The electrochemical behavior of redox centers in the active site of amine oxidases from lentil seedlings and *Euphorbia characias* latex was investigated using a mercury film electrode. Tyrosine-derived 6-hydroxydopa quinone (TPQ) and copper ions in the active site are redox centers of these amine oxidases. The enzymes undergo two reduction processes at negative potentials related to the reduction of the TPQ cofactor to the corresponding hydroquinones and the reduction of copper ions, \((\text{Cu}(\text{II}) \rightarrow \text{Cu}(\text{I}))\). Copper depleted enzymes, prepared by reduction with dithionite followed by dialysis against cyanide, undergo only one reduction process. Nyquist diagrams, recorded at potentials corresponding to the reduction of cofactors as dc-offset, represent charge transfer impedance followed by a Warburg-type line at low frequencies, indicating the occurrence of a diffusion controlled process in the rate-limiting step of the reduction process.

**Key words:** amine oxidase; lentil seedlings; *Euphorbia* latex; topa quinone; mercury film electrode

Amine oxidases belong to a heterogeneous superfamily of enzymes catalyzing the deamination of biogenic amines. Two main types of these enzymes are (i) copper/6-hydroxydopa quinone (TPQ)-containing amine oxidases (amine:oxygen oxidoreductase, deaminating; EC 1.4.3.6)\(^1\)\(^2\) and (ii) flavin-containing amine oxidases. Several Cu/TPQ plant amine oxidases have been purified and characterized. The best known and studied are those from seedlings of the pulses lentil (\(*\text{Lens esculenta}\); LSAO)\(^3\) and pea (\(*\text{Pisum sativum}\); PSAO)\(^4\) and from latex of *Euphorbia characias*, a perennial Mediterranean shrub (ELAO)\(^5\).

Plant Cu/TPQ amine oxidases catalyze the oxidation of the primary amino group of mono-, di- and polyamines with the formation of the corresponding aldehydes, ammonia, and hydrogen peroxide, subtracting two electrons from amines and transferring them to molecular oxygen. These enzymes can flip-flop from oxidized to reduced states through chemical reaction with amine, and come up oxidized by molecular oxygen, as in equations 1 and 2:

\[
\begin{align*}
\text{Enzyme(ox)} + \text{RCH}_2\text{NH}_2 & \rightarrow \text{Enzyme(red)-NH}_3^+ + \text{RCHO} \\
\text{Enzyme(red)-NH}_3^+ + \text{O}_2 & \rightarrow \text{Enzyme(ox)} + \text{NH}_4^+ + \text{H}_2\text{O}_2
\end{align*}
\]

Through this mechanism, the bond of amine substrate to TPQ in the resting oxidized enzyme forms the substrate Schiff base (Cu(II)-quinone ketimine), which, after proton abstraction by a conserved aspartate residue acting as general base, gives rise to the product Schiff base (Cu(II)-quinolaldimine). After hydrolysis and release of product aldehyde, a bleached Cu(II)-aminoquinol species is formed that is in equilibrium with the yellow Cu(I)-semiquinolamine intermediate. Cu(I)-semiquinolamine, reacting with O\(_2\), releases H\(_2\)O\(_2\) and ammonia, thereby regenerating the Cu(II)-quinone species.

Electrochemical studies of enzymes have yielded to an enhanced understanding of the role of these macromolecules in biological energy conversion the respiratory chain, in metabolism and in the redox switches involved. The direct and unmediated charge transfer process between the electrode surface and the redox center in an enzyme has now advanced from the viewpoint of detailed kinetic and thermodynamic investigations. It is also of particular interest in building
biosensors and biofuel cells and in bioelectroorganic synthesis. Moreover, probe systems have included redox proteins, such as cytochromes, hemoglobin and myoglobin, blue copper proteins, and amine oxidases.

