Anal. Chem. **2000,** *72,* 5988−5993

Electrooxidation Mechanism of Biogenic Amines at Amine Oxidase Modified Graphite Electrode

Mihaela Niculescu,[†,‡] Tautgirdas Ruzgas,[‡] Catalin Nistor,[‡] Ivo Frébort,[§] Marek Šebela,[§] Pavel Peč,[§] and Elisabeth Csöregi*[,†]

Department of Biotechnology, Lund University, P.O. Box 124, S-22100, Lund, Sweden

Amine oxidase (AO, EC. 1.4.3.6) was previously shown to be a very efficient biological recognition element of amperometric biosensors for monitoring biogenic amines. The enzyme was effectively working in both mono- and bienzyme electrode designs, based on either a direct or a mediated electron-transfer pathway. This work focuses on the elucidation of the electron-transfer mechanism of the monoenzymatic unmediated AO-modified biosensor. The observed unmediated catalytic currents were assumed to be caused by (i) a direct electron-transfer process, (ii) the electrooxidation of the formed product, or (iii) their combination. Experiments supporting these assumptions are discussed in detail.

Interaction between biological molecules and electrodes is a subject that gained real scientific interest in recent years.[1−3] Direct and reversible electron transfer was, until recently, believed to be difficult to achieve for most of the redox enzymes, mainly due to three facts: (i) the active site of a redox enzyme is often deeply buried in the protein structure; (ii) the possibility of the enzyme to be oriented in an unfavorable direction on the electrode surface is high; (iii) inactivating conformational changes can occur when the enzyme is immobilized on the electrode surface.[4] Therefore, properties such as size of enzyme, distance between the active site and the surface of the molecule, and orientation of the enzyme on the electrode are always important aspects in the development of enzyme electrodes based on direct electron transfer (DET).[3]

Direct electron transfer has been, however, reported for peroxidases,[5−9] laccases,[10,11] glucose oxidase,[12] ascorbate oxidase,[13] fumarate reductase,[14] cellobiose dehydrogenase,[15] and alcohol dehydrogenase.[16,17] DET-based biosensors display some main advantages over the mediated ones, such as the following: (i) they can operate at a potential close to the formal potential of the enzyme's cofactor and, thus, are less prone to interfering reactions[18] (improved selectivity), and (ii) they are simple to construct since there is no need to add extra reagents required in the reaction sequence (reagentless biosensors). Moreover, DET-based systems allow the possibility of modulating certain desired properties of an analytical device using, for example, protein modification (genetic or chemical engineering)[19,20] and/or novel interfacial technologies.[21,22]

In this work, the possibility of a DET mechanism between a recently isolated and purified amine oxidase from grass pea (AO) and solid graphite electrodes has been investigated. Amine oxidases are enzymes often used for the development of biosensors for monitoring biogenic amines, with applications in the food industry (measuring freshness[23−26]). We recently reported on two different AO-based biosensors: a monoenzymatic[27] and a bienzymatic one,[26] the latter being based on coimmobilized AO and horseradish peroxidase (HRP). The present work describes our

* Corresponding author: (phone) +46-46-2224274; (fax) +46-46-2224713; (e-mail) Elisabeth.Csöregi@biotek.lu.se.

† Department of Biotechnology, Lund University.

‡ Department of Analytical Chemistry, Lund University.

§ Department of Biochemistry, Faculty of Science, Palacký University, Šlechtitelù 11, 78371 Olomouc, Czech Republic.

