Catalytic Properties and the Role of Copper in Bovine and Lentil Seedling Copper/Quinone-Containing Amine Oxidases: Controversial Opinions

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In this microreview, the differences in the catalytic cycle of two copper/quinone-containing amine oxidases, one from lentil seedlings, representative of plant enzymes, and the other from bovine serum, typical of mammalian enzymes, are discussed. Although both enzymes are involved in the control of the levels of mono-, di-, and polyamines, and contain the same organic cofactor, the quinone of 2,4,5-trihydroxyphenylalanine, known as TOPA or TPQ, lentil amine oxidase operates in a different way and with a much higher catalytic activity than the bovine serum enzyme. The role of copper in the two enzymes is also discussed.

Abbreviations: TPQ or TOPA: quinone of 2,4,5-trihydroxyphenylalanine (6-hydroxydopa quinone). Cu/TPQ-AO: copper/TPQ-containing amine oxidase. LSAO: Cu/TPQ-AO from lentil seedlings. BSAO: Cu/TPQ-AO from bovine serum.

Plant and mammalian plasma amine oxidases [amine:oxygen oxidoreductase (deaminating) (copper containing); EC 1.4.3.6] (Cu/TPQ-AOs) belong to the heterogeneous superfamily of enzymes catalyzing the oxidative deamination of primary amino groups of mono-, di-, and polyamines. Cu/TPQ-AOs share some fundamental structural properties: these enzymes are homodimers, with each subunit containing one tightly bound Cu^{II} ion and one quinone of 2,4,5-trihydroxyphenylalanine (TPQ) as cofactors.[1] These enzymes operate by abstracting two electrons from amines and transferring them to molecular oxygen to form the corresponding aldehyde, ammonia, and hydrogen peroxide. The ping-pong catalytic mechanism of Cu/TPQ-AOs can be divided into two half-reactions: (1) enzyme reduction by substrate at the quinone moiety (TPQ → $TPQH_2$) and (2) its reoxidation by molecular oxygen as shown below:

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

$$E_{red}\text{-}NH_3^+ + O_2 + H_2O \rightarrow E_{ox} + NH_4^+ + H_2O_2 \quad (2)$$

Plant amine oxidases are intracellular enzymes, the best known and studied being those from seedlings of lentil (*Lens esculenta*)[2] and pea (*Pisum sativum*),[3] and from the latex of the shrub *Euphorbia characias*.[4] These enzymes prefer short aliphatic diamines, like putrescine (1,4-diaminobutane) and cadaverine (1,5-diaminopentane), as substrates. Plant AOs have an important role in cell growth by regulating the intracellular di- and polyamine levels, while the aldehyde products might have a key role in the biosynthesis of some alkaloids.

Mammalian plasma AOs are extracellular enzymes that are able to metabolize mono-, di-, and polyamines, even though in vitro they are preferentially active towards non-physiological amines like benzylamine. Plasma AOs are generally termed either plasma or serum AOs or benzylamine oxidases. The best known plasma AOs are those from bovine,[5,6] pig,[7] and equine plasma.[8]

In this microreview, a number of controversial questions about the structure and function of AOs are discussed, and

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the characteristics of the enzyme from lentil seedlings (LSAO), as representative of plant AOs, are compared with the corresponding features of bovine serum enzyme (BSAO), typical of mammalian proteins.

1. The Organic Cofactor

The first controversial matter involving all Cu/TPQ AOs has been the identification of the organic carbonyl cofactor. For many years, the most likely candidate was considered an unusual covalently bound form of pyridoxal 5-phosphate, but confirmation of such a structure was not forthcoming. In 1984[9,10] two independent reports seemed to have solved this controversial problem by suggesting that pyrroloquinoline quinone might be the active organic cofactor of a variety of eukaryotic proteins, including copper AOs from bovine serum, pig kidney, and from plants. Finally, in 1990 Janes et al. demonstrated that the redox-active cofactor of eukaryotic amine oxidases is the 2,4,5-trihydroxyphenylalanine quinone (6-hydroxydopa; Figure 1).[1]

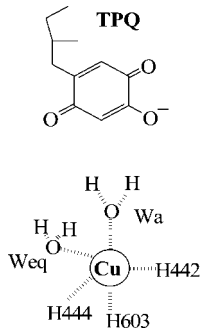

Figure 1. Structure of oxidized form of TPQ and the copper center.

