\text{SCHEME 1}

where $k_r$ and $k_1$ are bimolecular steps, depending on substrate and oxygen concentrations, respectively, whereas $k_{r-1}$, $k_2$ and $k_4$ are monomolecular, first order reactions; although in principle all the reactions are irreversible, we decided to make steps 2, 3, and 4 irreversible, step 4 because of the large difference in redox potential of the couple $\text{O}_2/\text{H}_2\text{O}$, steps 2 and 3 because of the observation that the oxidized enzyme can be anaerobically titrated with stoichiometric amounts of either putrescine or p-DABA, which implies that at least one of the two backward reactions must be extremely slow as compared with the onward reaction (1). This assumption allows an unequivocal fit of our experimental data.

Species $\text{Eox}$ is the resting enzyme in the presence of oxygen (see also Scheme 2) and has been characterized spectroscopically (1); $\text{ER}$ is the equilibrium species in an anaerobic enzyme substrate mixture. The conversion of $\text{ER}$ into $\text{Eox}$ observed upon mixing the substrate-reduced enzyme with oxygenated buffer (step 4) is a fast bimolecular reaction (6) and is associated, in similar enzymes, with release of...
hydrogen peroxide and ammonia (8). The Michaelis complex ES formed in step 1 has a bleached spectrum, as recorded in stopped flow experiments (Ref. 6; see also below). The EP intermediate cannot be resolved directly with putrescine, but since the oxidation of the chromogenic substrate p-DABA is not synchronous with either the bleaching (step 1) or the reduction of LSAO (step 3), this species was introduced and corresponds to an enzyme-product complex, although it is at present impossible to state whether the aldehyde is released at step 2 or 3. At least two or three wavelengths were simultaneously simulated. The values of $K_r$ and $k_{cat}$, neglecting the terms which do not contain $k_i$, are as follows.

$$k_{cat} = k_2 k_3/(k_3 + k_2)$$

$$K_r = k_{cat} (k_2 + k_3)/(k_3 k_2)$$

The parameters are highly correlated with each other and with the extinction coefficients of the enzyme intermediates. Therefore some constraints were applied to simplify the search of significant sets of parameters.

(i) The extinctions of Eox and ER were taken directly from the experimental values; the extinctions of ES and EP were assumed to be equal (at least in the visible range, where no contribution is to be attributed to the product) and were taken to correspond to those of ES and EP, neglecting the terms which do not contain $k_i$ (10), as follows.

(ii) The extinctions of all enzyme intermediates were requested to be substrate independent, even though in some instances it was necessary to add a constant absorbance to compensate for small baseline drifts.

(iii) The individual rate constants were requested to generate values of $K_r$ and $k_{cat}$, comparable with those measured by steady state techniques.

(iv) The rate constants $k_i$ and $k_r$ were requested to be independent of the amine substrates. This is a very stringent requirement and depends on the assumption that the aldehyde is released at step 2, which is not proven. It will be noticed that whereas the monomolecular steps are difficult to resolve, most notably in the case of putrescine, whose aldehyde is colorless and is formed at high rate, the bimolecular steps are both associated with clearcut absorbance transitions and can be resolved with great precision and confidence.

The scheme describes satisfactorily the reaction of LSAO and p-DABA and putrescine under all but the most extreme experimental conditions, as reported below. Very large amounts of oxygen and either amine cause a progressive reduction of the catalytic rate, which we attribute to product inhibition, also observed under steady state conditions, or to degradation of the enzyme by $\text{H}_2\text{O}_2$. Furthermore, when the oxidized enzyme is mixed with p-DABA in the complete absence of oxygen a stoichiometric amount of aldehyde is formed (reaction followed at 250 nm) at a slightly faster rate than one would predict using the rate constants determined in the presence of oxygen.