(1) Cass, A. E. G. In *Biosensors: A Practical Approach*; Rickwood, D., Hames, B. D., Eds; IRL Press: Oxford, 1990; pp 1−262.
(2) Mulchandani, A.; Rogers, K. R. In *Enzyme and Microbial Biosensors: Techniques and Protocols*; Humana Press Inc.: Totowa, NJ, 1998; pp 1−256.
(3) Ghindilis, A. L.; Atanasov, P.; Wilkins, E. *Electroanalysis* **1997**, *9*, 661.
(4) Burestedt, E. Enzymatic and Immunological Detection Principles for Environmental and Biological Applications. Doctoral Thesis, Lund University, Lund, 1999; pp 6−21.
(5) Jönsson, G.; Gorton, L. *Electroanalysis* **1989**, *1*, 465.
(6) Wollenberger, U.; Wang, J.; Ozsoz, M.; Gonzales Romero, E.; Scheller, F. *Bioelectrochem. Bioenerg.* **1991**, *26*, 287.
(7) Mondal, M. S.; Fuller, H. A.; Armstrong, F. A. *J. Am. Chem. Soc.* **1996**, *118*, 263.
(8) Ruzgas, T.; Gorton, L.; Emnéus, J.; Csöregi, E.; Marko-Varga, G. *Anal. Proc.* **1995**, *6*, 207.
(9) Ruzgas, T.; Csöregi, E.; Emnéus, J.; Gorton, L.; Marko-Varga, G. *Anal. Chim. Acta* **1996**, *330*, 123.
(10) Tarasevich, M. R.; Yaropolov, A. I.; Bogdanovskaya, V. A.; Varfolomeev, S. D. *Bioelectrochem. Bioenerg.* **1979**, *6*, 393.
(11) Yaropolov, A. I.; Kharybin, A. N.; Emnéus, J.; Marko-Varga, G.; Gorton, L. *Bioelectrochem. Bioenerg.* **1996**, *40*, 49.
(12) Alvarez-Icaza, M.; Schmid, R. D. *Bioelectrochem. Bioenerg.* **1994**, *33*, 191.
(13) Sakurai, T. *Chem. Lett.* **1996**, 481.
(14) Sucheta, A.; Cammack, R.; Weiner, J.; Armstrong, F. A. *Biochemistry* **1993**, *32*, 5455.
(15) Larsson, T.; Elmgren, M.; Lindquist, S. E.; Tessema, M.; Gorton, L.; Henriksson, G. *Anal. Chim. Acta* **1996**, *331*, 227.
(16) Ikeda, T. *Bull. Electrochem.* **1992**, *8*, 145.
(17) Ikeda, T.; Kobayashi, D.; Matsushita, S.; Sagara, T.; Niki, K. *J. Electroanal. Chem.* **1993**, *361*, 221.
(18) Gorton, L. *Electroanalysis* **1995**, *7*, 23.
(19) Heller, A.; Degani, Y. *J. Chem. Soc* **1988**, *110*, 2615.
(20) Willner, I.; Katz, E.; Willner, B. *Electroanalysis* **1997**, *9*, 965.
(21) Whitesides, G. M. *Science* **1991**, *254*, 1312.
(22) Finklea, H. O. In *Electroanalytical Chemistry*; Bard, A. J., Ed.; Marcel Dekker: New York, 1996; pp 114−335.
(23) Chemnitius, G. C.; Suzuki, M.; Isobe, K. *Anal. Chim. Acta* **1992**, *263*, 93.
(24) Chemnitius, G. C.; Bilitewski, U. *Sens. Actuators* **1996**, *B 32*, 107.
(25) Volpe, G.; Mascini, M. *Talanta* **1996**, *43*, 283.
(26) Niculescu, M.; Nistor, C.; Frébort, I.; Peč, P.; Mattiasson, B.; Csöregi, E. *Anal. Chem.* **2000**, *72*, 1591.
(27) Niculescu, M.; Frébort, I.; Peč, P.; Galuska, P.; Mattiasson, B.; Csöregi, E. *Electroanalysis* **2000**, *12*, 369.

10.1021/ac0001380 CCC: $19.00
Published on Web 11/16/2000

efforts to elucidate the working mechanism of the AO-based monoenzymatic electrodes.

EXPERIMENTAL SECTION

Materials. Amine oxidase (EC. 1.4.3.6) from grass pea was isolated and purified according to a previously published protocol.[28] Peroxidase from horseradish (EC 1.11.1.7) was purchased from Sigma Chemical Co. (St. Louis, MO) as a lyophilized powder with a declared activity of 1100 units/mg of solid. Putrescine dihydrochloride, histamine dihydrochloride, tyramine hydrochloride, cystamine dihydrochloride, agmatine sulfate, and spermidine phosphate salt were from ICN Biochemicals Inc. (Aurora, OH). Ethylenediamine was from Merck (Darmstadt, Germany). Cadaverine dihydrochloride was purchased from Sigma. Diethyldithiocarbamic acid, sodium salt trihydrate, was from ACROS (Geel, Belgium). Potassium dihydrogenphosphate and disodium hydrogen phosphate dihydrate, both from Merck, were used for the preparation of the phosphate buffer solution, 0.1 M, pH 7.2 (PB). Water purified in a Milli-Q system (Millipore, Bedford, MA) was used to prepare PB used as the carrier solution and supporting electrolyte in the flow injection and batch experiments, if not otherwise stated.

All experiments were performed at room temperature, and before use, all substrate solutions were freshly prepared using PB, filtered through a 0.45-μm filter (type HA, Millipore, Molshem, France) and degassed.

Instruments. Chronoamperometry (CA) and cyclic voltammetry (CV) experiments were carried out in a voltammetric cell, using an Ag/AgCl (0.1 M KCl), and a platinum wire as the reference and counter electrodes, respectively. PB was the supporting electrolyte, and the AO biosensor was the working electrode. The system was controlled by a BAS 100W electrochemical analyzer (Bioanalytical System Inc., West Lafayette, IN) connected to a computer. Cyclic voltammograms were recorded by scanning the working potential between −100 and +400 mV vs Ag/AgCl, and chronoamperometric experiments were performed at +200 mV vs Ag/AgCl. The solution was deaerated with nitrogen for 60 min prior to and also during the batch experiments, if not otherwise stated.

