Transient kinetics of copper-containing lentil (*Lens culinaris*) seedling amine oxidase

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The reaction between lentil (*Lens culinaris*) seedling amine oxidase and its chromogenic substrate, *p*-dimethylaminomethylbenzylamine, has been studied by the stopped-flow technique. Upon being mixed with substrate in the absence of oxygen, the enzyme is bleached in a complex kinetic process. A yellow intermediate absorbing at 464 nm and the first product (aldehyde) are formed in subsequent steps. When oxygenated buffer is mixed with substrate-reduced amine oxidase, the 496 nm absorption of the oxidized enzyme is very rapidly restored in a second-order process ($k = 2.5 \times 10^7$ $M^{-1} \cdot s^{-1}$). This reaction is appreciable even at very low oxygen concentration, in keeping with the fairly low K_m for O_2 measured by steady-state kinetics.

INTRODUCTION

Copper-containing amine oxidases are ubiquitous enzymes in eukaryotes (Mondoví, 1985; Mondoví & Finazzi-Agró, 1982) that oxidize only primary amines, producing an aldehyde, NH_3 and H_2O_2. Their specificity may vary between different organisms, and in the same organism between tissues. Some of these enzymes are strictly specific for diamines or polyamines, whereas others can oxidize several different substrates like monoamines and histamine.

Besides copper, amine oxidases contain an organic cofactor that confers on them a characteristic pink colour and has been identified either as pyridoxal phosphate (Finazzi-Agró *et al.*, 1977) or more recently as pyrroloquinoline quinone (Lobenstein-Veerbeek *et al.*, 1984).

These enzymes follow a double-displacement mechanism (Ping Pong Bi Ter), releasing one product, aldehyde, in the absence of air and and the other two products, NH_3 and H_2O_2 upon reaction with O_2 (Mondoví, 1985). Plant amine oxidases generally display higher catalytic activities than do those of animals and form a spectroscopically distinct intermediate with maxima at 460, 430 and 370 nm when they react with the substrate in the absence of O_2.

Taking advantage of this feature and using an artificial chromogenic substrate, *p*-dimethylaminomethylbenzylamine (DABA) (Bardsley *et al.*, 1972), we investigated the transient kinetics of pure lentil seedling amine oxidase by the stopped-flow technique.

MATERIALS AND METHODS

The chemicals used were all commercially purchased and used as they were, except for DABA, which was synthesized as described by Bardsley *et al.* (1972). This compound is oxidized by lentil amine oxidase with a $V_{max.}$ one-fifth of that shown by the reaction with the purported natural substrate, putrescine. DABA yields an aldehyde that has an absorption maximum at 250 nm (ϵ 11000 $M^{-1} \cdot cm^{-1}$, see Fig 1 below).

Lentil seedling amine oxidase was purified to electrophoretic homogeneity (Floris *et al.*, 1983).

Steady-state experiments were conducted either spectrophotometrically or with an O_2-sensitive electrode. Rapid-mixing experiments were carried out with a Gibson–Durrum stopped-flow apparatus, which has a dead time of about 3.5 ms (Gibson & Milnes, 1964). Anaerobiosis was obtained by several cycles of evacuation followed by flushing with O_2-free N_2. The experiments at variable O_2 concentration were done by adding aerated buffer to the anaerobic sample with the aid of syringes.

Computer simulations were carried out on a HP 87 desk-top computer.

RESULTS

The static reduction of lentil seedling amine oxidase with DABA in the absence of O_2 results in a bleaching of the broad visible absorption of the resting enzyme and in the formation of new absorption bands at 464, 432 and 360 nm (Fig. 1). It is therefore possible to monitor, in a rapid-mixing experiment, the bleaching of the enzyme absorption at 496 nm, where the newly formed transitions do not contribute appreciably. On the other hand, if the observation is made at 464 nm, one should take into account the contribution of the disappearance of the resting absorption, which corresponds to 40% of the total absorbance change. Fig. 1 also shows that the formation of the DABA aldehyde (P1, see below) can be monitored at 250 nm.

