

Minireview

Cu^I-semiquinone radical species in plant copper-amine oxidases

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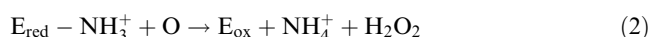
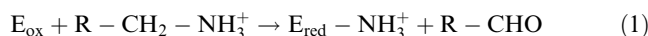
Abstract The intermediate Cu^I-semiquinone radical species in the catalytic mechanism of copper-amine oxidase from *Lens esculenta* and *Pisum sativum* seedlings has been studied by optical, Raman resonance and ESR spectroscopies and by stopped-flow and temperature-jump measurements. Treatment of highly purified enzyme preparations with good, poor or suicide substrates, under anaerobic and aerobic conditions, at different pH values and temperatures, makes it possible to generate, detect and characterize this free radical intermediate.

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Key words: Lentil; Amine oxidase; Copper; Cofactor; 6-Hydroxydopa; *Lens esculenta*

1. Introduction

Copper-containing amine oxidases (amine:oxygen oxidoreductase (deaminating) (copper-containing): EC 1.4.3.6) catalyze the oxidative deamination of primary amines subtracting two electrons from amines and transferring them to molecular oxygen. The catalytic mechanism can be divided into two half-reactions, (Eq. 1) enzyme reduction by substrate followed by (Eq. 2) enzyme re-oxidation by molecular oxygen:



In the plant kingdom, the presence of amine oxidases was first demonstrated in 1948 by Werle and co-workers [1]. Afterwards, plant amine oxidases from different species of several families have been purified to homogeneity and characterized [2].

Plant amine oxidases are homodimers, each subunit (molecular weight ≈ 70 kDa) contains one tightly bound Cu^{II} and one 6-hydroxydopa quinone (TPQ) [3] as organic prosthetic group formed by post-translational modification of a tyrosinyl

residue conserved in all copper-containing amine oxidases [4–7].

1.1. The reaction mechanism

The catalytic mechanism of copper-amine oxidases (Cu-AOs) is reported in Scheme 1. The amine substrate binds to the organic cofactor of the resting oxidized enzyme (Cu^{II}-TPQ, a) to form a Schiff base (Cu^{II}-quinone ketimine, b) [8,9]. Both these intermediates are thought to have a 498 nm absorption band. The oxidation of the amine substrate occurs via a base-catalyzed abstraction of a proton at C1, tentatively identified as Asp-300 in pea seedling amine oxidase (PSAO) [10] or as a histidine residue in lentil seedling amine oxidase (LSAO) [11], with the formation of a Cu^{II}-carbanion species (c). Transformation of the Cu^{II}-carbanion (c) into the Cu^{II}-quinolaldimine (d) is associated with the bleaching of the 498 nm absorption band. Oxidation of the bound substrate (followed by hydrolysis) releases the aldehydic product, leaving the Cu^{II}-aminoresorcinol derivative (e), which has a bound ammonia molecule. This species is still colorless. In plant amine oxidases, the Cu^{II}-aminoresorcinol is in equilibrium with the yellow, EPR-detectable Cu^I-semiquinolamine radical (f), containing the substrate-derived nitrogen covalently bound to the aromatic ring system [12–14], and characterized by absorption bands at 464, 434 and 360 nm [15,16]. The electron transfer rate between Cu^{II}-aminoresorcinol and the radical species is fast and involves a Cu^{II} → Cu^I transition [15]. Both forms of the reduced enzyme (e and f) can react with O₂ to release H₂O₂ and ammonia, thereby regenerating the Cu^{II}-quinone species [8,17,18].

In consideration of the catalytic mechanism, plant amine oxidase may be called a 'protein radical enzyme' [19], operating through a free radical located on a modified amino acid residue as a cofactor. A description of the characteristic free radical state of the best known enzyme radical amine oxidases from *Pisum* and *Lens* seedlings follows.