Experiments using an in-house-built electrochemical cell with two working electrodes (dual electrode flow cell) were performed in a single-channel flow injection system containing a manual sample injection valve (Valco Instruments Co. Inc., Houston, TX) equipped with a 50-μL injection loop. A peristaltic pump (Alitea AB, Stockholm, Sweden) transported the carrier solution at desired flow rates through Teflon tubing (0.5 mm i.d.) to the flow cell, and a bipotentiostat (LC-3D Petit Ampère, Bioanalytical System Inc.) maintained a constant potential between the working and the reference electrodes. The resulting currents were monitored with two single-channel recorders (model BD 111, Kipp & Zonen, Delft, The Netherlands). Selectivity experiments were performed in a similar system, but using a cell with only one working electrode, an Ag/AgCl and a Pt wire as the reference and counter electrodes, respectively.

Methods. 1. Electrode Preparation. AO-modified graphite electrodes were prepared as follows:

(A) For monoenzymatic electrodes, rods of spectroscopic graphite (Ringsdorff Werke GmbH., Bonn, Germany, type RW001, 3.05 mm diameter) were cut and wet polished on fine emery paper (Tufback, Durite P1200, Allar, Sterling Heights, MI). After rinsing the electrode surface with water and drying at room temperature, 6 μL of an AO stock solution (5 mg/mL) was placed on the surface of the electrode.

(B) Bienzymatic electrodes were prepared using the same procedure as given in (A), but placing 6 μL of a premixed solution containing 80% AO and 20% HRP (w/w)[26] on the graphite electrode surface.

All presented results are the mean of three equally prepared electrodes, if not otherwise stated.

2. Preparation of Cu-Free Amine Oxidase. Cu-free AO was prepared according to Rinaldi et al.[29] using sodium diethyldithiocarbamate (DDC) for the precipitation of cupric ions. AO (0.1 mM) and DDC (60 mM) recrystallized from ethanol were mixed in a molar ratio of 1:100. This mixture was left to stand at 4 °C for 24 h. Next, the brown-black Cu−DDC complex precipitate was removed by centrifugation and the supernatant was immediately passed through Sephadex G-25 column (Pharmacia Biotech., Uppsala, Sweden, 2.5 cm diameter × 20 cm), previously equilibrated with 20 mM PB. The protein fractions were cooled and concentrated using an ultrafiltration cell (Amicon, Denver, MA). Finally, the copper-free enzyme was further purified by gel permeation chromatography at a flow rate of 1.2 mL/min, using a Sephacryl S-300 HR column (Pharmacia Biotech., 2.5 cm diameter × 50 cm) and the same buffer conditions. The final preparation of copper-free AO was homogeneous and showed an absorption spectrum with a maximum at 500 nm.

RESULTS AND DISCUSSION

Amine oxidases represent a class of enzymes widely distributed in mammals, plants, and microorganisms.[30,31] However, their structure, selectivity, and biological functions are very different, depending on the isolation source; i.e., in prokaryotes, the enzyme allows the microorganisms to use amines as carbon and nitrogen sources, while in both plants and animals, their main role is in the detoxification processes by catabolizing the toxic mono- and diamines and in the regulation of fundamental cellular processes, such as tissue differentiation, tumor growth, and programmed cell death.[32,33]

The copper-containing amine oxidases (amine oxygen oxidoreductase deaminating) catalyze the oxidative deamination of primary amines on the basis of oxygen reduction, generating the corresponding aldehyde, according to the following reaction:[34]

$$RCH_2NH_2 + H_2O + O_2 \rightarrow RCHO + H_2O_2 + NH_3$$

Besides copper, the presence of an organic cofactor with a

(28) Šebela, M.; Luhová, L.; Frébort, I.; Faulhammer, H. G.; Hirota, S.; Zajoncová, L.; Stu ka, V.; Peč, P. *Phytochem. Anal.* **1998**, *9*, 211−222.

(29) Rinaldi, A.; Giartosio, A.; Floris, G.; Medda, R.; Finazzi Agro, A. *Biochim. Biophys. Res. Commun.* **1984**, *120*, 242.

(30) Rinaldi, A.; Floris, G.; Giartosio, A. In *Structure and functions of amine oxidases*; Mondovi, B., Ed.; CRC Press: Boca Raton, FL, 1985; pp 51−66.

(31) Knowles, P. F.; Dooley, D. M. In *Metal ions in biological systems*; Sigel, H., Sigel A., Eds; Marcel Dekker: New York, 1994; pp 361−403.

(32) McIntire, S. W.; Hartmann, C. In *Principles and applications of quinoproteins*; Davidson, V. L., Ed.; Dekker: New York, 1992; pp 97−152.

(33) Li, R.; Klinman, J. P.; Scott Mathews, F. *Res. Article* **1998**, *6*, 293.

(34) Bachrach, U. In *Structure and Functions of Amine Oxidases*; Mondovi, B., Ed.; CRC Press: Boca Raton, FL, 1985; pp 5−20.