The time course of the absorbance change observed at 496 nm and at 464 nm after mixing of the enzyme and DABA is shown in Fig. 2. In the absence of O_2 the absorption band of the resting enzyme is fully bleached upon addition of excess substrate. The bleaching appears

Abbreviation used: DABA, *p*-dimethylaminomethylbenzylamine.

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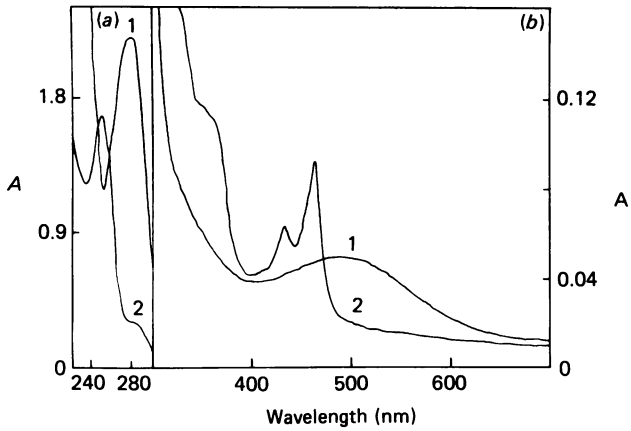

Fig. 1. Static optical spectra of lentil seedling amine oxidase

Spectra of the enzyme were run in the absence of DABA (1) and a few seconds after the addition of 2 mM-DABA (2). The conditions were as follows: $T = 50$ °C; buffer 0.1 M-potassium phosphate, pH 7; [enzyme] = 12 μM. Spectrum 2 in (*a*) was obtained after a 1:25 dilution.

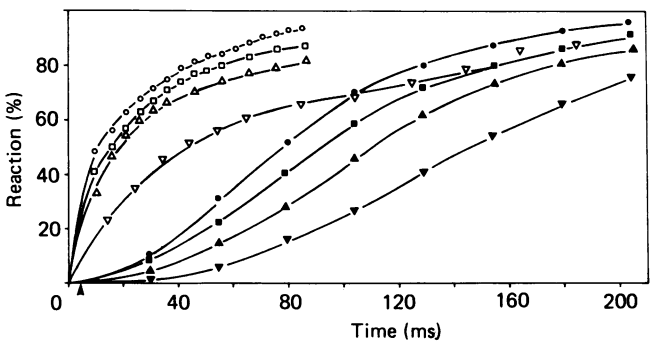

Fig. 2. Time course of the reaction of lentil seedling amine oxidase with DABA in anaerobiosis

The enzyme concentration was 19 μM; the substrate concentrations were as follows: ○, ●, 1.5 mM; □, ■, 0.5 mM; △, ▲, 0.15 mM; ▽, ▼, 0.05 mM. Open symbols refer to an observation wavelength of 496 nm; closed symbols refer to observations made at 464 nm. The buffer was 0.1 M-potassium phosphate, pH 7, and the temperature 20 °C.

to be biphasic, with a fast phase clearly dependent on the concentration of the substrate (Fig. 2, open symbols), although at the highest substrate concentrations a relevant fraction of the transmission increase is lost in the dead time of the instrument.

The slower phase seems to be independent of substrate concentration and is characterized by a rate similar to that observed for the formation of the yellow intermediate (Fig. 2, closed symbols).

The presence of O_2, even at a very low level (5 μM), enhances the heterogeneity of the bleaching, which shows an initial rapid decrease of the absorption, followed by a steady state, and finally by a complete bleaching if the ratio of substrate to O_2 is in favour of the substrate (Fig. 3). The amount of absorbance change obtained during the first, fast, phase appears to amount always to about half of the total bleaching.

