

## Copper/Topaquinone-Containing Amine Oxidase from Lentil Seedlings and Bovine Plasma: Catalytic Mechanism and Energetic Domains

R. Medda<sup>a</sup>, S. Longu<sup>a</sup>, E. Agostinelli<sup>b</sup>, L. Dalla Vedova<sup>b</sup>, J.Z. Pedersen<sup>c</sup>, G. Floris<sup>a</sup>, A.A. Moosavi-Movahedi<sup>d</sup> and A. Padiglia<sup>a,\*</sup>

<sup>a</sup> Department of Applied Sciences in Biosystems, University of Cagliari, Cagliari, Italy

<sup>b</sup> Department of Biochemical Sciences, University of Rome "La Sapienza", and CNR Biology and Molecular Pathology Institutes, Piazzale A. Moro 5, 00185 Rome, Italy

<sup>c</sup> Department of Biology, University of Rome "Tor Vergata", Rome, Italy

<sup>d</sup> Institute of Biochemistry and Biophysics, University of Tehran, Tehran, Iran

(Received 27 December 2003, Accepted 6 March 2004)

In this review the characteristics of the prosthetic group and the role of copper in amine oxidase purified from lentil seedlings are compared with the corresponding features of the amine oxidase isolated from bovine serum. Although both enzymes contain the same organic cofactor, the 6-hydroxydopa (2,4,5-trihydroxyphenethylamine) quinone, the catalytic cycle of lentil seedling amine oxidase operates through a Cu(I)-free-radical intermediate of the cofactor, whereas in bovine serum enzyme the radical form was not observed. The role of the metal in the catalytic mechanism of the two enzymes is also discussed. Moreover, the energetic domains and the effect of the temperature on activity, for both enzymes, are examined using differential scanning calorimetry.

**Keywords:** Amine oxidase, Copper, 6-Hydroxydopa, Differential scanning calorimetry, Deconvolution

### INTRODUCTION

Copper/TPQ-containing amine oxidases [amine:oxygen oxidoreductase (deaminating); EC 1.4.3.6] are mostly homodimers in which each subunit of 70-90 kDa contains one tightly bound Cu(II) ion and one tyrosine-derived 6-hydroxydopa quinone (TPQ) moiety as prosthetic groups [1-3]. These enzymes catalyse the oxidative deamination of primary amines subtracting two electrons from amines and transferring them to molecular oxygen. The Ping-Pong catalytic mechanism occurs by two half-reactions, *i.e.* (1) enzyme reduction in the presence of substrate with release of

aldehyde, and (2) enzyme reoxidation by molecular oxygen with release of ammonia and hydrogen peroxide:



Within the class of Cu/TPQ amine oxidases are included:

- The intracellular soluble AOs, ubiquitous enzymes occurring in microorganisms (fungi and bacteria), plants, and mammals. Plant amine oxidases from various species of several families have been purified to homogeneity and characterized. Among these enzymes, the best known and studied are those isolated from seedlings of the pulses

\* Corresponding author. E-mail: padiglia@unica.it

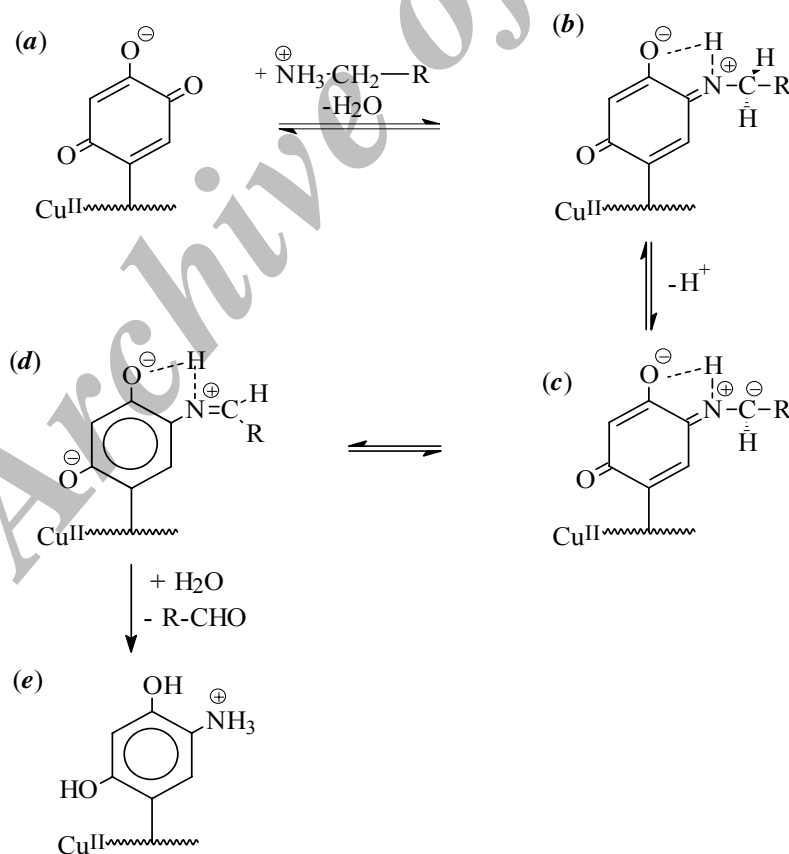
- lentil (*Lens esculenta*) [4], pea (*Pisum sativum*) [5] and *Euphorbia characias* (a shrub) latex [6].
- The mammalian plasma soluble AOs, well-studied examples being those purified from bovine serum [7,8], pig plasma [9] and equine plasma [10].
  - Tissue-bound AOs, often indicated as semicarbazide-sensitive amine oxidases (SSAOs) [11].