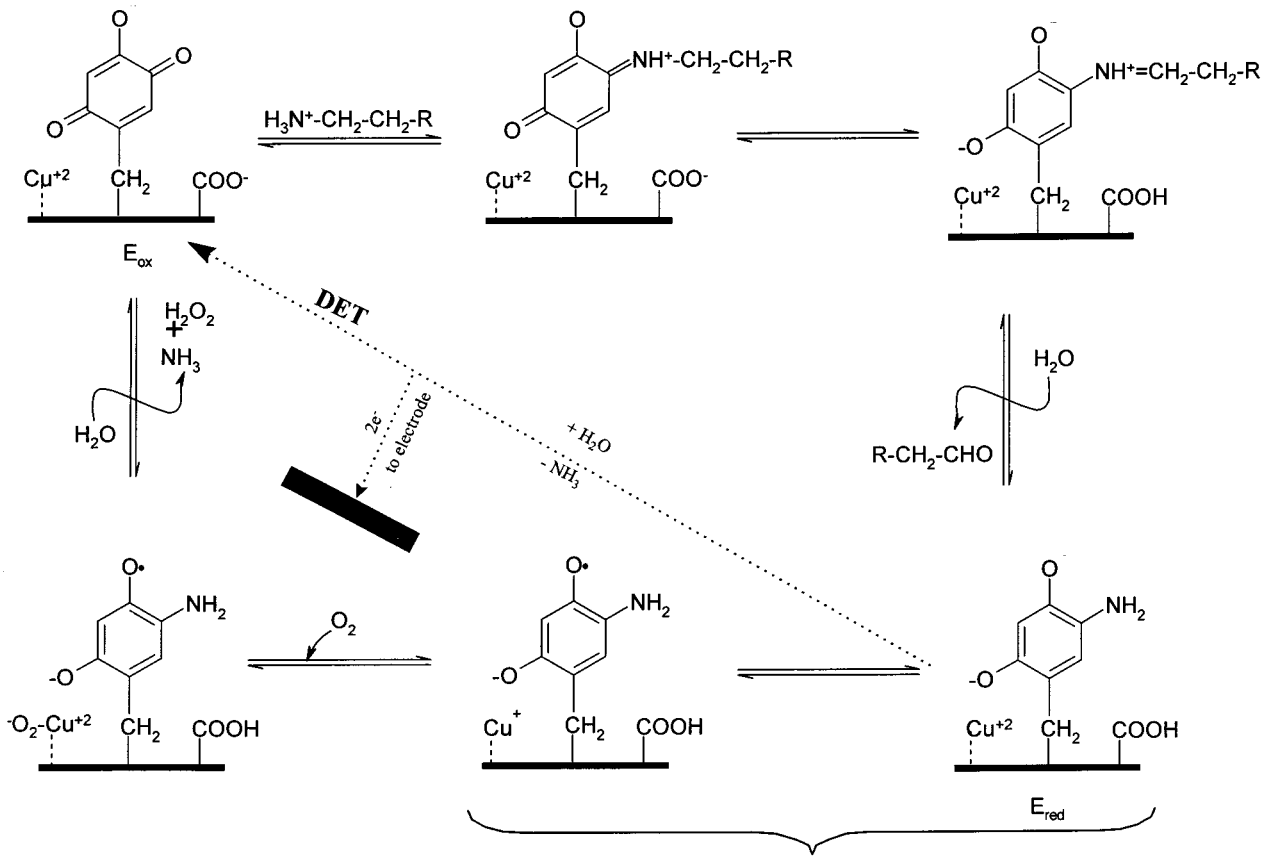

Figure 1. Mechanism of the reaction catalyzed by plant copper amine oxidases.[39] The dashed line shows the proposed "short circuit" in the catalytic mechanism caused by a DET between the enzyme's active site and an oxidative electrode.

quinoide structure (topa quinone, a modified tyrosine side chain) in the catalytic site has also been demonstrated,[28,35−36] most of the proposed reaction mechanisms being related to the structure of this cofactor.[37−39]

The mechanism of the reaction catalyzed by AOs in solution has been studied extensively, but the final elucidation was only recently accomplished[38] (see Figure 1). Accordingly, one of the enzyme cofactors, topa quinone, is responsible for binding the primary substrate, the amine, while the other cofactor, Cu^{2+}, complexes the secondary substrate, dioxygen, in two sequential steps. First, the amine substrate binds to the C4 quinone group of topa forming a ketimine that undergoes a proton abstraction from the α-carbon of the amine by a nucleophilic agent (a side-chain carboxylate group of an Asp residue—present in the enzyme's active site). Next, the structure turns into an aldimine that is easily hydrolyzed, releasing the aldehyde product. In the following step, the transfer of an electron between the reduced topa and Cu^{2+} takes place, being mediated by an integrated network of water. Finally, molecular oxygen forms a complex with Cu^{+}, and in the presence of water environment, the other two products, hydrogen peroxide and ammonia, are released, while the oxidized form of the topa quinone is regenerated.