Fig. 2 also shows that the formation of the yellow

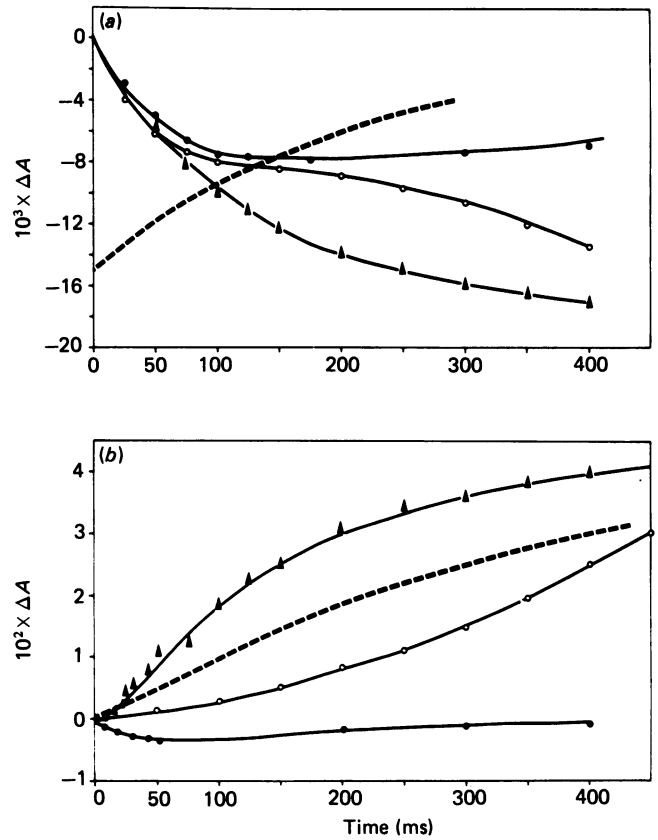

Fig. 3. Time course of the reaction of lentil seedling amine oxidase with DABA in the presence of O_2

The enzyme (19 μM) was mixed with DABA (50 μM) in the presence of the following O_2 concentrations: ▲, no oxygen (dithionite added); ○, 5 μM-O_2; ●, 270 μM-O_2. (*a*) Observations made at 496 nm; (*b*) observations made at 464 nm. In both panels the broken line indicates the time course of anaerobic aldehyde production observed at 250 nm (arbitrary units). Other conditions were as in Fig. 2.

intermediate always parallels (with respect to time) the bleaching of the enzyme. The lag preceding the formation of this new absorption band is present even in the absence of O_2, but it lasts longer when O_2 is present in the solution. At high [O_2] the concentration of the yellow intermediate at steady state is very low, since at 464 nm the increase in the absorption is undetectable and only a decrease due to the bleaching of the resting enzyme is observed (Fig. 3). Fig. 3 shows that the production of aldehyde proceeds at a rate that is slower than, or at most equal to, the formation of the yellow intermediate.

Steady-state experiments carried out at constant [substrate] ([DABA] = 1 mM) and variable O_2 (from 5 to 270 μM) concentration, and measuring the rate of formation of the aldehyde at 250 nm, gave an upper limit of 6 μM for $K_m(O_2)$. The reoxidation of the amine oxidase was studied by mixing the anaerobically substrate-reduced enzyme with O_2-containing buffer in the stopped-flow apparatus. The reoxidation step, giving rise to the absorption at 496 nm, is fast. Two experiments conducted at different O_2 concentrations are reported in Fig. 4. Fig. 4(*b*) shows that the enzyme recovers very rapidly its absorption in the presence of O_2, then reaches a steady