2. The radical species

2.1. Optical spectroscopy

In addition to the protein absorbance maximum at 278 nm, the oxidized Cu-AO absorbs in the visible region with a maximum at 498 nm and has a distinctive pink color. The extinction coefficients at 498 and 280 nm are determined to be 4.9 mM⁻¹ cm⁻¹ and 300 mM⁻¹ cm⁻¹ for PSAO [20], 4.5 mM⁻¹ cm⁻¹ and 245 mM⁻¹ cm⁻¹ for LSAO [21]. When a good substrate like putrescine, *p*-((dimethylamino)methyl)-

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Abbreviations: Cu-AO, copper-amine oxidase; GABA, γ -aminobutyric acid; LSAO, lentil seedling amine oxidase; *p*-DABA, *p*-(dimethylamino)-benzylamine; *p*-DAMBA, *p*-((dimethylamino)methyl)-benzylamine; PSAO, pea seedling amine oxidase; TPQ, 6-hydroxydopa (2,4,5-trihydroxyphenethylamine) quinone

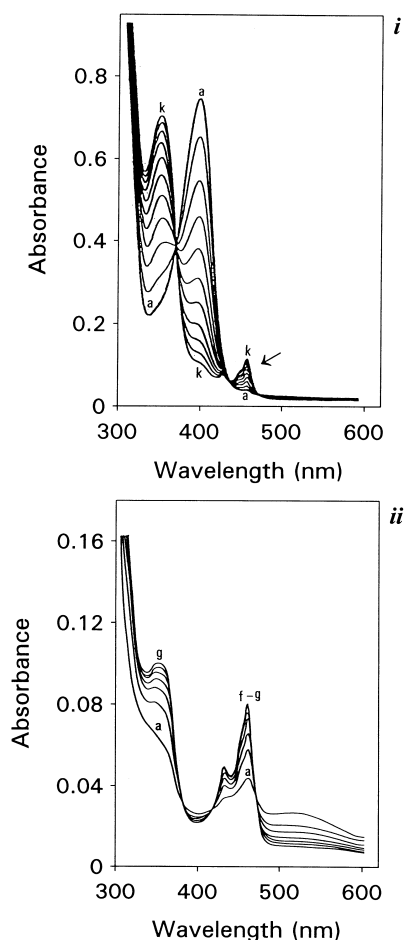


Fig. 1. i: Spectral change during the reaction of 11 μM LSAO with 1 mM *p*-DABA in 100 mM phosphate buffer, pH 7, under anaerobic conditions. Spectra were recorded from 1 min (a) to 300 min (k) with intervals of 30 min. The band centered at 400 nm is assigned to the protonated tautomeric form of the quinolaldimine, whereas the band at 350 nm corresponds to *p*-(dimethylamino)benzaldehyde (see text). The arrow indicates the radical species. ii: Spectral changes during the reaction of 11 μM LSAO with 1 mM GABA in 100 mM phosphate buffer, pH 7, under anaerobic conditions. Spectra were recorded from 4 min (a) to 28 min (g) at intervals of 4 min.

spectral change, isosbestic points at 474, 414 and 380 nm are observed (Fig. 1ii).

5. Catalysis in the presence of suicide substrates: disappearance of the radical species

The haloamine 2-Br-ethylamine [29] and tryptamine [30] are found to be both substrates and irreversible inhibitors of lentil amine oxidase. The addition of 2-Br-ethylamine or tryptamine to LSAO under anaerobic conditions results in a rapid bleaching of the broad absorption at 498 nm and new sharp bands at 434 and 464 nm appear in the spectrum, indicative of the free radical intermediate species (f). The radical species obtained upon reaction with good substrates such as putrescine is stable in anaerobiosis for many hours ($t_{1/2} = 36$ h) and re-admission of oxygen restores the pink-red color to its original intensity. In contrast, the radical obtained with 2-Br-ethylamine and with tryptamine decays even under anaerobic con-

ditions, with half-lives of 10 and 90 min, respectively. In parallel, the enzyme becomes irreversibly inactivated.

