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.**



*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:

$$E_{ox} + R - CH_2 - NH_3^+ \rightarrow E_{red} - NH_3^+ + R - CHO \quad (1)$$

$$E_{red} - NH_3^+ + O \rightarrow E_{ox} + NH_4^+ + H_2O_2 \quad (2)$$

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 $\cong$ 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 $Cu^{II} \rightarrow Cu^{I}$ transition [15]. Both forms of the reduced enzyme (e and f) can react with $O_2$ to release $H_2O_2$ 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


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benzylamine (*p*-DAMBA) or benzylamine is added to LSAO, the broad absorption band at 498 nm disappears instantaneously, indicating the rapid conversion of the TPQ cofactor to a colorless species, the $Cu^{II}$-quinolaldimine (d). If the amine substrate concentration is high enough to exhaust oxygen, the solution turns yellow as a result of the formation of new absorption bands centered at 464, 434 and 360 nm, indicative of the free radical intermediate species (f) [13,15,22]. The extinction coefficients of reduced LSAO at pH 7 and 298 K are: $\varepsilon_{464} = 7.1\ mM^{-1}\ cm^{-1}$, $\varepsilon_{434} = 4.6\ mM^{-1}\ cm^{-1}$ [8].

### 2.2. Raman resonance spectroscopy

The aromatic hydrazone derivatives of the TPQ cofactor show characteristic resonance-enhanced Raman bands which have been used to probe its chemical structure by comparison with model compounds [5,23]. Raman resonance spectroscopy with excitation in the 450–500 nm absorption bands also proves suitable to explore and characterize the quinone and semiquinolamine states of non-derivatized TPQ [24,25]. The majority of the peaks in the Raman resonance spectrum of both the oxidized and reduced states of underivatized Cu-AO from pea seedlings present shifts of 1200–1700 $cm^{-1}$. Some of the peaks attributed specifically to the semiquinolamine radical are the 1647 $cm^{-1}$ (assigned to a C-C stretching mode) and the 1469 $cm^{-1}$ ones (assigned to a C-O or C-N stretching mode) [25]. Comparison with the Raman resonance spectra of model compounds has confirmed that the yellow radical species is indeed a semiquinolamine. Moreover, strong coupling with the $Cu^{I}$-ion is demonstrated by the perturbation of the Raman resonance spectrum in the presence of cyanide, which is known to bind to the metal [24].

### 2.3. ESR spectroscopy

The ESR parameters of plant Cu-AOs fall in the category of so-called type 2 Cu-EPR spectra. Native enzymes (LSAO and PSAO) have a typical type 2 copper spectrum, characterized by $g_{\|} = 2.32$ and $A_{\|} = 153$ G and by the absence of a superhyperfine structure [15,26]. The addition of substrate to AO in anaerobic conditions markedly decreases the ESR signal attributed to $Cu^{II}$, whereas a new signal appears at $g \sim 2$, which increases in intensity with time and reaches a maximal height after 5 min incubation at room temperature and is then stable in frozen samples or in anaerobic liquid solutions. Admission of air leads to a gradual disappearance of this signal. At room temperature and low microwave power, only the signal at $g \sim 2$ is detectable and, at a field modulation amplitude lower than 1 G, it shows a well-resolved hyperfine spectrum. These features are indicative of a free radical.

Comparison of the $Cu^{II}$ spectrum for the resting and substrate-reduced PSAO indicates that $\sim 40\%$ $Cu^{II}$ is reduced [15]. Thus, the 'yellow' reduced enzyme is an approximately stoichiometric mixture of $Cu^{I}$-semiquinolamine and $Cu^{II}$-aminoresorcinol. It is still uncertain whether the radical state is randomly distributed amongst individual molecules of the enzyme or only one of the two monomers in the dimer may generate the radical.