In this review, the intermediates in the catalytic mechanism of an intracellular soluble plant copper-amine oxidase from lentil seedlings are compared with the corresponding ones of mammalian plasma soluble amine oxidase from bovine serum. The role of copper in the two enzymes is discussed and the energetic domains and effect of the temperature on activity for both enzymes are also examined using differential scanning calorimetry.

## THE REACTION MECHANISM: THE REDUCTIVE HALF-REACTION

Due to the presence of TPQ, the oxidized forms of Cu/TPQ-AOs have a distinctive pink color and they show, in addition to the protein absorbance maximum at 278 nm, a broad absorption band in the visible region. The maximum absorbance observed for BSAO is at 476 nm, whereas in lentil seedling enzyme the red band is shifted to 498 nm; this difference is probably due to specific interactions with amino acid residues in the active sites. The extinction coefficients were determined to be  $\epsilon_{476} = 3.8 \text{ mM}^{-1} \text{ cm}^{-1}$  and  $\epsilon_{278} = 261 \text{ mM}^{-1} \text{ cm}^{-1}$  for BSAO [12,13], while for LSAO the corresponding values were  $\epsilon_{498} = 4.1 \text{ mM}^{-1} \text{ cm}^{-1}$  and  $\epsilon_{278} = 245 \text{ mM}^{-1} \text{ cm}^{-1}$  [14].

Circular dichroism (CD) studies demonstrated that the broad



Scheme 1. Catalytic mechanism of Cu-AOs: the reductive half-reaction. (a) Resting oxidized enzyme; (b) Cu(II)-quinone ketimine, the substrate Schiff base; (c) Cu(II)-carbanion species; (d) Cu(II)-quinolaldimine, the product Schiff base; (e) Cu(II)-aminoresorcinol.

visible absorption band depends on several electronic transitions. BSAO shows a CD spectrum in the range between 300 and 700 nm with two positive extrema around 370 nm and 650 nm, a negative extremum at 450 nm, and a negative shoulder at about 525 nm [12], while LSAO shows two major CD bands at 360 (-) and 520 (+) nm, with two shoulders at about 430 and 580 nm [15].

A plausible catalytic mechanism for the reductive half-reaction (Equation [1]) in the Cu-AOs is described below and reported in Scheme 1.

### Step 1: Cu(II)-Quinone Ketimine

The amine substrate binds to a carbonyl function of TPQ in the resting oxidized enzyme [Cu(II)-TPQ, (*a*)] to form a substrate Schiff base [Cu(II)-quinone ketimine, (*b*)]. Hartmann and co-workers [16] demonstrated by single-wavelength stopped-flow data, in conjunction with rapid-scanning stopped-flow experiments, that the reaction of BSAO with benzylamine as substrate in anaerobic conditions generates a detectable relaxation at 340 nm which was attributed to the quinone ketimine. This substrate Schiff base complex is stabilized by electrostatic interactions between the protonated imine nitrogen at C5 and the C4 oxoanion of TPQ. Thus the quinone and the quinone ketimine, oxidized forms of the enzyme, both of which absorb at 480 nm, are spectroscopically distinguishable since only *b* shows a shoulder at 340 nm.

In order to maximize the population of the quinone ketimine species in the catalytic cycle of LSAO, Floris and co-workers [17] used a poor substrate,  $\gamma$ -aminobutanoic acid, with  $k_{\text{cat}} = 8.3 \times 10^{-4} \text{ s}^{-1}$  ( $k_{\text{cat}}$  represents mol of substrate consumed/mol of active sites in 1 s). When  $\gamma$ -aminobutanoic acid was added to LSAO in the absence of air, there was a lag period (4 min) before the disappearance of the 498 nm band, indicating that the formation of the reduced Cu(II)-quinolaldimine was slow (see Step 3). The species that accumulates during the lag phase was assumed to be the quinone ketimine intermediate, to which was assigned the same “pink” spectrum, as that of the native enzyme.

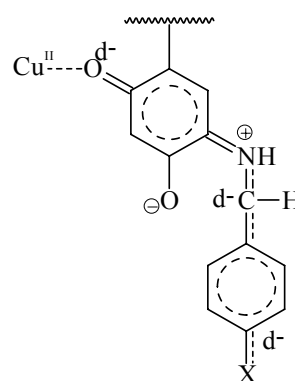
### Step 2: Cu(II)-Carbanion Species

The oxidation of the amine substrate involves base-catalyzed hydrogen abstraction from C1 leading to the

formation of a Cu(II)-carbanion species (*c*). The active site base was identified as Asp 300 in LSAO [18] and the conserved Asp381 seems to play a similar role in BSAO [19]. The formation of the carbanion species was demonstrated in the case of BSAO using *para*-substituted benzylamines [20], and the resulting negative charge is delocalized between the substituted benzene ring of the substrate and the quinone ring of TPQ (Fig. 1). The catalytic cycle of LSAO involves a carbanion intermediate (*c*), that could be detected through its reaction with the electrophilic reagent tetranitromethane to generate the nitroform anion [21]. The initial rates for nitroform production are linearly related to the concentration of functional active sites of the enzyme.