Since the invention of classical polarography by Heyrovsky, mercury has been established as an electrode material representing a potential window expanded to high negative potentials. In this context, successful electrodes are composed of a thin layer of metallic mercury electrodeposited on a conventional solid surface, e.g., Au, Pt, and glassy carbon. These electrodes are commonly termed mercury film electrodes (MFEs). MFEs offer several advantages: small size, a large surface-to-volume ratio, no need for ancillaries (fine capillaries, a mercury reservoir, etc.), and higher mechanical stability than mercury drops, and easier chemical modification of the surface. Moreover, the consumption of metallic mercury is minimized.

Following our recent studies of the activity, stability, and energetic domains of LSAO and ELAO, in this study the electrochemical behavior of redox centers in the active sites of these enzymes on MFE was scrutinized. Based on our knowledge, this received little attention to date.

Materials and Methods

All chemicals used were of analytical grade, and were from Merck (Darmstadt, Germany). They were used without further purification. All solutions were prepared with doubly distilled deionized water.

LSAO and ELAO were purified as previously reported. Only high-quality protein was used, on the basis of 2 ± 0.1 titratable TPQ per dimer. The concentration of the enzymes was determined using ε_{278} = 2.46 × 10^{5} M^{-1} cm^{-1} and ε_{278} = 3.78 × 10^{5} M^{-1} cm^{-1} for LSAO and ELAO respectively.

Copper-depleted enzymes were prepared as previously described. Briefly, dithionite was added to solutions of the enzymes under anaerobic conditions, followed by dialysis against a solution of 1.0 mM sodium cyanide in 100 mM potassium phosphate buffer, pH 7.0.

Electrochemical studies were carried out in a 250μl all-glass cell incorporating the three electrode configuration (Goldis, Iran), containing 100 mM potassium phosphate buffer, pH 7.0.

Fig. 1. Linear Sweep Voltammograms of Solutions of 455μM ELAO and 97μM LSAO in 100 mM Potassium Phosphate Buffer, pH 7.0 the Background Signal Using a MFE Is Also Depicted.

The potential sweep rate was 5 mV s^{-1}. Inset: Similar to the main panel, except for zooming in the c2 region.

Fig. 2. Linear Sweep Voltammograms for 455 and 97 μM Copper-Depleted ELAO and LSAO in 100 mM Potassium Phosphate Buffer, pH 7.0, Using a Potential Sweep Rate of 5 mV s^{-1}.
phosphate buffer, pH 7.0, as running electrolyte, powered by an electrochemical system comprised of the AUTOLAB system with PGSTAT30 and FRA2 boards (Eco Chemie, Utrecht, Netherlands). In impedance measurements, a frequency range of 100 kHz to 25 mHz was employed, and the a.c. voltage amplitude was 10 mV and the equilibrium time 5 s. The system was run on a PC by FRA and GPES 4.9 software. In all voltammetric measurements, the IR drop compensation was performed by positive feedback. An Ag/AgCl and a platinum wire were used as reference and counter electrodes respectively. Platinum wire was fitted into glass as a micro-disk exposing a circular area 200 μm in diameter to prepare the working electrode. The Pt electrode was further polished with 0.05 μm α-alumina powder on damp cotton wool and rinsed thoroughly with doubly distilled water prior to modification. A Film of mercury formed on the Pt surface by potentiostatic electrodeposition from an Hg2+ solution. All experiments were carried out at room temperature.

Results and Discussion

In 100 mM of potassium phosphate buffer, pH 7.0, ELAO and LSAO underwent two reduction processes. Figure 1 represents typical linear-sweep voltammograms of 455 mM ELAO and 97 mM LSAO containing solutions using MFE. A potential sweep rate of 5 mV s⁻¹ was employed. The voltammogram of the supporting electrolyte is also depicted in Fig. 1 for the sake of comparison. Both the enzymes suffered reduction processes, with current peak positions, at about 36 and 16 V (ELAO) and 42 and 21 V (LSAO). Similar current peak positions have been reported for other amine oxidases related to reduction of the quinine moiety and the metal center in the active site of the enzyme.15) Figure 1, inset, shows the enlarged C2 potential region of the main panel.