## 3. Interconversion of the radical and aminoresorcinol species: pH and temperature dependence

The static spectrum of reduced LSAO is shown to be pH dependent [8], because of the opposite effects of two titratable groups (with p*K*s = 5.7 and 7.9, respectively) on the equilibrium between the $Cu^{II}$-aminoresorcinol and the $Cu^{I}$-semiquinolamine. The reduced TPQ has a maximum absorbance at 464, 434 and 360 nm at pH 7, which decreases both at acidic or alkaline pH values and fades out at extreme pH values (5 and 10). Hence, a spectrum and a rate constant could be assigned to these two species [8] which reflect the equilibrium between $Cu^{I}$-semiquinolamine and the bleached $Cu^{II}$- aminoresorcinol as:

$$\underset{\text{pH 7}}{Cu^{I} - \text{semiquinolamine}} \rightleftharpoons \underset{\text{pH 5 or 10}}{Cu^{II} - \text{aminoresorcinol}}$$

A similar behavior is observed as a function of temperature. In fact, the $Cu^{I}$-semiquinolamine species, monitored as a function of temperature, is not detected below about 258 K and may be observed in an increasing amount from 283 to 298 K, the highest temperature investigated [27,28].

Temperature-jump relaxation experiments have provided an estimate of the rate constant of intramolecular electron transfer between the reduced quinone and $Cu^{II}$ [27]. It is shown that electron transfer (eT) between the TPQ-aminoresorcinol and the copper ion is fast and complex, the relaxation being described by three first order processes with apparent rates of 43 000, 4300 and 600 $s^{-1}$, the first of which accounts for over 50% of the total optical density change. While the reasons behind this complex time course are unclear, it is evident that the eT rate is fast and, taking into account that the measured rate corresponds to the sum of the approximately equally fast forward and backward reactions, corresponds to approximately 20 000 $s^{-1}$.

## 4. Catalysis in the presence of poor substrates: tardive radical species formation

*p*-(Dimethylamino)benzylamine (*p*-DABA) [16] and γ-aminobutyric acid (GABA) [8] are poor substrates for LSAO. The catalytic activity (defined as mol of substrate consumed/mol of active sites in 1 s) with *p*-DABA is $2.3 \times 10^{-4}\ s^{-1}$, while with GABA, it is $8.3 \times 10^{-4}\ s^{-1}$, very small values when compared with that found for putrescine (155 $s^{-1}$). However, *p*-DABA and GABA behave differently when added to LSAO in anaerobic conditions. In the presence of *p*-DABA [16], the broad absorption band of LSAO at 498 nm disappears instantaneously, indicating the rapid conversion of the TPQ cofactor to a bleached species, the quinolaldimine (d). Together with the formation of the quinolaldimine, a new band centered at 400 nm appears. This band is assigned to the protonated tautomeric form of the quinolaldimine (a 'quino'-imine). Under anaerobic conditions, this species decays slowly ($t_{1/2} = 120$ min), in parallel with formation of the yellow radical intermediate and the liberation of the corresponding *p*-(dimethylamino)benzaldehyde directly observable by the increase in absorbance at 350 nm. In this process, isosbestic points at 372, 440 and 478 nm can be observed (Fig. 1i).

Using GABA as a substrate, a marked lag ($\sim 4$ min) precedes the bleaching of the cofactor, indicative of the formation of the quinoneketimine intermediate, the 'pink' spectrum of which the authors were not able to discriminate from that of the quinone species [8]. After the lag phase, the appearance of the spectral features of the $Cu^{I}$-semiquinolamine is synchronous with the bleaching of the 498 nm band. In this

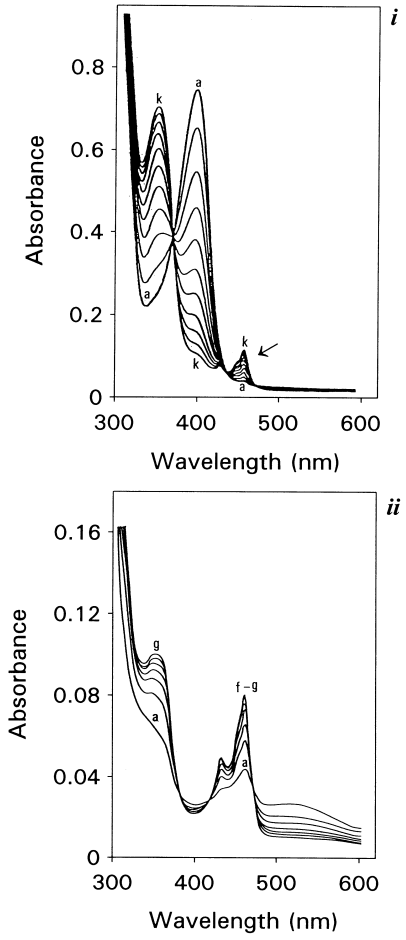



Fig. 1. i: Spectral change during the reaction of 11 μM LSAO with 1 mM *p*-DABA in 100 mM phosphate buffer, pH 7, under anaerobic conditions. Spectra were recorded from 1 min (a) to 300 min (k) with intervals of 30 min. The band centered at 400 nm is assigned to the protonated tautomeric form of the 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 readmission 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 conditions, 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 $[O_2] = 135$ μM and [*p*-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-