### Step 3: Cu(II)-Quinolaldimine

Transformation of the Cu(II)-carbanion (*c*) into the product Schiff base, the Cu(II)-quinolaldimine (*d*), involves the transfer of two electrons from the substrate amine to the oxidized form of TPQ, and is associated with the bleaching of the 498 nm absorption band. With normal AO substrates it is not possible to observe any optical spectrum of the Cu(II)-quinolaldimine species. However, in LSAO using the poor substrate *p*-(dimethylamino)benzylamine ( $k_{\text{cat}} = 2.3 \times 10^{-4} \text{ s}^{-1}$ ) [4] a new intense band centered at 400 nm appears. This band is assigned to the protonated tautomeric form of the



**Fig. 1.** The oxidation of the amine substrate involves base-catalyzed hydrogen abstraction from C1 leading to the formation of a Cu(II)-carbanion species. The resulting negative charge is delocalized between the substituted benzene ring of the substrate and the benzene ring of TPQ.

quinolaldimine (a “quino-imine”; Fig. 2). The quino-imine form was also identified in BSAO [16] having a broad absorption band at 460 nm. The reason why LSAO and the bovine serum enzyme give different spectral changes is not clear.

#### Step 4: Cu(II)-Aminoresorcinol

Oxidation of the bound substrate (followed by hydrolysis) releases the aldehyde product, leaving the Cu(II)-aminoresorcinol derivative (*e*). This species is apparently colorless. Hartmann and co-workers [16] demonstrated a complex time evolution of the colorless component in BSAO, attributed to the transformation of quinolaldimine into aminoresorcinol, although they were unable to assign a defined difference spectrum to this reaction. It has been demonstrated in both bovine serum [22] and lentil seedling [4,23] AOs that the aldehyde is released after hydrolysis of the imine, leaving the amino group bound to the cofactor.

In conclusion the reductive half-reaction is quite similar in BSAO and LSAO, and in consideration of the spectroscopic changes described above, in both enzymes the species *a* and *b* in Scheme 1 are believed to be pink, albeit with slightly different spectra, whereas species *d* and *e* are colorless.

### THE OXIDATIVE HALF-REACTION

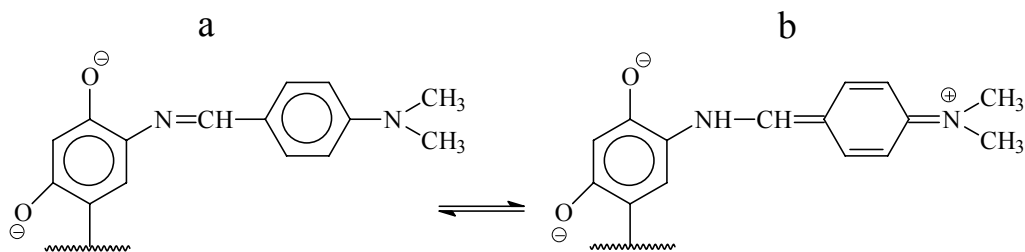
The oxidative half-reaction (Equation [2]) in LSAO is very different compared to that of the BSAO.

#### The Oxidative Half-Reaction in LSAO: The Radical Species

When a good substrate like putrescine ( $k_{\text{cat}} = 155 \text{ s}^{-1}$ ) is added to LSAO in anaerobiosis, the broad absorption band at