In the presence of Br-ethylamine or tryptamine as substrates, the decay of the free radical species could also be followed through its characteristic ESR signal. No other spectral component could be detected during the process, indicating that the reaction leading to inactivation of the enzyme does not involve other free radicals (Fig. 2) [30].

6. Catalysis in the presence of oxygen: time-resolved spectroscopy

Since some of the TPQ derivatives formed during the catalytic cycle are short-lived and elusive, transient spectroscopy (i.e. stopped-flow) has been exploited in an effort to characterize the spectrum and reactivity of as many intermediates as possible. In a typical stopped-flow experiment, the three spectroscopic components pink, colorless and yellow are easily resolved. Pre-steady state and steady state experiments are carried out either by mixing the amine-reduced Cu-AO with oxygen-containing buffer or by mixing the oxidized Cu-AO with the amine substrate in the presence of oxygen. In either case, a bimolecular reaction takes place which leads to the steady state condition and, after that, to the exhaustion of either substrate. Although this experiment is difficult to analyze because of the many overlapping processes, it is easy to carry out and conveys a large amount of information. When oxidized LSAO is mixed with very efficient substrates, such as putrescine or the chromogenic substrate *p*-DAMBA, the bleaching of the 498 nm band is only partial. As the steady state mixture contains a significant amount of the quinone and aminoresorcinol derivatives (e.g. with $[\text{O}_2] = 135$ μM and $[p\text{-DAMBA}] = 100$ μM), the TPQ derivative accounts for approximately 1/3 of the total enzyme and the quinolaldimine for 2/3, all other species adding up to less than 5%. On the other hand, if the substrate is putrescine, the much faster conversion of the quinolaldimine into the aminoresorcinol allows the population of this species and the semiquinolamine radical to become significant [31].

An important point is that the stopped-flow experiments make it possible to estimate all the rate constants of the catalytic cycle (albeit with a large uncertainty) and, thus, to cal-

