PHENOL-SENSITIVE ENZYME ELECTRODE WITH SUBSTRATE CYCLING FOR QUANTIFICATION OF CERTAIN INHIBITORY AROMATIC ACIDS AND THIO COMPOUNDS

Lumír MACHOLÁN

Department of Biochemistry, Masaryk University, 611 37 Brno

Received December 21, 1989
Accepted February 5, 1990

A Clark oxygen electrode coated with a membrane of crosslinked champignon phenol oxidase (tyrosinase) is able to detect phenol containing analytical samples 3—5 times more sensitively after the admixture of hydrazine hydrochloride to the reaction buffer. This compound will recycle the substrate via chemical reduction of the quinoid product thus simultaneously preventing the membrane from rapid blackening. The decrease of oxygen in the membrane due to phenol oxidation is rapidly compensated by diffusion from the bulk solution when an inhibitor of the membrane-bound phenol oxidase is added to the reaction medium. This effect has been utilized for the measurement of low concentrations of compounds decreasing the sensitivity of the bioelectrode either reversibly (benzoic acid, 3- and 4-aminobenzoic acid, salicylic acid, 4-aminosalicylic acid, nicotinic acid, 4-nitrophenol, 1-naphthol) or irreversibly (phenylthiourea, thioacetamide, L-cysteine, reduced glutathione, 2-mercaptoethanol, 2,3-dimercaptopropanol and others). Benzoate as a potent reversible inhibitor markedly stabilizes the phenol oxidase reaction membrane during its storage either in the buffer or in dry state at 4°C for one year at least.

Phenol-sensitive bioelectrodes with a phenol oxidase (tyrosinase) reaction layer are usually constructed as amperometric oxygen sensors since hydrogen peroxide is not formed during substrate oxidation. The enzyme isolated from champignons was immobilized for these purposes by crosslinking using glutaraldehyde on a polyamide mesh[1] or by gel-entrapment in polyacrylamide on a platinum mesh[2] or in gelatin[3]. The enzyme electrode was used either in the stationary or flow-through arrangement[4] and was employed to advantage for the activity assays of enzymes hydrolyzing different phenol conjugates[5], for the determination of phenol content of surface and waste water[6] and in a modified construction also for direct determination of *p*-cresol in chloroform extracts[7]. The purified enzyme immobilized in the biocatalytic layer can be replaced also by other less usual materials, such as thin slices of champignon fruit-body[8], sugar beet[9], banana[10], walnut pericarp[11] and crosslinked insect hemolymph[11].

Based on the well-known fact that reversible inhibitors often protect enzymes from denaturation we introduced stabilization of phenol oxidase membranes by benzoate. To increase the sensitivity of the bioelectrode we propose to carry out the analyses

in the presence of hydrazine which can chemically recycle phenol in the sensor membrane via reduction of the quinoid product. The improved phenol-sensitive electrode can receive new application in the quantification of several aromatic acids and thio compounds. The method is based on the finding that these substances decrease the ability of the phenol oxidase membrane to oxidize the phenolic substrate thus increasing the oxygen content in the membrane with respect to the control and also the current intensity of the cathodic oxygen reduction. The plot of the electrode current versus concentration of the inhibiting compound is hyperbolic and can be linearized in an appropriate manner.

EXPERIMENTAL

Material and methods: Champignon phenol oxidase (EC 1.14.18.1) was purified to step 6 according to Kertesz and Zito[12]. The concentrated enzyme solution of activity 0·95 IU μl^{-1} (15·8 nkat μl^{-1}) can be stored at −20°C for several years without considerable loss of activity. Tissue slices 0·3 mm thick were cut from commercial mushroom (*Agaricus bisporus*) as described elsewhere[8]. The following commercial preparations were used: bovine serum albumin (Mann Research Labs), 25% glutaraldehyde (Fluka), hydrazine hydrochloride (Chemapol, Prague), 4-aminobenzoic acid (Merck), sodium 4-aminosalycilate (Cambrian Chemicals), nicotinic acid, reduced glutathione (Lachema, Brno), 2-mercaptoethanol (Koch–Light), and phenylthiourea (Reachim, U.S.S.R.). *p*-Benzoquinone was purified by steam distillation. The remaining chemicals were of reagent purity and deionized water was used throughout.