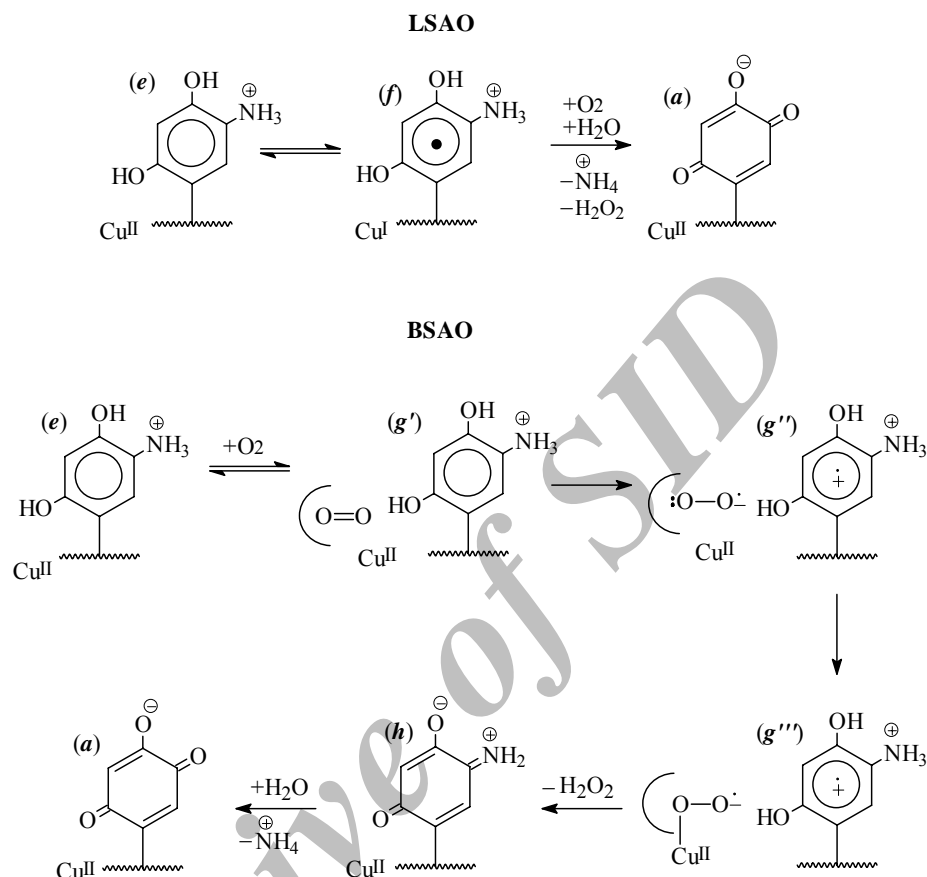
498 nm disappears instantaneously, indicating the rapid conversion of the TPQ cofactor to the colorless Cu(II)-aminoresorcinol (Scheme 2, **a**). The solution turns yellow as a result of the formation of new absorption bands centred at 464, 434 and 360 nm, indicative of the free radical intermediate species (Scheme 2, **b**) [24]. The extinction coefficients of reduced LSAO at pH 7 and 298 °K are:  $\epsilon_{464} = 7.1 \text{ mM}^{-1} \text{ cm}^{-1}$ ;  $\epsilon_{434} = 4.6 \text{ mM}^{-1} \text{ cm}^{-1}$  [17]. The electron transfer rate between Cu(II)-aminoresorcinol and the radical species is fast and involves a Cu(II)  $\rightarrow$  Cu(I) transition. The radical species so obtained is stable in anaerobiosis for many hours ( $t_{1/2} = 36 \text{ h}$ ); after readmission of oxygen it releases  $\text{H}_2\text{O}_2$  and ammonia, thereby regenerating the Cu(II)-quinone species [4,17].

The ESR parameters of plant copper amine oxidases fall in the category of so-called type 2 Cu-EPR spectra. Native LSAO have a typical type 2 copper spectrum, characterised by  $g_{\parallel} = 2.32$  and  $A_{\parallel} = 153 \text{ G}$ , and by the absence of superhyperfine structure [25]. The addition of substrate to LSAO in anaerobic conditions markedly decreases the ESR signal attributed to Cu(II), whereas a new narrow signal appears at  $g \sim 2.0$ , which increases in intensity with time and reaches a maximal high after 5 min incubation at room temperature, and then remains stable. Admission of air leads to disappearance of this signal. At room temperature and low microwave power only the signal at  $g \sim 2.0$  is detectable and, at field modulation amplitudes lower than 1 G, it shows a well-resolved hyperfine spectrum. These features are indicative of a free radical. The hyperfine splittings show that different substrates give rise to the same radical species, and  $^{15}\text{N}$ -isotope experiments demonstrate that the amino group from the substrate is still bound to the TPQ ring structure [26]. Comparison of Cu(II) spectra for the resting and substrate-reduced LSAO indicates that  $\sim 20\%$  Cu(II) is reduced. Thus



**Fig. 2.** Oxidation of *p*-(dimethylamino)benzylamine by lentil enzyme. The quinolaldimine species (**a**) is in equilibrium with its tautomeric form, the quino-imine (**b**), which has an absorbance maximum at 400 nm.

Copper/Topaquinone-Containing Amine Oxidases



*Scheme 2.* Catalytic mechanism of Cu-AOs: the oxidative half-reaction. (e) Cu(II)-aminoresorcinol; (f) Cu(I)-semiquinolamine radical; (g', g'', g''') Cu(II)-aminoresorcinol-oxygen complex; (h)

the “yellow” reduced enzyme is a mixture of Cu(I)-semiquinolamine and Cu(II)-aminoresorcinol [4,17].

The static spectrum of reduced LSAO is shown to be pH dependent [17], reflecting opposite effects of two titratable groups (with pKs = 5.7 and 7.9 respectively) on the equilibrium between the Cu(II)-aminoresorcinol and the Cu(I)-semiquinolamine. The latter has a maximum absorbance at 464, 434 and 360 nm at pH 7, which decreases both at acidic and alkaline pH values, and fades out at extreme pH values (5 and 10). The equilibrium also depends strongly on the 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 increasing amounts from 283 to 298 K, the highest temperature investigated [27].

Interestingly, in the presence of sodium dithionite the

spectrum of the reduced enzyme is not stable but decays with a time course consistent with a pseudo-first order reaction [17]. Since in anaerobiosis ammonia remains bound to the reduced enzyme, further reduction of the fully reduced enzyme is assigned to the formation of Cu(I)-aminoresorcinol, the optical spectrum of which is fully bleached and indistinguishable from that of the Cu(II)-aminoresorcinol.

Using  $\gamma$ -aminobutanoic acid as a substrate [17], a marked lag (~4 min) precedes the bleaching of the cofactor (see “The reductive half-reaction”, Step 1). After the lag phase, in anaerobic conditions, the appearance of the spectral features of the Cu(I)-semiquinolamine is synchronous with the bleaching of the 498 nm band. During this spectral change, isosbestic points at 474, 414 and 380 nm are observed. In the presence of *p*-(dimethylamino)benzylamine as substrate [4] the broad

absorption band of LSAO at 498 nm disappears instantaneously (see “The reductive half-reaction”, Step 3) and a new band centred at 400 nm appears (the quinone-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.