TPQ is derived from the copper-catalyzed oxidation of a post-translationally modified tyrosine residue in the consensus sequence Asn-y-Asp/Glu of the polypeptide chain[11,12] common to plant and mammalian CuAOs analyzed so far.[13] Due to the presence of TPQ, the oxidized form of both LSAO and BSAO has a distinctive pink color and, in addition to the protein absorbance maximum at 278 nm, absorbs in the visible region: BSAO shows the electronic absorption band at 476 nm (ε_{476} = 3800 M^{-1} cm^{-1})[5] whereas

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in LSAO the visible absorption band is centered at 498 nm (ε_{498} = 4100 $M^{-1}cm^{-1}$).[2]

2. The Number of Active Sites

Since all Cu/TPQAOs are homodimers of apparently identical subunits, although the molecular size of the BSAO subunit (85 KDa) is larger than that of LSAO (70 KDa), a problem arose concerning the number of active sites present in native proteins. Thus, a controversial matter on whether one[5,14] or two[15,16] TPQs per dimer are active in AOs from different sources is still debated. The conflicting results obtained on titrating AOs with carbonyl reagents, such as phenylhydrazine, have been rationalized for LSAO.[17] The titration of highly purified samples extrapolated to 1 mol of inhibitor per enzyme subunit indicated the presence of two active quinone moieties per dimer. With carbonyl reagents, such as phenylhydrazine, a stoichiometry of one organic cofactor per protein subunit/mol of inhibitor has been reported for highly purified samples of BSAO,[15] although other research groups, using similar methods, had previously determined a single carbonyl cofactor per dimer.[18,19] Taking into account the results obtained by Janes and Klinman,[15] a spectroscopic study was carefully undertaken on the reaction of BSAO with some inhibitors for determining the stoichiometry of binding.[5] The results showed that BSAO samples having a specific activity comparable to that used by Janes and Klinman react with a stoichiometry of one phenylhydrazine per BSAO dimer. The lower value for the phenylhydrazine/BSAO ratio was mainly attributed to the lower quality of phenylhydrazine being used in the titration, which is reflected by the different molar extinction coefficient of the adduct at 447 nm, corresponding to 41 500 $M^{-1}cm^{-1}$[5] vs. 32 400 $M^{-1}cm^{-1}$.[15] Moreover, reaction of the bovine serum enzyme with other inhibitors like hydrazines and hydrazides[20] led to the proposal of a so-called "half of the sites" reactivity mechanism.[5,21]

3. The Catalytic Cycle Of Cu/TPQ–AOs

Several differences are worth specific mention concerning the catalytic mechanism of LSAO and BSAO.

The Reductive Half-Reaction

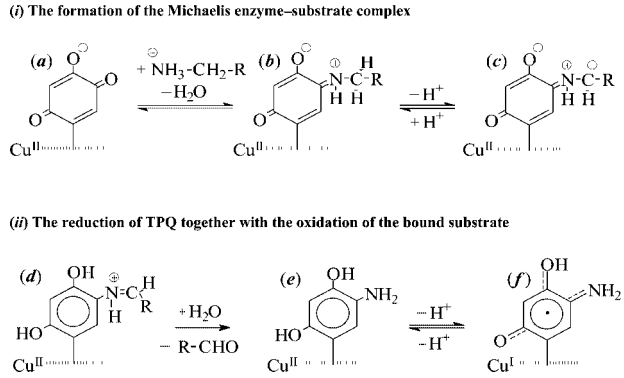
As shown in Scheme 1, at least three separate kinetic steps participate in the reductive half cycle: (*i*) the formation of the Michaelis enzyme–substrate complex, (*ii*) the reduction of TPQ together with the oxidation of the bound substrate to yield the enzyme–product complex; (*iii*) the hydrolysis of the enzyme–product complex with the release of the aldehyde and the formation of the aminoquinol derivative.