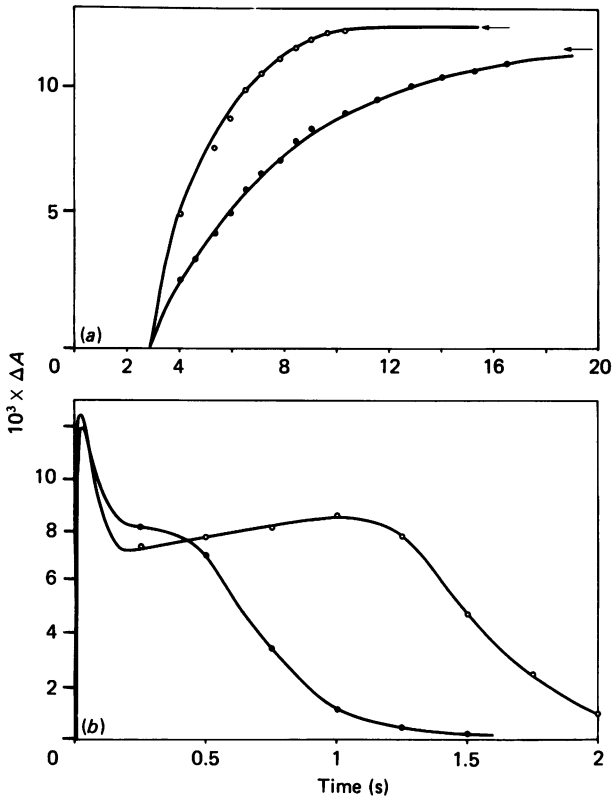

Fig. 4. Time course of the reaction of reduced lentil seedling amine oxidase with O_2

The enzyme (14 μM) was mixed anaerobically with 100 μM-DABA. The reduced enzyme was then mixed with oxygenated buffer containing (after mixing) ○, 54 μM- or ●, 27 μM-O_2. The wavelength of observation was 496 nm. The time scale in (*a*) is 100 times expanded with respect to that of (*b*) in order to show the fast phase of reoxidation. The arrow indicates the extrapolated end point of the reaction. Other conditions were as in Fig. 2.

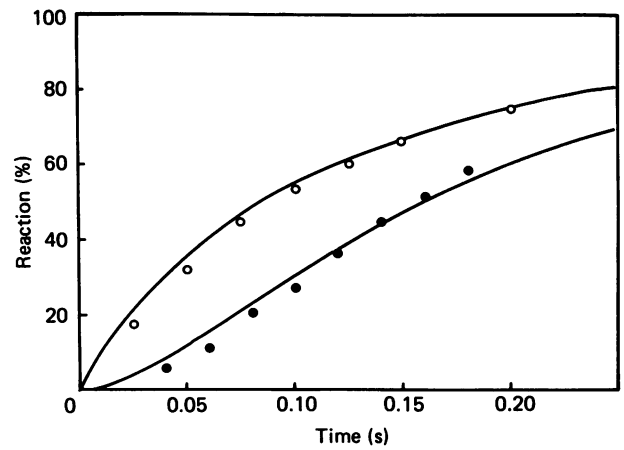

Fig. 5. Computer simulation of the lentil seedling amine oxidase reaction with DABA

Experimentally determined (○, 496 nm; ●, 464 nm) and simulated (continuous lines) time courses of the reaction of the enzyme with DABA in the absence of O_2 are shown. The experimental conditions were as in Fig. 2. Computer simultion was made according to Scheme 1 by using the following values for the rate constants: $k_{-1} = 3\ s^{-1}$, $k_{+1} = 2 \times 10^5\ M^{-1} \cdot s^{-1}$, $k_{+2} = 15\ s^{-1}$, $k_{+3} = 12\ s^{-1}$, $k_{+4} = 2 \times 10^7\ M^{-1} \cdot s^{-1}$. The values of $k_{+1} - k_{+4}$ were found experimentally, whereas that of k_{-1} is obtained from the fit. The simulated time courses reflect the disappearance of species E and the appearance of species EP + ER at 496 and 464 nm respectively. No correction has been used to take into account the contribution of the bleaching at 464 nm (see the text).

state, which is followed by bleaching if the substrate concentration exceeds that of O_2. The fast phase is better seen in Fig. 4(*a*), where the progress curves of the O_2 reaction is extrapolated, the dead time of the mixing device being taken into account.

The experiments at two different O_2 concentrations indicate that the affinity of the reduced enzyme for O_2 is very high ($K > 10^5\ M^{-1}$), and allows a rough estimate of the second-order rate constant for oxidation, namely $\sim 2.5 \times 10^7\ M^{-1} \cdot s^{-1}$.