The haloamine 2-Br-ethylamine [28] and tryptamine [29] 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. The radical obtained with 2-Br-ethylamine and with tryptamine decays even under anaerobic conditions, with half-lives of 10 min and 90 min, respectively. In parallel, the enzyme becomes irreversibly inactivated.

### **The Oxidative Half-Reaction in BSAO: Mechanism of Proton-Coupled Electron-Transfer to Dioxygen**

In an extensive study of the re-oxidation of bovine serum Cu-amine oxidase, an enzyme which does not normally populate the Cu(I)-semiquinolamine radical, Su and Klinman [30] reported a detailed analysis of chemical and kinetic mechanism of dioxygen conversion into hydrogen peroxide not requiring the conversion of Cu(II) to Cu(I). Oxygen initially binds to a non-metal site of the enzyme and the reduced TPQ starts the reaction by transferring one electron to dioxygen to form the superoxide anion. This superoxide may bind to Cu(II) facilitating the transfer of a second electron and two protons from the semiquinolamine radical coupled to the release of H<sub>2</sub>O<sub>2</sub> (Scheme 2). In conclusion, a dominant species of the reduced TPQ is the Cu(II)-aminoresorcinol form and not Cu(II)-semiquinone radical species. The successive release of ammonia regenerates the Cu(II)-quinone species.

## **THE ROLE OF COPPER**

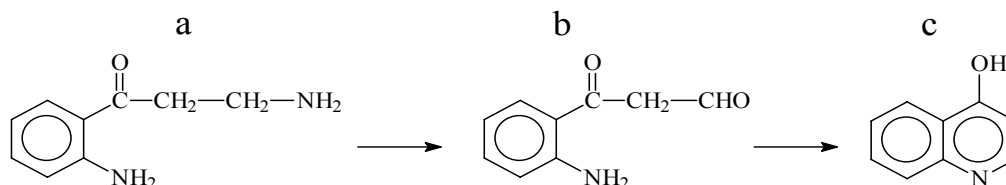
Apparently all TPQ amine oxidases contain copper in a 1:1

stoichiometric ratio with the organic cofactor. In the lentil enzyme the copper site is located very close to the TPQ cofactor and is essential for the re-oxidation of the substrate-reduced enzyme in the presence of oxygen. It has been unequivocally demonstrated that copper ion reduction occurs in LSAO [4,17] and that this is coupled to the appearance of the characteristic radical species of the reduced TPQ (the Cu(I)-semiquinolamine).

### **Copper-Free Lentil Seedling and Bovine Serum Amine Oxidases**

A crucial question is the role of copper ions in the catalytic mechanism of amine oxidases and whether the apoenzyme (copper-free) can oxidize the substrate. This problem has been solved in LSAO using kynuramine as substrate [23]. While Cu(II) in BSAO was only partially removed (10-15% remaining) by treatment with recrystallized diethyldithiocarbamate in the absence of a reductant, in lentil seedling AO, using the same experimental conditions, the metal was almost completely removed [31]. The residual copper in the LSAO apoenzyme, measured by atomic absorption spectroscopy, is normally  $0.2 \pm 0.2\%$  of the original content. The treatment did not significantly affect the position or the intensity of the absorption band in the visible range. In fact copper-free LSAO is still pink and shows a broad absorption peak in the visible region at 480 nm, shifted toward shorter wavelengths with respect to the native enzyme, but with similar intensity. Apo-LSAO has been shown to be able to oxidize one equivalent of kynuramine under single turnover conditions and release one equivalent of aldehyde per enzyme subunit under anaerobic as well as aerobic conditions. The aldehyde released spontaneously converts to 4-hydroxyquinoline (Fig. 3); this rearrangement proves the product has effectively been released from the enzyme. The particular different absorption properties of kynuramine and 4-hydroxyquinoline makes the substrate useful in precision studies of the catalytic cycle of apo-LSAO.

Through a detailed analysis of fully copper depleted, half reconstituted or fully reconstituted lentil amine oxidase, and of the Co-, Ni- and Zn-substituted enzyme, Floris and Co-workers [32] have shown that copper ions are essential for the fast catalytic rate of lentil enzyme. Using various substrates, only the Co-substituted enzyme retains some activity but the



**Fig. 3.** Oxidation of kynuramine (a) by lentil amine oxidase. The released aldehyde (b) spontaneously converts to 4-hydroxyquinoline (c).

$k_c$  value is much lower than that of native enzyme. In contrast the Ni- and Zn-substituted forms are catalytically inactive.