Formation of the Michaelis Enzyme–Substrate Complex

Step 1: The Substrate Schiff-Base Cu^{II}–Quinone Ketimine

The amine substrate binds to a carbonyl function of TPQ in the resting oxidized enzyme [Cu^{II}–TPQ, (see *a* in Scheme 1; Figure 2)] to form a substrate Schiff base [Cu^{II}–quinone ketimine; (see *b* in Scheme 1)]. Hartmann and coworkers[22] have demonstrated that the reaction of BSAO with benzylamine as substrate under anaerobic conditions generates a detectable relaxation at 340 nm, which was attributed to the quinone ketimine. Thus, the quinone and the quinone ketimine oxidized forms of the enzyme, which both absorb at 476 nm, are spectroscopically distinguishable since only *b* shows a shoulder at 340 nm.

In LSAO, as previously demonstrated,[23] the quinone ketimine intermediate has the same "pink" spectrum as that of the native enzyme



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}–aminoquinol; (*f*) Cu^{I}–semiquinolamine radical.

Step 2: Cu^{II}–Carbanion Species

The oxidation of the amine substrate involves a base-catalyzed hydrogen abstraction from the C bound to nitrogen, leading to the formation of a Cu^{II}–carbanion species (see *c* in Scheme 1). The formation of the carbanion species has been demonstrated, in the case of BSAO, using *para*-substituted benzylamines,[24] and, in LSAO, through the reaction with the electrophilic reagent tetranitromethane to generate the nitroform anion.[25] The active-site base was identified as Asp_{300} in LSAO[26] and Asp_{381} in BSAO.[27]

The Reduction of TPQ Together With the Oxidation of the Bound Substrate

Step 3: The Product Schiff base Cu^{II}–Quinolaldimine

Transformation of the Cu^{II}–carbanion (*c*) into the product Schiff base Cu^{II}–quinolaldimine (see *d* in Scheme 1) involves the transfer of two electrons from the substrate amine to the oxidized form of TPQ, and is associated with the bleaching of the visible absorption band. In LSAO, after addition of the poor substrate *p*-(dimethylamino)benzylamine ($k_{cat} = 2.3 \times 10^{-4}$ s^{-1}) a new intense band centered at 400 nm appears.[2] This band is assigned to the protonated tautomeric form of the quinolaldimine (a "quino"-imine; Figure 3). The quino-imine form was also identified