In this work the possibility of a "short circuit" in the catalytic mechanism, caused by a DET between the enzyme's active site and the electrode, was investigated (see Figure 1, DET).

Figure 2 shows the cyclic voltammograms obtained using the AO-modified graphite as the working electrode, immersed in histamine (A), putrescine (B), and PB (C) solutions, respectively. As seen, the catalytic wave appears only in the presence of the substrates, with a half-wave potential of about +50 ± 10 mV vs Ag/AgCl (+270 ± 10 mV vs NHE) for histamine (A) and putrescine (B), respectively. However, this effect was much more accentuated in the presence of histamine (A) than in the presence of putrescine (B), which is the main substrate of the enzyme in solution.[28] Typical hydrodynamic voltammograms of unmodified (A, B) and AO-modified graphite electrodes (C, D), recorded for histamine (A, C) and putrescine (B, D) are shown in Figure 3. Clearly, only the AO-modified biosensors displayed a catalytic current, starting at around −50 mV vs Ag/AgCl, increasing with increasing potential, and reaching a plateau at around +250 mV. The half-wave potential appears for both substrates around +50 mV vs Ag/AgCl (+270 mV vs NHE), these values being in good correlation with the ones reported for free topa (+300 mV vs NHE)[28] and the reduced form of grass pea AO (+250 mV vs NHE),[28] respectively.

According to the obtained results, a possible explanation of the recorded catalytic current in the presence of AO substrates

(35) Janes, S. M.; Mu, D.; Wemmer, D.; Smith, A. J.; Kaur, S.; Maltby, D.; Burlingame, A. L.; Klinman, J. P. *Science* **1990**, *248*, 981.
(36) Šebela, M.; Luhová, L.; Frébort, I.; Hirota, S.; Faulhammer, H. G.; Stuka, V.; Pec, P. *J. Exp. Bot.* **1997**, *48*, 1897.
(37) Medda, R.; Padiglia, A.; Pedersen, J. Z.; Rotilio, G.; Finazzi Agro, A.; G., F. *Biochemistry* **1995**, *34*, 16375.
(38) Su, Q.; Klinman, P. *Biochemistry* **1998**, *37*, 12513.
(39) Padiglia, A.; Medda, R.; Pedersen, J. Z.; Lorrai, A.; Peè, P.; Frébort, I.; Floris, G. *J. Enzyme Inhib.* **1998**, *13*, 311.

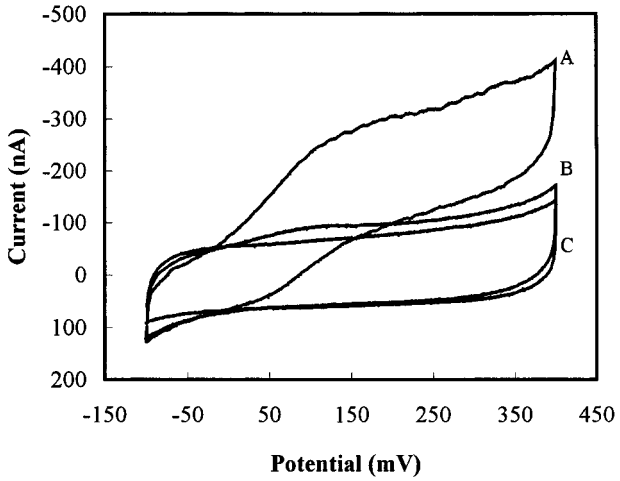

Figure 2. Cyclic voltammograms obtained using AO-modified electrodes in the presence of histamine (A), putrescine (B), and 0.1 M PB (C). Other experimental conditions: scan rates 1 mV/s, substrate concentration 1 mM, deaeration (N_2 bubbling) for 60 min prior the experiment.

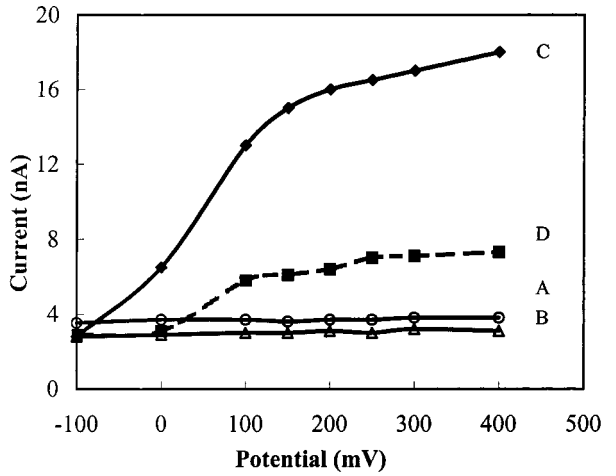

Figure 3. Hydrodynamic voltammograms recorded in a flow injection system for unmodified (A, B) and AO-modified graphite electrodes (C, D), in the presence of histamine (A, C) and in the presence of putrescine (B, D). Conditions: flow rate 0.5 mL/min, injection volume 100 μL, concentration of histamine 100 μM and putrescine 1 mM.