**RESULTS**

**Steady State Analysis**—The steady state parameters $K_r$ and $k_{cat}$ for p-DABA were measured spectroscopically, following the absorbance increase at 250 nm due to the formation of the aromatic aldehyde. The experiments were carried out in air-equilibrated phosphate buffer, and the substrate was varied over a wide range of concentrations (10-670 µM). The concentration of the enzyme was varied between 10 and 500 nM to explore possible concentration-dependent phenomena (e.g. dissociation of the dimer). This was demonstrated not to be the case.

The steady state parameters for putrescine have been determined earlier (1) and confirmed in this work. The $K_r$ value for oxygen was estimated spectroscopically using p-DABA as substrate.

The steady state kinetic parameters for oxygen, p-DABA, and putrescine are reported in Table I.

**Stopped-flow Experiments on Substrate Binding and Degradation**—Reduced LSAO (obtained under anaerobic conditions in the presence of an excess of putrescine or p-DABA) was mixed with oxygen-containing buffer; the oxidation of the enzyme was found to follow bimolecular kinetics. The oxidized enzyme catalyzes the aldehyde formation with a time course equal to that observed when the reaction was started by mixing the resting enzyme (in the presence of oxygen) with its substrate.

The reaction of oxidized LSAO with the two substrates was followed in the absence and in the presence of 25-270 µM
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TABLE I
Fast kinetic and steady state parameters for LSAO
Experimental (exptl.) conditions: 0.1 M phosphate buffer, pH 7, t = 20 °C, substrate concentrations 25-500 μM, and oxygen concentration 0-135 μM. The values of $K_m$ and $k_{cat}$ are calculated from the individual rate constants $k_1$, $k_2$, $k_3$, and $k_4$. The standard deviation was calculated on six independent determinations.

| Substrate | $K_m$ (μM) | $k_{cat}$ | $k_{cat}$ | $k_1$ | $k_2$ | $k_3$ | $k_4$
|-----------|------------|-----------|-----------|-------|-------|-------|-------|
| Oxygen    | <6         | 1         | Not rate-limiting | 3     | 2.5 x 10⁶ | 8     | 200   | 2 x 10⁷ |
| p-DABA    | 29 ± 4     | 37        |           | 5.5 ± 0.1 | 7.5   | 3     | 4 x 10⁵ |
| Putrescine| 230 ± 30   | 250       |           | 92 ± 15 | 100   | 3     | 200   | 2 x 10⁷ |

Fig. 1. Time course of the reaction of LSAO and p-DABA as followed at 464 (□) and 496 (+) nm. Experimental conditions: 10 μM enzyme, 500 μM substrate, and 27 μM oxygen; buffer 0.1 M phosphate, pH 7.0, 20 °C. The inset shows a three-dimensional reconstruction of the raw absorbance data.

In the presence of oxygen, the time course of the reaction of LSAO with putrescine is more complex and the spectrum of the steady state enzyme is easily recorded (Figs. 2 and 3B). Interestingly this spectrum is only satisfactorily simulated as a mixture of oxidized, reduced, and radical species. This result provides evidence for the presence of the same spectroscopic components in the reaction with the two substrates and offers an estimate for the relative amounts of the different enzyme species under steady state conditions (Fig. 3, A and B).

The rate constants for each step of Scheme 1 can be obtained by numerical integration of the differential equations and fitting to the experimental data (Table I). The steady state parameters were calculated from these values according to the procedure described by Fromm (10) and compared with the experimental ones (Table I). The time course described by the fitted parameters is reported as continuous lines in Figs. 1 and 2. The set of rate constants is not unequivocally determined in the case of putrescine, mainly because of the lack of independent information on the time course of aldehyde production. Scheme 1 in this case would be redundant and the values reported in Table I for $k_2$ and $k_3$ do not represent a unique set of parameters. By contrast the value of $k_4$ and $k_4$ corresponding to fast bimolecular reactions associated (in the scheme) with spectroscopic transitions, are determined with sufficient precision.

The parameters for putrescine reported in Table I allow to calculate reasonable steady state constants $K_m$ and $k_{cat}$ and satisfactorily reproduce the spectrum of the intermediates present in the steady state mixture.