Fig. 3. Alignment of ELAO Sequence versus PSAO.
The computation was performed at the SIB using the blast network service. Cysteine residues are shown in grey.
using a potential sweep rate of 5 mV s\(^{-1}\), are depicted in Fig. 2. Only one reduction peak for C2 appears (at \(-16 V\) for ELAO and \(-21 V\) for LSAO), whereas the C1 peak disappears for both enzymes. These results suggest that C1 peaks, for which the corresponding currents are proportional to the concentrations of the enzymes, reflect a reduction of the Cu(II) center. The appearance of a cathodic wave at the negative edge of working window, related to reduction of the Cu(II) center, is in agreement with that reported for PSAO.\(^{15}\) Reduction of the Cu(II) center in both enzymes occurred at highly negative potentials, observed also for carbonic anhydrase\(^{21}\) and PSAO,\(^{15}\) since the metal is strongly bound to the active site and its thermodynamic potential shifts negatively. Moreover, in the voltammograms represented in Fig. 1 of the C1 region, some current noise is observed. This may be due to the simultaneous occurrence of a hydrogen evolution reaction via presodium catalysis.\(^{22}\) Since the enzymes did not have any other electro-reactive center and disulfide bridges were not accessible (see below), the second reduction peak (C2 in Figs. 1 and 2) might be related to the reduction in TPQ occurring for both native and copper-depleted enzymes, because the dithionite procedure removes only the metal center of the enzymes. The reduction of TPQ is depicted in Scheme 1. It should be noted that this process is electrochemical and occurred via an external circuit at the surface of the electrode in the absence of the substrate, while the reduced and oxidized forms of the enzyme participating in equations 1 and 2 were formed via reaction with substrates.

It has been reported that some disulfide-containing proteins were adsorbed on a mercury surface and that the disulfide bond was consequently reduced as a reduction peak in the voltammograms.\(^{23}\) In the voltammograms represented in Fig. 1, however, no peak was related to this process, and the reduction of the disulfide bond did not occur in the case of ELAO and LSAO. In order to inspect the accessibility of the disulfide bridges of ELAO and LSAO and to confirm that disulfide bonds are not reducible on the working electrode, we used the X-ray crystallography structure of PSAO as a model.

Fig. 4. Alignment of LSAO Sequence versus PSAO.

The computation was performed at the SIB using the blast network service. Cysteine residues are shown in grey.
Fig. 5. Nyquist Diagrams for ELAO (455 μm) and LSAO (97 μm) in 100 Mm Potassium Phosphate Buffer, pH 7.0, Using a MFE at Biases $-1.16$ and $-1.21$ V.

Continuous lines indicate fitting data by Randles equivalent circuit. Inset A, corresponding Bode magnitude and Bode phase plots. Inset b, real and imaginary parts of impedance vs. minus square root of frequency at low frequencies (warburg region).
because nucleotide sequence alignment of ELAO (accession no., Q9SW90) and LSAO (accession no., P49252) versus the PSAO (accession no., Q9SXW5) showed very similar sequence identities and homology (Figs. 3 and 4), and also because it has been reported that PSAO does not undergo the S-S reduction process on the mercury electrode.\(^{15}\) In the sequence of the PSAO, there are five cysteine residues, named cysteine 132, cysteine 153, cysteine 314, cysteine 340, and cysteine 642. From these residues, cysteine 132 with cysteine 153, and cysteine 314 with cysteine 340 make S-S bonds, and cysteine 642 has free sulfhydryl group. The sequence alignment of PSAO versus ELAO shows that residues cysteine 153 and tryptophan 639 in PSAO were replaced by phenylalanine and cysteine in ELAO respectively. The other cysteine residues were conserved. Moreover, the sequence alignment of PSAO versus LSAO shows that cysteine residues were conserved. Along these lines, the PDB files of ELAO and LSAO were made using the Swiss model tools in the Expasy site\(^{24-26}\) then the accessibility of different residual atoms were indicated by GETAREA software\(^{27}\) by radius of the water probe in 1.4 Å. We used the SPDB viewer to detect residues with accessible areas of more than 4%. The both methods indicated that all atoms in the S-S bonds were buried and inaccessible. Therefore, theses enzymes did not bear the reducible sulfide-sulfide segment linkers. It should be added that both ELAO and LSAO have two subunits and dimer structures. It has been reported that enzymes that bear subunits and are folded into a globular conformation shows small adsorption at the mercury surface.\(^{23}\) This results the negligible surface denaturing.\(^{23}\) Also, impedance measurements did not imply any surface process (see below). However, reduction of the enzymes did not result in the surface denaturing.