The effect of copper ion removal and Co(II) substitution have been extensively studied also for bovine serum amine oxidase. The Cu(II)-depleted BSAO was obtained as a pale yellow solution by treatment of native enzyme with sodium dithionite in the presence of potassium cyanide [12] or benzylamine, under anaerobic conditions [13]. The two different derivatives resulted to be totally devoid of catalytic activity; they contained about 0.50 mol/dimer of phenylhydrazine-reactive TPQ cofactor and about 0.22 or 0.70 residual Cu(II)/dimer, depending on the reagent added (potassium cyanide or benzylamine). In the Cu-depleted protein the rate of TPQ reduction by benzylamine was decreased by a factor of about  $10^3$  without a substantial change in the enzyme-substrate affinity; the rate of binding to hydrazine inhibitors was substantially reduced, and the re-oxidation process was no longer observed [33]. Co(II)-derivative BSAO partially restored the catalytic activity up to 26%, showing that the substitution of copper with cobalt, in the native enzyme, does not substantially impair the enzymatic activity [34]. Upon reaction with different amines the kinetic parameters  $K_M$  and  $k_c$  resulted different from those measured for the copper native enzyme. Co(II)-substituted BSAO decreased  $K_M$  to an extent depending on the amino substrate and restored the reactivity towards hydrazine inhibitors to almost the native level [13]. Therefore, the catalytic efficiency was not greatly impaired by Co(II)-substitution as a lower  $K_M$  value compensated in part the decrease in  $k_c$  [19]. Moreover, the Co(II)-derivative also led to an almost 50-fold increase of the oxygen  $K_M$  [19].

The Co(II)-BSAO represents a distinct enzyme form different from the native one, with quite different kinetic

parameters. This implies that the metal centre has no redox role in BSAO ([12,33,5], but it might have a structural role and may act as a Lewis acid by modulating the  $pK_a$  of the coordinated water molecule, through which it is bonded to TPQ, thus facilitating the proton transfer to O<sub>2</sub> [19]. Since Co(II)-containing BSAO, like Co(II)-substituted LSAO, cannot form a stable semiquinone radical, it can be excluded that the formation of the Cu(I)-semiquinone is an essential step of the BSAO catalytic pathway [13]. That the metal in BSAO is not involved in amine oxidation process, but shows a structural role, was also confirmed by the similar behaviour of Cu(II)- and Co(II)-BSAO derivatives in differential scanning calorimetry studies [13,36].

## DIFFERENTIAL SCANNING CALORIMETRY

The activity of LSAO versus the temperature shows two phases, the first from 17 to 37 °C (phase I), and the second one from 42 to 57 °C (phase II); between 37 and 42 °C the activity is independent of the temperature. Thus the Arrhenius plot shows a discontinuity which depicts two distinct linear lines with different steepness [37]. The change in LSAO activity at different temperatures and the Arrhenius plot show a similar behaviour as the curves measured for BSAO [38].

The temperature dependence of the molar heat capacity ( $C_p$ ) of native LSAO, obtained by calorimetric results, shows that a broad transition starts at 16 °C and ends at 37 °C, whereas the thermal profile begins to increase at 45 °C, with a  $T_m = 65.18$  °C [37]. The thermal denaturation that starts at 55 °C corresponds to decreasing activity. The DSC curve for LSAO shows a small and broad transition below 55 °C, the enthalpy of which is consistent with a conformational change in the native enzyme, whereas for BSAO there is negligible variation in the same range of temperature [37,39], which is in

**Table 1.** Thermodynamic Parameters of Two-State Transitions Obtained by Deconvolution of Native BSAO and LSAO Thermograms.  $T_m$  = Temperature of Maximum Excess Heat Capacity for Each Simulated Transition;  $\Delta H$  = Denaturation Enthalpy for Each Simulated Transition.

	Subpeak	$\Delta H$ (kJ mol <sup>-1</sup> )	$T_m$ (°C)
LSAO	I	388	61.9
	II	667	64.3
	III	879	66.1
BSAO	I	473	58.6
	II	728	68.4
	III	1201	70.0
	IV	1268	71.3
	V	703	77.0

agreement with the specific activity variation studies previously reported [40]. The deconvolution of the excess molar heat capacity reveals in LSAO and BSAO three subpeaks (transitions I-III) and five subpeaks respectively [37,36]. In LSAO the subpeaks (energetic domains, transitions I,II and III) [41] may belong to the three hypothesized structural domains for each subunit of LSAO (D3, D2 and D4 respectively) [5]. There are great differences in the energetic domains in the DSC profiles for LSAO and BSAO. The deconvolution of thermal profiles shows three and five subpeaks in LSAO and BSAO, respectively. In BSAO the lowest and the highest temperature peaks are both represented by a single two-state transitions, while the central peak may be deconvoluted into three two-state transitions. The thermodynamic parameters of the fitted curves, *i.e.* the values of  $\Delta H_{cal}$  and  $T_m$ , for the subpeaks of LSAO compared to those of the BSAO are reported in Table 1. The calorimetric profile of the fully-Cu(II)-depleted BSAO showed that the thermogram deconvolution required at least six two-state transitions. Copper removal did not induce the drastic effect on the thermal stability observed in other copper proteins after depletion of the metal [42-44]. In this study were also carried out calorimetric measurements of both Cu(II) and Co(II)-reconstituted BSAO. The thermal profiles of Co(II)-reconstituted enzyme were very similar to those of the Cu(II)-reconstituted BSAO, except for a slightly lower overall  $T_m$ . The thermograms of both derivatives can be deconvoluted into

five two-state transitions that characterize the native enzyme.

LSAO may be considered to have three energetic domains [37,41] that may belong to three hypothesized structural domains for each subunit of the enzyme. Reactivity with hydrazine derivatives and assays of the enzymic activity showed that in Co(II)-derivative the TPQ cofactor was reactive and the enzyme was catalytically competent. This was in agreement with the suggestions above reported, *i.e.* for a copper conformational role in the native protein, namely of maintaining a functional conformation at the active site. In addition these studies suggest that while LSAO subunits react independently, in BSAO the two subunits are identical but interdependent in activity [45]. This means that each subunit contains five energetic domains and the binding of the substrate or inhibitors to the active site of each subunit blocks the binding of the above molecules to the active site of the other subunit [7,46].