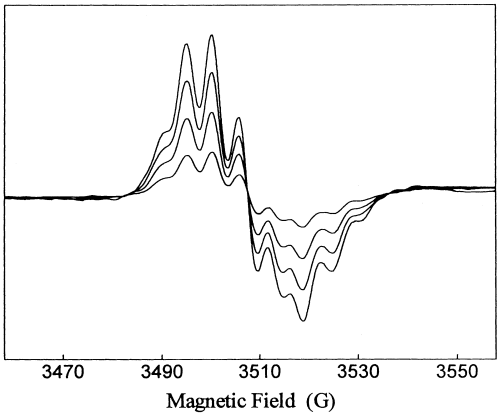
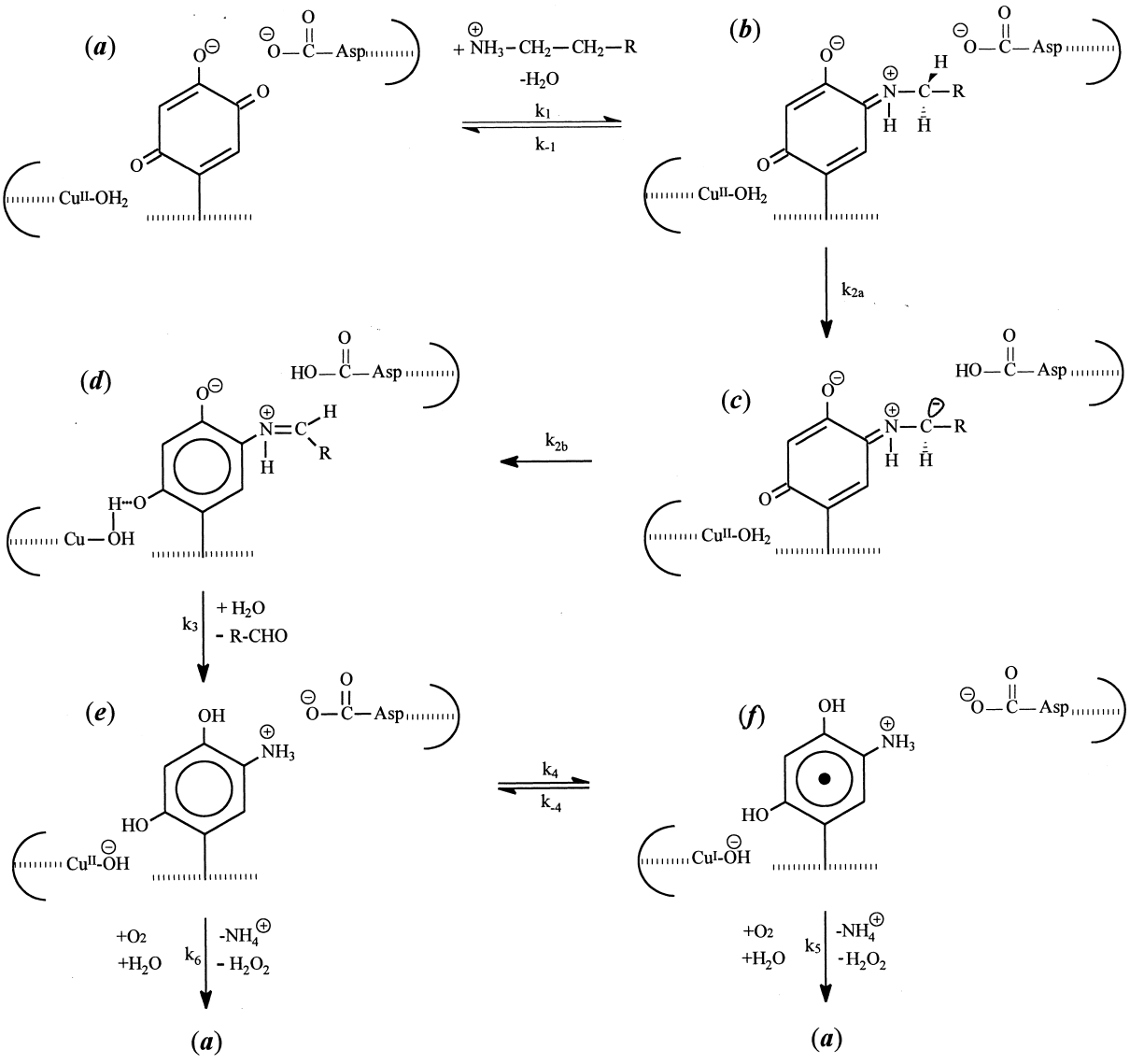
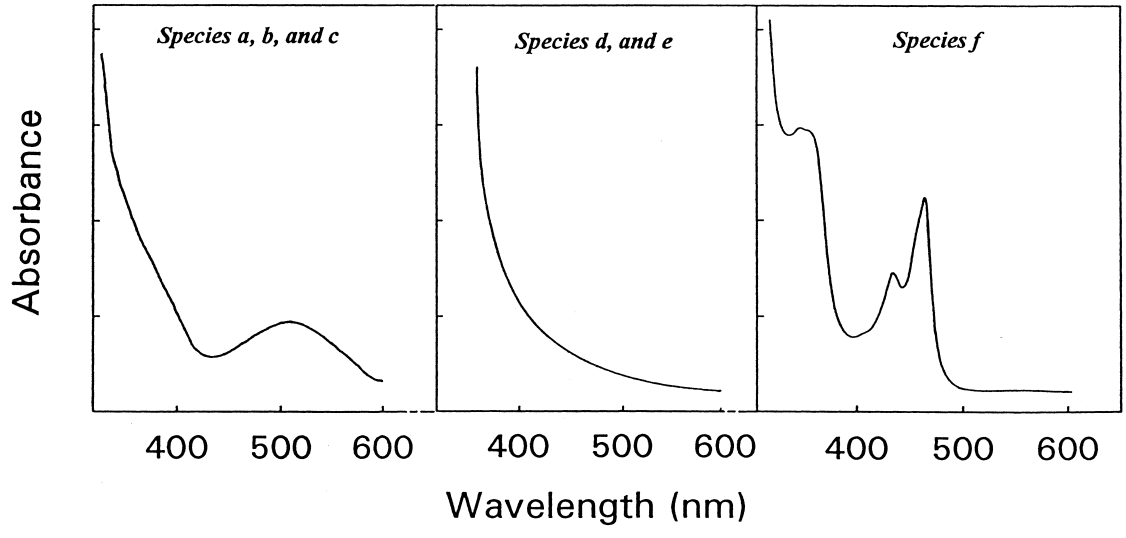