DISCUSSION

The results reported above justify some important conclusions about the intermediate species in the catalytic cycle of oxygen; the concentration of putrescine and p-DABA was varied over the range 50–500 μM. Due to the low $K_m$ for oxygen, even a small contamination of gas in solution appreciably affects the time course of the reaction. Therefore a control experiment was run on an enzyme solution chemically deoxygenated just before the experiment with dithionite (which reduces the enzyme very slowly and only when present in large excess).

When the oxidized enzyme is mixed with p-DABA, three distinct spectroscopic components and two well separated processes are detected (Fig. 1). The initial species, showing a broad band centered around 500 nm, is rapidly converted to a bleached species, whose spectrum is very similar to that of the steady state mixture. Upon depletion of oxygen a yellow intermediate is formed; eventually, if the initial oxygen concentration exceeds that of p-DABA, the oxidized spectrum is obtained.

The first kinetic process, poorly resolved in Fig. 1, is bi-molecular and represents the formation of the enzyme-substrate complex followed by the reduction of the organic cofactor; it is monitored by an absorbance reduction (bleaching phase, see Ref. 6). The second phase corresponds to the formation of a radical species, the yellow intermediate.

Aldehyde formation as observed by mixing the oxidized enzyme with p-DABA in the absence of free oxygen is stoichiometric with the (monomer) enzyme concentration and is not synchronous with any kinetic process observed in the visible range; this observation demands that an additional chemical species, possibly the enzyme-product complex, is considered. The reactions of LSAO with putrescine in the absence of oxygen is observed to be a single exponential step; the rate constant of this process depends on substrate concentration with an order lower than 2.
amine oxidase from lentil seedlings.

(i) Only four spectroscopic species (including the optical contribution of the chromogenic substrate) can be observed, under the most favorable conditions. Two of these are indistinguishable if observation is carried out in the visible wavelength range.

(ii) The three basic spectra recorded in the visible range (Fig. 1) are substrate-independent.

(iii) The same simple kinetic scheme proposed to describe the reaction of this enzyme with p-DABA also describes the reaction with the physiological substrate.

(iv) The difference between putrescine and p-DABA does not lie in the recognition step (as demonstrated by the relevant rate constant, $k_i$ in Table I) but in the intramolecular steps.

Scheme 2 represents a possible mechanism of amine oxidase reaction pathway. The resting (oxidized) enzyme, $E_{ox}$, reacts with the amine substrate in a bimolecular step giving rise to a covalent enzyme-substrate complex ($E\cdot S^*$, a ketimine). This rapidly converts to a bleached form ($ES$, an aldimine), which in turn may release the aldehyde product leading to $EP$ also colorless and thus indistinguishable from $ES$. Thereafter an internal redox reaction, perhaps between the hydroxydopa and copper, produces a radical (ER) species, observed by EPR, which can rapidly react with oxygen to restore the oxidized enzyme liberating NH$_3$ and H$_2$O$_2$.

The different rate between p-DABA and putrescine as substrates appears to be limited to $k_2$ (Table I). The last conclusion could have been anticipated from the steady state parameters and the kinetic constants already reported for p-DABA (6). It is probably related to the $\alpha$-proton abstraction needed to transform the ketimine into an aldimine. A clear isotopic effect at the level of $\alpha$-protons has been reported (11).

Analysis of other substrates and similar enzymes will allow to define the possible general validity of this scheme.

It is important to recall that Dooley et al. (12) have demonstrated that several amine oxidases are able to generate a radical in the reaction with their substrates when copper is reduced to Cu$^+$ and have attributed to this radical the copper(I) semiquinone spectrum of the oxygen reacting species, with peaks at 464 and 432 nm.

As shown in Scheme 2 our data substantially agree with this interpretation, attributing the same spectrum to the species ER, an iminosemiquinone. Analysis of other substrates and inhibitors, and comparison with similar enzymes, will allow to further test the validity of this scheme and possibly suggest some modifications to take into account the small discrepancies observed under particular experimental conditions, such as the reduction of the catalytic rate in the presence of large amounts of products, tentatively attributed to product inhibition.

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