## ACTIVATION ENERGY

As mentioned above the activity temperature dependences of LSAO and BSAO are similar, producing biphasic Arrhenius plots [37,40]. The activation energies and  $Q_{10}$  for two phases for both enzymes are given in Table 2. The activation energies of the second phase as well as DSC profile provide evidence for temperature-induced transformation of LSAO from one stable form to another, each form possessing catalytic activity, but differing in activation energy. The difference in activation energies of two phases in BSAO is much less than in LSAO. Thus, the two conformations for BSAO do not differ considerably while LSAO undergoes a marked change in conformation in the same range of temperatures. It is worth concluding that the physiological temperature range for mammals is 37-42 °C and this is exactly the part of Arrhenius curve where the specific activity of BSAO is independent of temperature, as previously reported [40]. This means that the physiological status of mammalian amine oxidase is protected in this range of temperatures. In plants, the physiological temperature range is extended up to 50 °C that can modulate the physiological role for plant amine oxidase at higher temperatures probably because the dimeric structure confers to LSAO a structural potency to tolerate higher temperatures, due to conformational lock [47]. A reversible-dissociative thermal



**Table 2.** Activation Energy and  $Q_{10}$  of LSAO and BSAO in Various Temperatures Ranges

Enzyme	Temperature range (°C)	Activation energy Ea (kJ mol <sup>-1</sup> K <sup>-1</sup> )	Temperature (°C)	$Q_{10}$
LSAO <sup>a</sup>	17-37	63.8	27-37	2.28
	42-57	40.3	47-57	1.58
BSAO <sup>b</sup>	15-38	33.2	25-35	1.56
	42-55	32.6	45-55	1.55

<sup>a</sup>The data are taken from Ref. [37]. <sup>b</sup>The data are taken from Ref. [40].

inactivation (three-state of denaturation) can cause an extra resistance of the LSAO at higher temperatures. Therefore, LSAO can retain its structure and activity under heat stress.

## ACKNOWLEDGEMENTS

This study was supported partially by FIRB (Fondo per gli investimenti della ricerca di base), by C.N.R. Target Project on Biotechnology funds, by the Italian MIUR (Ministero dell'Istruzione, dell'Università e della Ricerca), the Ministero della Salute (1% Fondo Sanitario Nazionale), the MIUR-PRIN 2003, and the Research Council of the University of Tehran for partial support.

## REFERENCES

- [1] S.M. Janes, D. Mu, D. Wemmer, A.J. Smith, S. Kaur, D. Maltby, A.L. Burlingame, J.P. Klinman, *Sci.* 248 (1990) 981.
- [2] S.M. Janes, M.M. Palcic, C.H. Scaman, A.J. Smith, D.E. Brown, D.M. Dooley, M. Mure, J.P. Klinman, *Biochemistry* 31 (1992) 12147.
- [3] D. Mu, S.M. Janes, A.J. Smith, D.E. Brown, D.M. Dooley, J.P. Klinman, *J. Biol. Chem.* 267 (1992) 7979.
- [4] R. Medda, A. Padiglia, J.Z. Pedersen, G. Rotilio, A. Finazzi Agrò, G. Floris, *Biochemistry* 34 (1995) 16375.
- [5] V. Kumar, D.M. Dooley, H.C. Freeman, J.M. Guss, I. Harvey, M.A. McGuirl, M.C.J. Wilce, V. Zubak, *Structure* 4 (1996) 943.
- [6] A. Padiglia, R. Medda, A. Lorrain, B. Murgia, J.Z. Pedersen, A. Finazzi Agrò, G. Floris, *Plant Physiol.* 117 (1998) 363.
- [7] L. Morpurgo, E. Agostinelli, B. Mondovì, L. Avigliano, R. Silvestri, G. Stefanich, M. Artico, *Biochemistry* 31 (1992) 2615.
- [8] J.P. Klinman, D. Mu, *Annu. Rev. Biochem.* 63 (1994) 299.
- [9] K.D.S. Yadav, P.F. Knowles, *Eur. J. Biochem.* 114 (1981) 139.
- [10] S.R. Carter, M.A. McGuirl, D.E. Brown, D.M. Dooley, *J. Inorg. Biochem.* 56 (1994) 127.
- [11] G.A. Lyles, *J. Neural Transm.* 41 (1994) 387.
- [12] S. Suzuki, T. Sakurai, A. Nakahara, T. Manabe, T. Okuyama, *Biochemistry* 22 (1983) 1630.
- [13] E. Agostinelli, G. De Matteis, B. Mondovì, L. Morpurgo, *Biochem. J.* 330 (1998) 383.
- [14] A. Padiglia, R. Medda, G. Floris, *Biochem. Int.* 28 (1992) 1097.
- [15] A. Rinaldi, G. Floris, A. Giartosio, in: B. Mondovì (Ed.), *Structure and functions of amine oxidases*, CRC Press, Boca Raton, 1985.
- [16] C. Hartmann, P. Brzovic, J.P. Klinman, *Biochemistry* 32 (1993) 2234.
- [17] R. Medda, A. Padiglia, A. Bellelli, P. Sarti, S. Santanchè, A. Finazzi Agrò, G. Floris, *Biochem. J.* 332 (1998) 431.
- [18] A. Rossi, M. Petruzzelli, A. Finazzi Agrò, *FEBS Lett.* 301 (1992) 253.
- [19] G. De Matteis, E. Agostinelli, B. Mondovì, L. Morpurgo, *J. Biol. Inorg. Chem.* 4 (1999) 348.
- [20] C. Hartmann, J.P. Klinman, *Biochemistry* 30 (1991) 4605.
- [21] R. Medda, A. Padiglia, J.Z. Pedersen, G. Floris, *Biochem. Biophys. Res. Comm.* 196 (1993) 1349.
- [22] S.M. Janes, J.P. Klinman, *Biochem.* 30 (1991) 4599.
- [23] A. Padiglia, R. Medda, A. Lorrain, D. Congiu, J.Z.