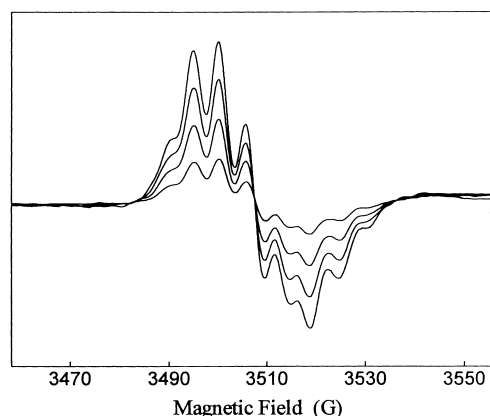
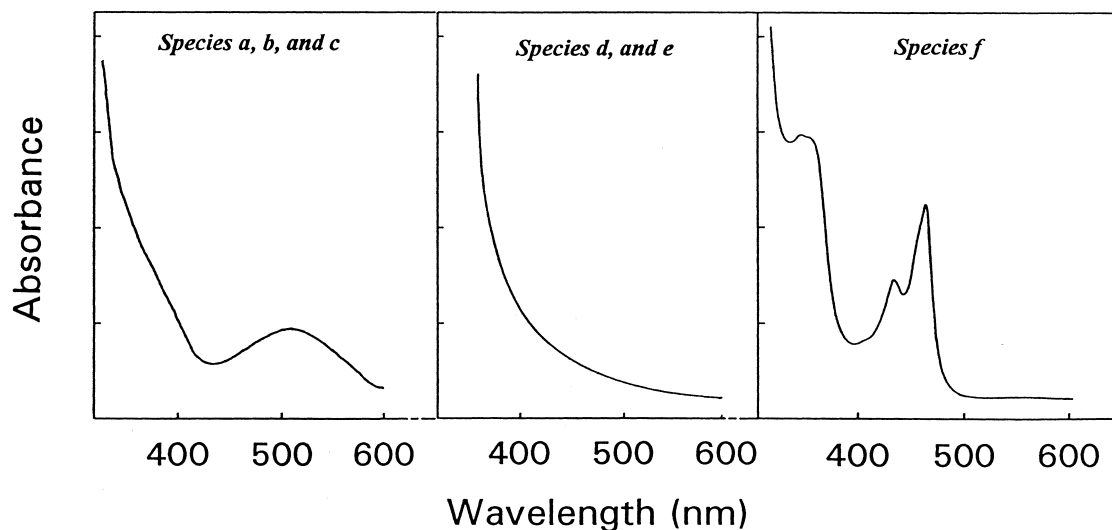
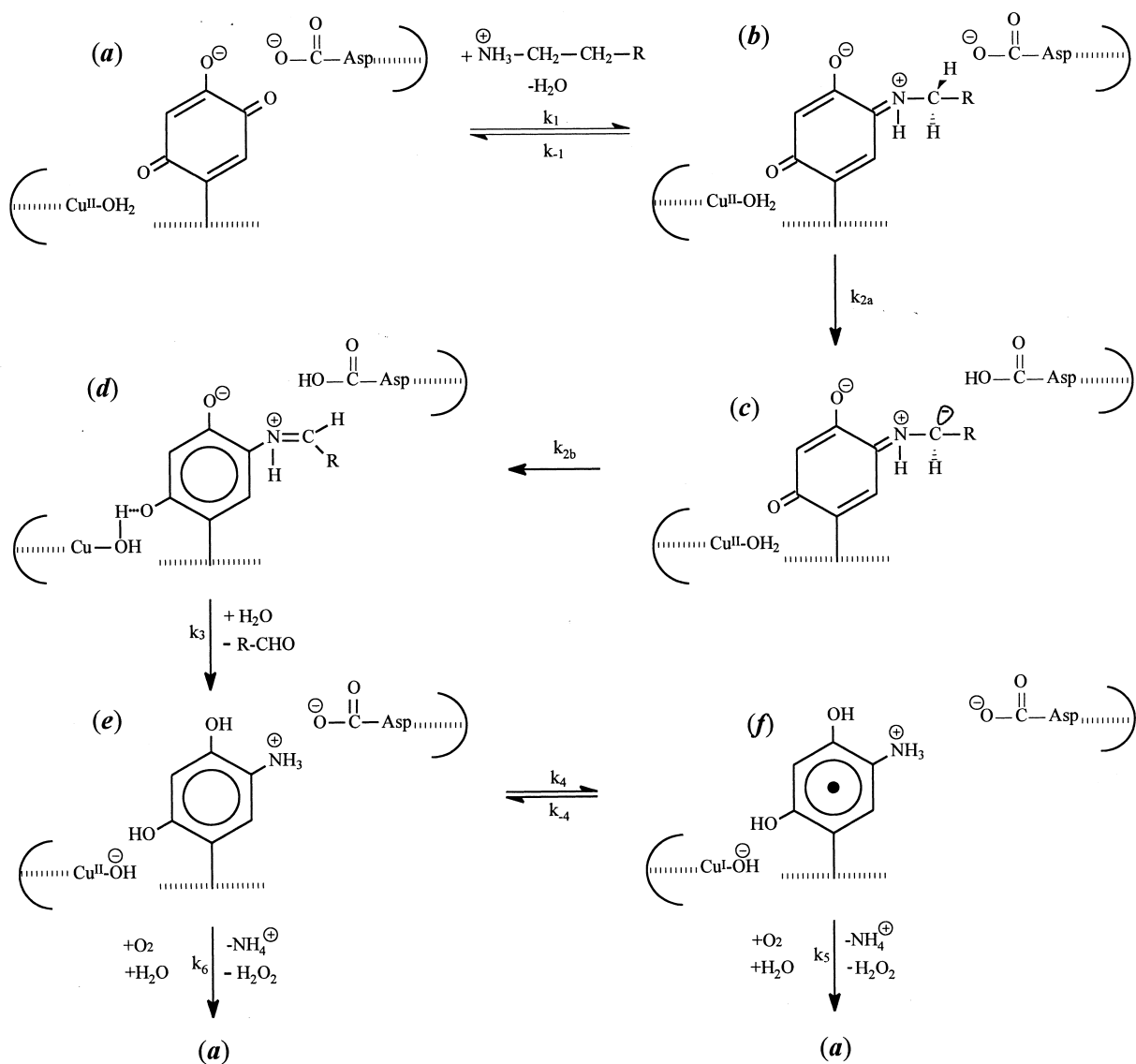