DISCUSSION

The data reported above indicate that a stopped-flow study of the reaction of lentil seedling amine oxidase with DABA can give more direct information than such studies of other amine oxidases, thanks to the formation of a yellow reaction intermediate not observable with enzymes of animal origin at room temperature or in the absence of inhibitors (Finazzi-Agró *et al.*, 1977). Nevertheless the main kinetic features appear to be similar to those reported for pig plasma (Pettersson, 1985) and pig kidney (Mondoví *et al.*, 1971) amine oxidases.

In particular, lentil seedling amine oxidase was shown to react rapidly with the substrate in the absence of air, generating a bleached species, presumably the Michaelis complex. The reaction between enzyme and substrate appears to be bimolecular (as expected) only in the initial stages of the progress curve; the calculated second-order rate constant is about $2 \times 10^5\ M^{-1} \cdot s^{-1}$. As the reaction proceeds this complex undergoes further transformations, giving rise to a yellow intermediate of a yet unknown chemical nature; the first product, aldehyde, is formed at a comparable rate. These observations may be described by Scheme 1:

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES \overset{k_{+2}}{\rightarrow} EP \overset{k_{+3}}{\rightarrow} ER + P1$$

$$ER + P_2 \overset{k_{+4}}{\rightleftharpoons} E + P2 + P3$$

Scheme 1.

where ES is the bleached form, EP is the yellow intermediate, ER (absorbing at 464 nm, like EP) is the species reacting with O_2, P1 is the aldehyde, and P2 and P3 are NH_3 and H_2O_2.

The time course of the reaction of the enzyme with the substrate and with O_2 (where present) has been simulated by employing the above kinetic scheme. As an example, Fig. 5 depicts the results of the simulation carried out under anaerobic conditions; Scheme 1 reproduces satisfactorily the experimental time course at both wavelengths (464 and 496 nm), using the parameters reported in the legend to Fig. 5. Of the five rate constants that appear in Scheme 1, four are estimated directly from the observations at the three wavelengths (including 250 nm).

Scheme 1 therefore accounts for the heterogeneous time course observed in the bleaching of the enzyme due to the presence of an equilibrium between ES and E+S, which is driven to the right by the formation of the intermediate, EP. This hypothesis is supported by the similarity of the formation rate of the yellow intermediate and of the slow phase of the bleaching. Since Scheme 1 is a minimal one, it is evident that the presence of an additional intermediate between ES and EP cannot be excluded. Likewise it has not been proven that the liberation of P1 occurs during the transition EP→ER.

The bleaching is complete only in the absence of O_2, whereas in air there is only a partial bleaching when the substrate concentration is lower than the O_2 concentration.

In Scheme 1 the formation of the aldehyde comes later with respect to the yellow intermediate, as supported by the experiments performed in the complete absence of O_2, since even in the presence of contaminating O_2 the formation of the yellow intermediate EP is kept at low steady-state levels (Fig. 3). In view of the very high affinity of the enzyme for O_2, the time course of the absorbance change (especially at 464 nm) is very sensitive to O_2 contamination, which leads to a lengthening of the lag preceding the formation of the 464 nm-absorbing species. The reoxidation of the substrate-treated enzyme by O_2 indicates that this step is fast and essentially irreversible, yielding the absorption spectrum of the resting enzyme. The fairly high rate of reaction of the reduced enzyme with O_2 is in keeping with the very high affinity for O_2 indicated by steady-state measurements. Independent experiments showed that the release of NH_3 and H_2O_2 (P2 and P3) only occurred in the reoxidation step (Rinaldi *et al.*, 1984).

All the absorbance changes observed in the interaction between lentil seedling amine oxidase and its substrate are linked to the organic cofactor.

Static experiments have shown that the copper-free enzyme is still able to release aldehyde and NH_3 from the substrate (Rinaldi *et al.*, 1984). For the same modified enzyme the visible absorption band, which is almost unaffected by copper removal, is bleached by substrate, but is not restored in the presence of O_2; furthermore, no yellow intermediate is formed during the reduction step. Copper therefore appears essential for at least some of the steps, both in the reduction and in the reoxidation of the enzyme.

Further work is required to identify the nature of the intermediate observed by fast kinetics and to trace out the electron pathway from the substrate to O_2.

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