- Pedersen, G. Floris, *Phytochem. Anal.* 9 (1998) 223.
- [24] A. Finazzi Agrò, A. Rinaldi, G. Floris, G. Rotilio, *FEBS Lett.* 176 (1984) 378.
- [25] G. Rotilio, in: B. Mondovì (Ed.), *Structure and functions of amine oxidases*, CRC Press, Boca Raton, 1985.
- [26] J.Z. Pedersen, S. El-Sherbini, A. Finazzi Agrò, G. Rotilio, *Biochemistry* 31 (1992) 8.
- [27] R. Medda, A. Padiglia, A. Bellelli, J.Z. Pedersen, A. Finazzi Agrò, G. Floris, *FEBS Lett.* 453 (1999) 1.
- [28] R. Medda, A. Padiglia, J.Z. Pedersen, A. Finazzi Agrò, G. Rotilio, G. Floris, *Biochemistry* 36 (1997) 2595.
- [29] R. Medda, A. Padiglia, A. Finazzi Agrò, J.Z. Pedersen, A. Lorrai, G. Floris, *Eur. J. Biochem.* 250, (1997) 377.
- [30] Q. Su, J.P. Klinman, *Biochemistry* 37 (1998) 12513.
- [31] A. Rinaldi, A. Giartosio, G. Floris, R. Medda, A. Finazzi Agrò, *Biochem. Biophys. Res. Comm.* 120 (1984) 242.
- [32] A. Padiglia, R. Medda, J.Z. Pedersen, A. Finazzi Agrò, A. Lorrai, B. Murgia, G. Floris, *J. Biol. Inorg. Chem.* 4 (1999) 608.
- [33] E. Agostinelli, G. De Matteis, A. Sinibaldi, B. Mondovì, L. Morpurgo, *Biochem. J.* 324 (1997) 497.
- [34] L. Morpurgo, E. Agostinelli, B. Mondovì, L. Avigliano, *Biol. Metals* 3 (1990) 114.
- [35] S. Suzuki, T. Sakurai, A. Nakahara, T. Manabe, T. Okuyama, *Biochemistry* 25 (1986) 338.
- [36] E. Agostinelli, L. Morpurgo, C. Wang, A. Giartosio, B. Mondovì, *Eur. J. Biochem.* 222 (1994) 727.
- [37] S.Z. Moosavi-Nejad, M. Rezaei-Tavirani, A. Padiglia, G. Floris, A.A. Moosavi-Movahedi, *J. Protein Chem.* 20 (2001) 405.
- [38] R. Stevanato, F. Vianello, A. Rigo, *Arch. Biochem. Biophys.* 324 (1995) 374.
- [39] A. Giartosio, E. Agostinelli, B. Mondovì, *Biochem. Biophys. Res. Comm.* 154 (1988) 66.
- [40] O. Befani, S. Sabatini, M.A. Mateescu, B. Mondovì, *Biochem. Biophys. Research Commun.* 163 (1989) 1168.
- [41] S.Z. Moosavi-Nejad, A.A. Moosavi-Movahedi, G. Floris, A. Padiglia, M. Rezaei-Tavirani, *Int. J. Biol. Macromol.* (2003) submitted.
- [42] J.A. Roe, A. Butler, D.M. Scholler, J.S. Valentine, L. Marky, K.J. Breslauer, *Biochemistry* 27 (1988) 950.
- [43] M.C. Bonaccorsi di Patti, G. Musci, A. Giartosio, S. D'Alessio, L. Calabrese, *J. Biol. Chem.* 265 (1990) 21016.
- [44] I. Savini, S. D'Alessio, A. Giartosio, L. Morpurgo, L. Avigliano, *Eur. J. Biochem.* 190 (1990) 491.
- [45] D. De Biase, E. Agostinelli, G. De Matteis, B. Mondovì, L. Morpurgo, *Eur. J. Biochem.* 237 (1996) 93.
- [46] D. Collison, P.F. Knowles, F.E. Mabbs, F.X. Rius, I. Sing, D.M. Dooley, C.E. Cote, M.A. McGuirl, *Biochem. J.* 264 (1989) 663.
- [47] S.Z. Moosavi-Nejad, A.A. Moosavi-Movahedi, M. Rezaei-Tavirani, G. Floris, R. Medda, *J. Biochem. Mol. Biol.* 36 (2003) 167.