Fig. 2. ESR spectra showing the decay of LSAO free radical intermediate formed upon reaction with tryptamine. Spectra were recorded after 8, 70, 170 and 290 min, the sample contained 60 μM enzyme and 1 mM tryptamine in 100 mM phosphate buffer, pH 7.




Scheme 1. Catalytic mechanism of Cu-AOs. (a) Resting oxidized enzyme, (b) $Cu^{II}$-quinone ketimine, (c) $Cu^{II}$-carbanion species, (d) $Cu^{II}$-quinol-aldimine, (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 | *p*-DAMBA | Substrate independent |
|---|---|---|---|
| $k_1$ | $4\times10^5$ $M^{-1}$ $s^{-1}$ | $2.5\times10^5$ $M^{-1}$ $s^{-1}$ | |
| $k_{-1}$ | 3 $s^{-1}$ (?) | 3 $s^{-1}$ (?) | |
| $k_{2\ (a\ and\ b)}$ | $>200$ $s^{-1}$ | $>200$ $s^{-1}$ | |
| $k_3$ | 200 $s^{-1}$ | 8 $s^{-1}$ | |
| $k_4$ | | | 20 000 $s^{-1}$ |
| $k_{-4}$ | | | 20 000 $s^{-1}$ |
| $k_5$ | | | $3\times10^7$ $M^{-1}$ $s^{-1}$ |
| $k_6$ | | | $5\times10^5$ $M^{-1}$ $s^{-1}$ |

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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