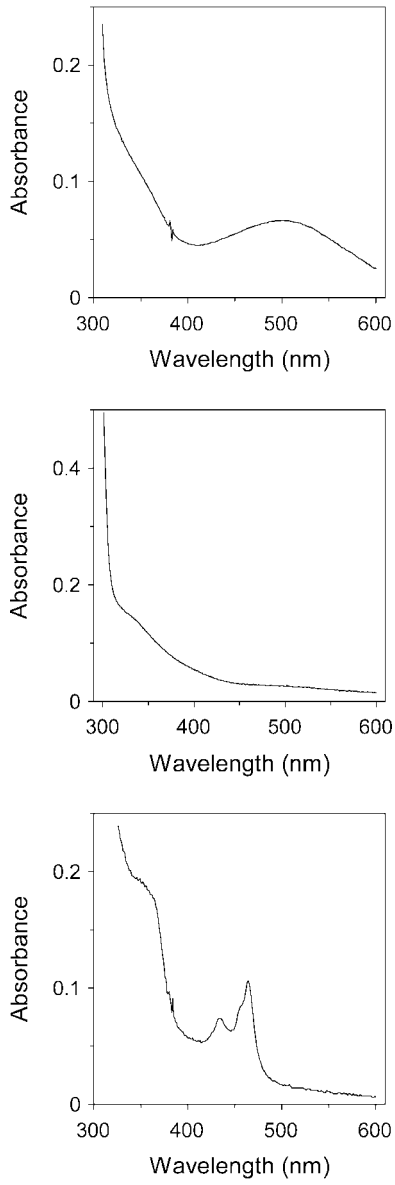


Figure 2. Visible absorption spectrum of LSAO. Top: resting oxidized enzyme; middle: the Cu^{II}–aminoquinol; bottom: Cu^{I}–semiquinolamine radical.

in BSAO[22] due to its broad absorption band at 460 nm. The reason why LSAO and the bovine serum enzyme give a different quino-imine spectrum is not clear.

Step 4: Cu^{II}–Aminoquinol

Oxidation of the bound substrate (followed by hydrolysis) releases the aldehyde product and gives the Cu^{II}–aminoquinol derivative (see *e* in Scheme 1). In both enzymes this species is colorless (Figure 2) and it was impossible to assign a defined difference spectrum attributed to the transformation of quinolaldimine into aminoquinol. In both enzymes the aldehyde is released after hydrolysis of the imine, and ^{15}N-isotope experiments have demonstrated that the amino group from the substrate is still bound to the TPQ ring structure.[28]

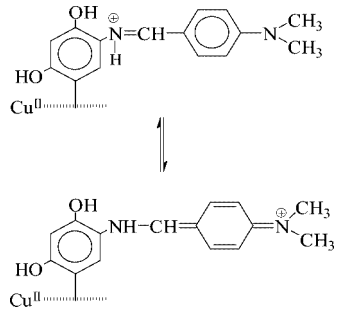


Figure 3. Oxidation of *p*-(dimethylamino)benzylamine by lentil enzyme. The quinolaldimine species is in equilibrium with its tautomeric form, the quino-imine.

Step 5: Cu^{I}–Semiquinolamine Cation Radical Species

This derivative is typical of plant AOs. In lentil enzyme, after the conversion of the TPQ cofactor to the substrate-reduced colorless Cu^{II}–aminoresorcinol, the solution turns yellow immediately as result of the formation of new absorption bands centered at 464, 434, and 360 nm, indicative of a second reduced derivative, the free radical Cu^{I}–semiquinolamine cation species (Scheme 1f; Figure 2).[29] It has been unequivocally demonstrated that copper ion reduction occurs.[2,23] The static spectrum of Cu^{I}–semiquinolamine has been shown to be pH-dependent.[23] The intensities of the absorbances of reduced LSAO observed at pH 7 decrease at both acidic and alkaline pH values, and disappear completely at extreme pH values (pH $<$ 5 or $>$ 10). The titration data have been fitted to an equilibrium between Cu^{I}–semiquinolamine and the bleached Cu^{II}–aminoquinol controlled by two independent ionizable groups with $pK_1 = 5.7$ and $pK_2 = 7.9$:

Cu^{I}–semiquinolamine (pH 7) $\rightleftharpoons$ Cu^{II}–aminoquinol (pH 5 or 10)

The radical species could not be detected below 258 K, and was observed in increasing amounts from 283 to 298 K, the highest temperature investigated.[30] Thus, in LSAO, as in other plant AOs, the Cu^{II}–aminoquinol derivative equilibrates rapidly with the Cu^{I}–semiquinolamine cation radical species by transferring one electron to copper, which in turn is reduced from the cupric to the cuprous state.[31] Even under the most favorable experimental conditions, the population of Cu^{I}–semiquinolamine radical does not exceed 30–40% of the total TPQ, as estimated by ESR spectroscopy.[32]

BSAO does not populate the Cu^{I}–semiquinolamine radical species, but the presence of about 1% of a radical derivative has been demonstrated by Su and Klinman[33] in the mechanism of dioxygen conversion into hydrogen peroxide without the conversion of Cu^{II} to Cu^{I} (see "the oxidative half-reaction" below).