For deeper investigation of the electrochemical behavior of the enzymes, electrochemical impedance spectroscopy was also employed. Figure 5 exhibits typical complex plane diagrams for 455 μM ELAO and 97μM LSAO in 100 mM potassium phosphate buffer, pH 7.0, recorded at the potential of reduction of cofactors as dc-offset (−2.16 and −2.21 V vs. Ag/AgCl respectively). The entire frequency range can be divided into two sub-regions, corresponding to two time constants. Along this line, a high frequencies semicircle and a Warburg-type line at low frequencies were observed. The high frequency semicircle was related to the charge transport process of the reduction of the TPQ cofactor (Scheme 1). The linear tail with a slope of near-unity at low frequencies corresponds to Warburg impedance, as confirmed by the linear dependency of both the real and the imaginary parts of impedance on the negative square root of the frequency. This was attributed to semi-infinitue diffusion of the enzymes from the bulk of the solution to the electrode surface. An equivalent circuit compatible with the results obtained is the well-known Randles-type equivalent circuit which comprises a parallel combination of double-layer capacitance with a series of combined charge transfer resistance-Warburg elements. The equivalent circuit is completed by supplementing the solution resistance (Scheme 2). A good fit of experimental results with the equivalent circuit is also indicated in Fig. 5 and Table 1 shows the values of the equivalent circuit elements obtained by fitting the experimental results, in which \(R_s\), \(Cdl\), \(Rct\) and \(Tw\) indicate the solution resistance, double layer capacitance, charge transfer resistance, and Warburg impedance coefficient respectively.

The alignment of amino acid sequences from ELAO (GB data bank accession no., AF171698) and LSAO (EMB data bank accession no., X64201) shows 93% homology, but these enzymes show different reactivity toward various substrates.\(^{28}\) Differences in the reactivity of the enzymes are perhaps explained by differences in the thermodynamic potentials of the corresponding cofactors.

### Conclusion

Redox centers of plant copper/TPQ amine oxidases from lentil seedlings and *Euphorbia characias* latex were investigated using a mercury film electrode in the regime of linear sweep voltammetry. The TPQ and copper entities of the enzymes are the electroactive sites that underwent the reduction process. The latter was evaluated by the method employed for the native and copper-depleted enzymes. The copper sites of the enzymes underwent reduction at higher negative potentials than the TPQ organic cofactor. Nevertheless, both
peaks (C1 and C2) related to the reduction of the electro-active sites of ELAO appeared at more positive potentials than LSAO. We conclude that in the process of oxidation of an amine substrate, ELAO deaminated the substrate more favorably thermodynamically than LSAO. That is the first step in the ping-pong mechanism (equation 1) is more favorable to ELAO. In contrast, LSAO, which represents the reduction of TPQ cofactor and copper entities at higher negative potentials, is favorable to the second step of the ping-pong mechanism. (equation 2) Therefore, ELAO oxidizes the substrates more easily than LSAO to form the reduced state and the reduced state of LSAO regenerates the resting enzyme more easily. Besides, impedance measurements reflected the occurrence of a diffusion-limited reduction process for the cofactors in the native enzymes.

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References


23) Honeychurch, M. J., The reduction of disulfide bonds in proteins at mercury electrodes. Bioelectrochem. Bioen-