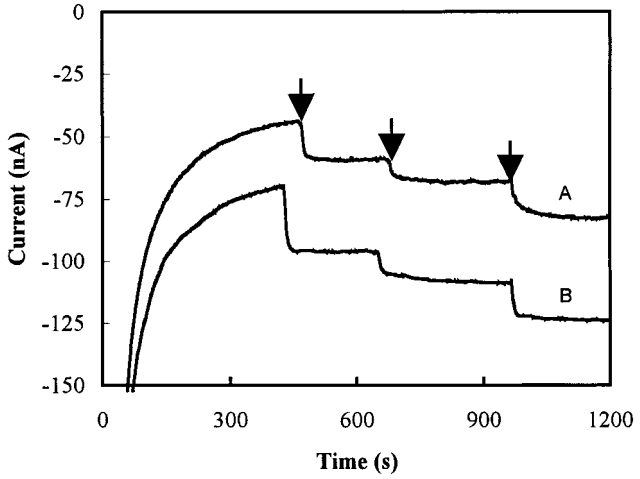

Figure 4. Chronoamperometric response of AO-modified electrodes to histamine in the presence of O_2 (A) and in the absence of O_2 (bubbling of N_2 for 60 min, B). Arrows indicate 100 μM additions of histamine.

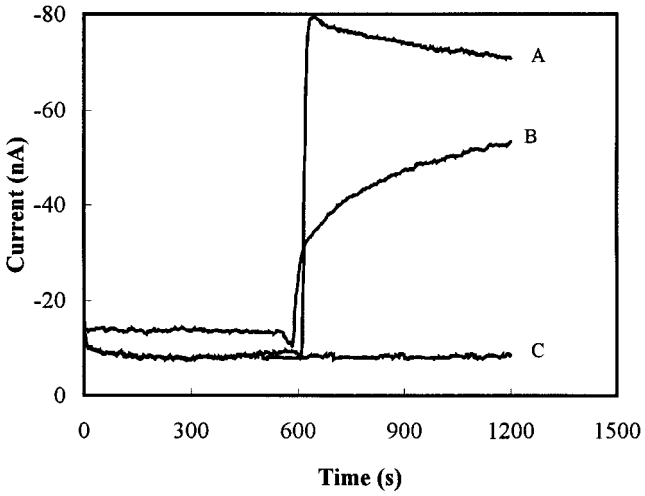

Figure 5. Chronoamperometric response of native AO- (A) and Cu-free AO-modified graphite electrodes (B) and unmodified electrodes (C) upon addition of histamine. Final histamine concentration in the voltammetric cell 1 mM.

is the reoxidation of the topa formed during the reaction with the amine substrates, via a direct regeneration of the active form of the enzyme. The half-wave potentials obtained in CV did not depend on the substrate and were in agreement with those in hydrodynamic voltammogram experiments, thus supporting a DET hypothesis.

The profiles of the chronoamperometric responses recorded for the AO biosensor using histamine as substrate, in the presence (A) and in the absence (B) of dissolved oxygen were similar (see Figure 4), demonstrating a possible replacement of the O_2 as final electron acceptor in the catalytic cycle by the polarized electrode (+200 mV vs Ag/AgCl). An efficient oxygen removal from both the supporting electrolyte and the substrate solution was achieved by bubbling N_2 for 60 min (<1 μM final concentration, measured with Clark-type oxygen electrode-results not shown).

In accordance with the theory of a DET, the exclusion of the O_2-binding site (Cu^{2+} ions in the enzyme structure) should not cause a deactivation of the enzyme because of the possibility of regenerating the active site of the enzyme by direct oxidation. Therefore, a Cu-free AO was studied in similar experimental conditions. Both the active and the Cu-free enzymes were immobilized on graphite electrodes, and their responses for histamine were analyzed by chronoamperometry (Figure 5). As seen, both sensors responded similarly; however, the Cu-free AO biosensor showed somewhat slower kinetics. This fact demonstrates the possibility of replacing O_2 in the catalytic mechanism and, thus, supports the DET hypothesis.

To elucidate whether any electroactive species could be produced during the reaction catalyzed by AO, a series of experiments were performed using a specially designed flow cell with two working electrodes. The dual electrode cell contained the studied electrode (the one first reached by the carrier flow) and the collecting electrode, placed in close proximity to the first one. To avoid contact of the sample with the counter electrode upstream, a Teflon membrane (1-mm thickness) has been applied