In conclusion, in the reductive half-reaction four steps are quite similar in BSAO and LSAO and, in consideration of the spectroscopic changes described above, in both enzymes the Cu^{II}–quinone ketimine and Cu^{II}–carbanion species are believed to be pink, whereas Cu^{II}–quinolaldimine and Cu^{II}–aminoquinol species are colorless. As described as step five, the yellow Cu^{I}–semiquinolamine cation radical

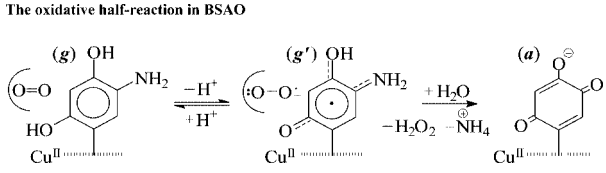
species is only present in plant enzymes and represents the biggest difference observed between LSAO and BSAO in the reductive catalytic mechanism.

The Oxidative Half-Reaction

The Oxidative Half-Reaction in BSAO is Very Different to That of LSAO

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

In an extensive study of the reoxidation of BSAO, an enzyme which does not normally populate the Cu^{I}–semiquinolamine radical, Su and Klinman[33] have reported a detailed analysis of a chemical and kinetic mechanism of dioxygen conversion into hydrogen peroxide that does not require the conversion of Cu^{II} into 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}, thereby facilitating the transfer of a second electron and two protons from the semiquinolamine radical and the release of H_2O_2 (see *g* and *g′* Scheme 2). Thus, during its reoxidation, the reduced BSAO populates a single intermediate species, the reversible oxygen complex $E_{red}O_2$, which has the same absorbance spectrum as E_{red}. The subsequent release of ammonia regenerates the Cu^{II}–quinone species.



Scheme 2. Catalytic mechanism of BSAO: the oxidative half-reaction. *g, g′*: Cu^{II}–aminoresorcinol–oxygen complex. *a*: Resting oxidized enzyme.

The Oxidative Half-Reaction in LSAO

The Cu^{I}–semiquinolamine cation radical described in "the reductive half-cycle" is the species that reacts rapidly with oxygen.[23,30] This observation has allowed Floris and co-workers[23] to determine the oxidation rate constant of reduced LSAO as a function of the semiquinolamine radical population. Their study demonstrates that both reduced derivatives − the Cu^{I}–semiquinolamine and the Cu^{II}–aminoquinol − are capable of reacting with oxygen, although with widely different rate constants, and that at neutral pH the reaction path involving the semiquinolamine radical intermediate largely predominates. Moreover, under experimental conditions that inhibit the conversion to the radical species, the apparent second-order rate constant for oxidation of LSAO closely approaches that of BSAO, although the stability of the $E_{red}O_2$ complex is much lower in the former enzyme, perhaps because of its faster conversion to the oxidized derivative (E_{ox}). In the same way as BSAO, the subsequent release of ammonia regenerates the Cu^{II}–quinone species.

4. The Role of Copper

The discussion of the oxidative half-reaction implies another controversial matter: the role of copper in the catalytic mechanism of AOs. All Cu/TPQ AOs contain a copper atom in a 1:1 stoichiometric ratio with the organic cofactor. The ESR parameters of copper/TPQ AOs fall in the category of so-called type-2 Cu ESR spectra.[34] The copper ion is coordinated to three hystidine residues and two water molecules (Figure 1) and is located very close to the TPQ cofactor. Copper is essential for the reoxidation of the substrate-reduced enzyme in the presence of oxygen and appears to control the transfer of substrates from a hydrophobic binding site near the protein surface[35] to the deeply buried active site.[3,36]