Fig. 2. ESR spectra showing the decay of LSAO free radical intermediate formed upon reaction with tryptamine. Spectra were recorded after 8, 70, 170 and 290 min, the sample contained 60 μM enzyme and 1 mM tryptamine in 100 mM phosphate buffer, pH 7.



Scheme 1. Catalytic mechanism of Cu-AOs. (a) Resting oxidized enzyme, (b) Cu^{II}-quinone ketimine, (c) Cu^{II}-carbanion species, (d) Cu^{II}-quinolaldimine, (e) Cu^{II}-aminoresorcinol, (f) Cu^I-semiquinolamine radical.

culate the values for K_m and k_{cat} , which can be compared with those obtained directly. This bridges the gap between the crude but accurate estimates obtained in a typical Michaelis-Menten experiment and the determination of individual rate constants in more selective experiments (see above). Inspection of Scheme 1 reveals that the reactions occurring after the aminoresorcinol derivative has been formed are independent of the amine substrate. Hence, in stopped-flow experiments, these are probed either when starting from the substrate-reduced enzyme or when using very efficient substrates (e.g. putrescine). Wrong estimates of the rate constants of the faster intramolecular steps may be obtained unless different substrates are compared and analyzed together (e.g. the rate of conversion of quinolaldimine to quinolamine was given a wrong value in our initial work on *p*-DAMBA) [32]. Table 1 reports a set of rate constants obtained using different substrates on LSAO.

7. Concluding remarks

Amine oxidases represent an interesting class of enzymes for at least two reasons. First, they are present in every biological organism because they are deputed to the control of the level of very active compounds, the primary (di)amines. The oxidation of these compounds generates the corresponding aldehydes, some of which have been found to either directly or indirectly influence the fate of the cell or tissue where they are formed. And, more important, hydrogen peroxide is always formed which is more and more considered to be a signal molecule or a crucial substrate rather than a noxious waste product. Second, the organic cofactor in amine oxidases is formed in a post-synthetic modification, namely the oxidation of a tyrosine residue to TPQ, which binds the primary amine and together with Cu^{II} oxidizes it to the expense of oxygen. The mechanism of the concerted electron transfer through two independent redox cofactors is not yet fully understood but certainly represents a new original pathway of oxidizing relatively inert organic compounds.

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Table 1
Rate constants for the elementary kinetic steps of the catalytic cycle of LSAO

Process	Putrescine	<i>p</i> -DAMBA	Substrate independent
k_1	$4 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$	$2.5 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$	
k_{-1}	3 s^{-1} (?)	3 s^{-1} (?)	
k_2 (a and b)	$> 200 \text{ s}^{-1}$	$> 200 \text{ s}^{-1}$	
k_3	200 s^{-1}	8 s^{-1}	
k_4			20000 s^{-1}
k_{-4}			20000 s^{-1}
k_5			$3 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$
k_6			$5 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$

All data from Bellelli et al. [32] and Medda et al. [8] except the values for k_4 and k_{-4} which are from Turowski et al. [27] (for PSAO). As discussed in the text, the backward rate constants for reactions 2, 3, 5 and 6 are negligible as compared to the corresponding forward rate constants, hence, these reactions were assumed to be essentially irreversible.

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