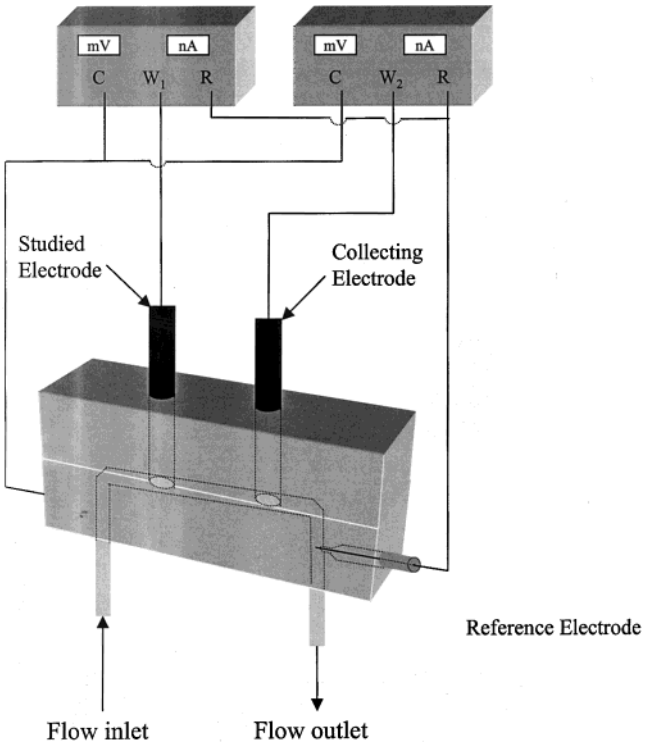

Figure 6. Schematic presentation of the specially designed dual flow cell with two working electrodes for the detection of biogenerated products. Distance between the two electrodes 3 mm.

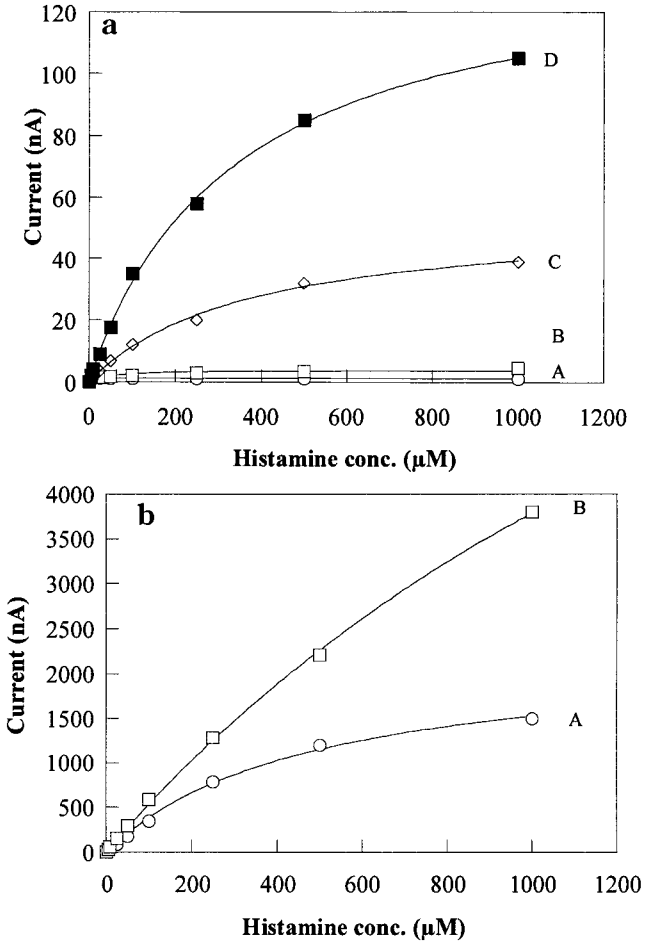

Figure 7. (a) Histamine responses recorded for (A) unmodified graphite, applied potential + 200 mV vs Ag/AgCl, flow cell with single working electrode; (B) unmodified graphite as the collecting electrode, AO biosensor as the studied electrode, both polarized at + 200 mV vs Ag/AgCl; (C) unmodified graphite as the collecting electrode, and not polarized AO biosensor as the studied electrode; and (D) AO biosensor, polarized at + 200 mV vs Ag/AgCl flow cell with single working electrode. (b) Histamine responses on the HRP-modified collecting electrode recorded for (A) AO biosensor polarized at +200 mV vs Ag/AgCl, collecting electrode polarized at −50 mV vs Ag/AgCl, and (B) AO electrode not polarized, collector electrode polarized at −50 mV vs Ag/AgCl.

on the top of the auxiliary electrode, so that the carrier flow touches the counter electrode only when exiting the flow cell (quasi-downstrem) (see Figure 6).

First, an AO biosensor was the studied electrode and an unmodified graphite was the collecting one. Figure 7 shows the results obtained for the injection of different concentrations of histamine in different situations.

The histamine was practically not detectable on a naked graphite electrode (Figure 7a, trace A), while in all other studied cases, the signal was proportional to the concentration of substrate injected into the carrier flow. The histamine response recorded for the case when both the AO-modified and unmodified graphite electrodes were polarized at +200 mV (Figure 7a, trace B) was lower than the one obtained when the same potential was applied only on the unmodified graphite (Figure 7a, trace C). Moreover, within a large potential scale (−50 and +300 mV vs Ag/AgCl), no concentration dependency could be observed for unmodified graphite electrodes upon injection of either histamine or H_2O_2 (experiments performed in a single working electrode cell, results not shown).