A Structural Role for Copper

The role of the metal cofactor in the catalytic mechanism of BSAO has partially been solved by studies using both copper-free enzyme[18] and enzymes in which the copper has been replaced with other metals.[37] Copper can be removed from BSAO by treatment with sodium dithionite in the presence of potassium cyanide[18] or benzylamine under anaerobic conditions.[37] The two derivatives contained about 0.22 or 0.70 residual Cu^{II} centers per dimer (corresponding to 10 and 35% residual Cu^{II} per dimer, respectively)[37] depending on the reagent added (potassium cyanide or benzylamine) and were found to be almost totally inactive. The Cu-depleted protein does not react with hydrazine inhibitors and, when reduced by substrate, does not undergo the reoxidation process.[35] After complete removal of copper, the metal binding site of BSAO may accept other metal ions, like Co^{II}, Ni^{II}, and Zn^{II}.[31,38] The addition of cobalt to Cu^{II}-depleted BSAO partially restores the activity of the enzyme (up to 25–40%, corresponding to a k_{cat} value of 0.31–0.49)[18,39] (Table 1), thus confirming that the enzyme does not require the conversion of the cupric copper ion to the cuprous state in the mechanism of dioxygen conversion into H_2O_2. Thus, in BSAO, the metal, whether Cu^{II} or Co^{II}, is not directly involved in amine oxidation, but may have a structural function, as confirmed by the closely similar behavior of Cu^{II} and Co^{II} BSAO derivatives upon deconvolution of their thermal profiles in DSC studies.[38] In these studies, the deconvolution of thermal profiles in native BSAO showed five subpeaks, while the fully-Cu-depleted BSAO required at least six two-state transitions. The thermal profiles of Co^{II}-reconstituted enzyme closely resemble those of the native and Cu^{II}-reconstituted BSAO. The thermograms of both BSAO derivatives can be deconvoluted into five two-state transitions that characterize the native enzyme.[38]

Table 1. Differences and common features between amine oxidase from bovine serum (BSAO) and lentil seedlings (LSAO).

	BSAO	LSAO
Molecular size subunits (KDa)	85	70
Copper ions (content)	2	2
Number of active sites (TPQ dimer)	2,[15,16] 1[5,14]	2[17]
Absorbance maximum (TPQ oxidized)	476 nm, ε_{476} = 3800 $M^{-1}cm^{-1}$[5]	498 nm, ε_{498} = 4100 $M^{-1}cm^{-1}$[2]
Reduced TPQ aminoquinol	colorless	colorless
Reduced semiquinolamine radical	1% of the total TPQ[33]	30–40% of the total TPQ[29] 464, 434, and 360 nm
Percent activity of Co^{II}-reconstituted enzyme	25–40	7
k_{cat} of native (a) and Co^{II}-substituted (b) enzyme	(a) 1.23,[37,39] (b) 0.31–0.49[37,39]	(a) 155,[44] (b) 11[44]

Reactivity with hydrazine derivatives and assays of the enzymatic activity have shown that in the Co^{II} derivative the TPQ is reactive and the enzyme is catalytically competent. The substitution of copper with cobalt does not greatly impair the enzymatic activity. The Co^{II} derivative has the peculiar effect of decreasing both the k_{cat} and the K_M values.[37,39] Thus, substantially smaller K_M values were obtained for all examined amines than for native BSAO, thus confirming that the catalytic activity of Co^{II}–BSAO is not due to residual (10 or 35%) or spurious copper.[27,37]

Furthermore, the CD spectrum of Co^{II}-reconstituted BSAO shows a band at 540 nm which, like the band at 640 nm of the native protein, is assigned to the metal d–d electronic transition.[19,40] The band at 440 nm, which appears in both spectra, is assigned to a TPQ transition.[40]