All these results support the conclusion that the signal measured on the unmodified electrode is caused by the oxidation of the primary product of the reaction catalyzed by AO, e.g., imidazole acetaldehyde.

Next, an HRP-modified graphite electrode was used as the collecting electrode to monitor the reduction current of the generated hydrogen peroxide, either when the AO biosensor (studied electrode) was polarized at + 200 mV vs Ag/AgCl (Figure 7b, trace A) or was not coupled to any potential source (Figure 7b, trace B). The signal recorded was higher when there was no potential applied on the AO-modified electrode, showing a higher production of H_2O_2, and clearly indicating the existence of a DET process between the AO and the graphite electrode. When the oxidative potential was applied, the competition between the electrode and oxygen for the regeneration of the reduced form of AO resulted in a decrease in the bioelectrocatalytical current caused by the reduction of H_2O_2 on the HRP-based biosensor.

In accordance with all these experiments, it was concluded that the current observed on the AO-modified electrode in the absence of an electrochemical mediator is probably due to a mixed ET process, where both a DET and the electrooxidation of the biogenerated aldehyde product occur simultaneously.

The relative selectivity of the AO-based monoenzymatic biosensor for 10 amine substrates was compared with the one obtained for a previously developed AO/HRP electrode[26] (see Figure 8). As seen, the AO monoenzymatic biosensor discriminates between the studied substrates, showing a higher selectivity. Actually, there are only three substrates (histamine, tyramine,

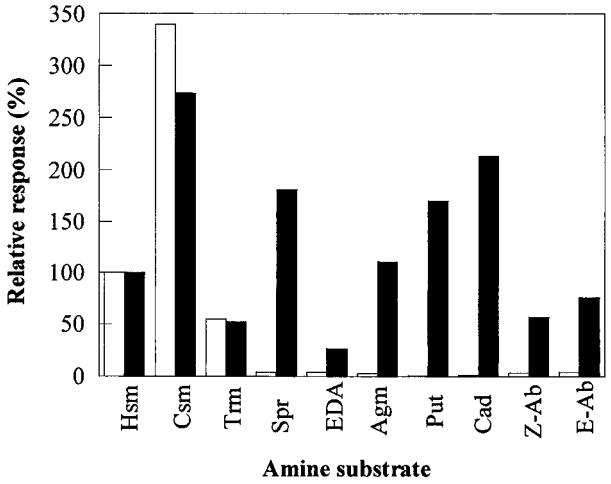

Figure 8. Relative selectivity for amine substrates using different types of AO-biosensors: (□) monoenzymatic and (■) bienzymatic. Histamine signals obtained for the respective electrodes were used as reference: Hsm, histamine; Csm, cystamine; Trm, tyramine; Spr, spermidine; EDA, ethylenediamine; Agm, agmatine; Put, putrescine; Cad, cadaverine.

cystamine) that are detected similarly with these electrodes. The responses of the studied electrodes for injection of 1 mM histamine were 108 (monoenzymatic) and 1900 nA, (bienzymatic), respectively. All other studied substrates are diamines, resulting in amino-aldehydes as bioreaction products, which can further undergo a very fast internal cyclization; therefore, product oxidation on the electrode surface is not possible anymore.[30,40] These facts possibly can explain the low selectivity of the monoenzymatic AO biosensors for putrescine, cadaverine, agmatine, and spermidine and also support the mixed ET pathway theory.

The relative selectivity of the developed biosensors is also quite different in comparison with the measurements using AO in solution;[28] indeed, such a dramatic change in the selectivity for similar electrode configurations (e.g., monoenzyme/bienzyme-based biosensors) has never been observed in previously reported studies.

CONCLUSIONS

This work was focused on the elucidation of the working mechanism of a biosensor based on immobilized AO from grass pea and graphite electrodes, to our knowledge the first reported AO-based biosensor showing an apparent direct electron-transfer mechanism. The results obtained during the present work were confronted with two proposed hypotheses, i.e., via a DET and/or via the product oxidation process. Unfortunately, none of them could be totally excluded, and accordingly, a new mixed ET pathway is proposed where both a DET and an aldehyde oxidation occur.

ACKNOWLEDGMENT

This work was financially supported by the European Commission (INCO-COPERNICUS, ERBIC 15CT96-1008), the Swedish Institute (M.N.), the Swedish Council for Forestry and Agricultural Research (SJSF, E.C.), the Swedish National Board for Industrial and Technical Development (NUTEK, E.C.), and the Ministry of Education (Grant VS96154, I.F.) Czech Republic. The authors thank to Prof. W. Schuhmann from Ruhr University, Bochum, Germany, for valuable discussions.

Received for review February 4, 2000. Accepted September 18, 2000.

AC0001380

(40) Medda, R.; Padiglia, A.; Floris, G. *Phytochemistry* **1995**, *39*, 1.