Thus, we can conclude that, in BSAO, Cu^{II} does not appear to have the usual redox role.[18,35,41] Rather, it seems to affect the redox potential of TPQ and/or modulate the pK_a of the coordinated water molecule, through which it is bonded to organic cofactor-O_2, thus facilitating the proton transfer to O_2. This makes it possible to identify the bridging water, in the place of Asp385, with the catalytic base with a pK_a of about 8.0 controlling BSAO catalytic activity. Such a function could also, in principle, be carried out by a different metal ion.[27,39]

Involvement of Copper in the Catalytic Cycle

In LSAO, copper can be almost completely removed by treatment with *N*,*N*-diethyldithiocarbamate.[42] The residual copper in the LSAO apoenzyme, measured by atomic absorption spectroscopy, is about 0.2 ± 0.02 residual Cu^{II} atoms per dimer (10% residual Cu^{II} per dimer). Copper-free LSAO is still pink and shows a broad absorption peak in the visible region at 480 nm that is shifted towards shorter wavelengths with respect to the native enzyme, but with similar intensity; apo-LSAO is able to oxidize one equivalent of substrate, although copper removal prevents the formation of the semiquinolamine radical and reoxidation of the enzyme. The oxidation occurs under single turnover conditions and releases one equivalent of aldehyde per enzyme subunit under anaerobic as well as aerobic conditions.[43] Moreover, apo-LSAO reacts with phenylhydrazine in a stoichiometry of two mol of inhibitor per mol of apoenzyme dimer, i.e. in a similar manner as the native enzyme.[42] This suggests that in LSAO copper has not only a structural function, as supposed for BSAO. Through a detailed analysis of fully copper depleted, half reconstituted, or fully reconstituted, and of the Co^{II}-substituted LSAO, Padiglia et al.[44] have shown that copper ions are essential for the fast catalytic rate of lentil enzyme. Although the Co^{II}-substituted enzyme retains some activity, the k_{cat} value for the best substrate (putrescine) is much lower than that of the native enzyme (11 vs. 155), and the formation of the semiquinolamine radical is not proven. Thus, in LSAO, copper is certainly involved in the amine oxidation process, and the formation of the Cu^{I}–semiquinone species is an essential step of the catalytic pathway. Cu^{I}–semiquinolamine radical is the species that reacts preferentially with O_2[2,23,30] and the lower catalytic activity of Co^{II}–LSAO may be ascribed to its slow reoxidation.

5. Concluding Remarks

Amine oxidases, a widespread class of enzymes, are present in all living systems, where they control the level of very active compounds, i.e. mono-, di-, and polyamines. The oxidation of these compounds may generate other biologically active substances, such as aldehydes, ammonia, and hydrogen peroxide. Both copper ions and TPQ are essential for the enzyme function. The findings for the catalytic cycle of BSAO[45] offer an interesting comparison with those reported for plant amine oxidase.[23] The catalytic mechanism for LSAO may be referred to as a "protein–radical enzyme", operating through a free radical located on the cofactor and the reduction of copper from the cupric to the cuprous state. This is in contrast to BSAO, which forms the free radical intermediate only to a minor extent in the course of the reoxidative step without the reduction of copper. Thus, two steps of the catalytic mechanism are significantly slower in BSAO than in LSAO, namely the reoxidation and the proton abstraction occurring in the enzyme substrate complex. The former difference is rationalized as being due to the low to zero concentration of the semiquinolamine-radical intermediate, while the latter is less easily interpreted. Copper, like TPQ, is an essential cofactor and its removal inactivates the enzymes. Moreover, a change in the valence of copper is found only in lentil enzyme, which is much more active than bovine AO by almost two orders of magnitude (k_{cat} LSAO = 155 s^{-1}; k_{cat} BSAO = 1.23 s^{-1}). In conclusion, the presence of two active sites, the formation of the radical species, and the copper reduction might

be the reason for the higher catalytic activity of LSAO with respect to BSAO (Table 1).

Acknowledgments

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