Apparatus: The oxygen uptake was measured by an oxygen electrode of the Clark type (Radiometer Electrochemical Instruments, Budapest) polarized at −650 mV versus an Ag/AgCl reference. The electrode was adapted to an enzyme pO_2 sensor by coating the measuring area with a wet phenol oxidase membrane. The electrode was connected through an convertor to TZ 4100 Linear Recorder (Laboratorní přístroje, Prague; scale 250 mm, chart speed 0·25 mm . . s^{-1}, range 5 V). The analyses were carried out with magnetic stirring (300 r.p.m.) in a jacketed glass vessel at 30 ± 0·1°C. The solutions of both the substrates and inhibitors were added by means of a Hamilton repeating dispenser.

Preparation of phenol oxidase membranes. Measured volumes of solutions of enzyme, bovine serum albumin and sodium benzoate were applied to an area of stretched polyamide mesh (Silon, Czechoslovakia, 25 mesh per mm^2, fiber thickness 40 μm) outlined in pencil. After mixing with glutaraldehyde the mixtures were spread on both sides of the mesh and allowed to dry at room temperature in horizontal position. The optimal amounts of components calculated per 1 cm^2 were 5–20 μl of enzyme solution (15·8 nkat μl^{-1}), 20 μl of 10% serum albumin, 5 μl of 0·1M sodium benzoate and 4 μl of 25% glutaraldehyde. The membranes were kept dry at 4°C or in 0·1M phosphate buffer, pH 7, containing 0·2% of benzoic acid. The membranes were exhaustively washed prior to use with the buffer not containing the inhibitor. The slices of the mushroom tissue were stored under similar conditions.

Measurement of activity of membranes. The bare pO_2 electrode polarized at −650 mV was placed into a thermostated vessel containing 2·4 ml of phosphate buffer, pH 7, and 0·6 ml of 0·05M catechol. After the current had stabilized under constant stirring the reaction membrane of known surface area cut off from the mesh polyamide was placed in the solution. The rate of oxygen uptake was calculated from the maximal slope of current decrease in the initial stage

of the reaction. It follows from the stoichiometry of the reaction that the uptake of 1 μmol of O_2 per min corresponds to the oxidation of 2 μmol of catechol, i.e. to an activity of 2 IU (33·4 nkat). The activity of the membrane measured was linearly dependent on the membrane surface area over the range of 25–200 mm^2 at least.

Measurement of inhibitor concentrations: The pO_2 electrode coated with the phenol oxidase membrane was placed in the vessel containing 3·0 ml of 0·1M phosphate buffer, pH 7. After the background current had stabilized (with constant stirring and at an external potential of −650 mV) either 0·05M phenol or catechol solution were added to the buffer to make the current drop to a value corresponding to 15–20% of the recorder scale and stabilize at this value. A 0·3 to 1M inhibitor solution was then added stepwise always after a new stabilized current value had been achieved. The admixtures varied between 1 and 50 μl so that the effect of dilution could be neglected. A typical electrode response is shown in Fig. 2 (A); see text and Fig. 3 for the method of evaluation.

RESULTS AND DISCUSSION

Improvement of Properties of Phenol-Sensitive Membranes

The crosslinking of mushroom phenol oxidase by glutaraldehyde on polyamide mesh matrix permits biosensor reaction membranes to be prepared in a manner which is technically simple and reproducible. It is, however, difficult to determine their actual activity since they are insoluble. It has been reported[13] that the crosslinkage of other enzymes results in a 30–80% drop of their activity. The activity of our membranes, however, was considerably lower except for the lowest quantity of phenol-oxidase used (ca 1 nkat cm^{-2}). The profile of the plot of activity measured versus the quantity of enzyme applied, provided that the amount of the other crosslinking components was constant, was hyperbolic. The profile of the percentage yield curve is the opposite (Fig. 1). It may be thus assumed that a considerable part

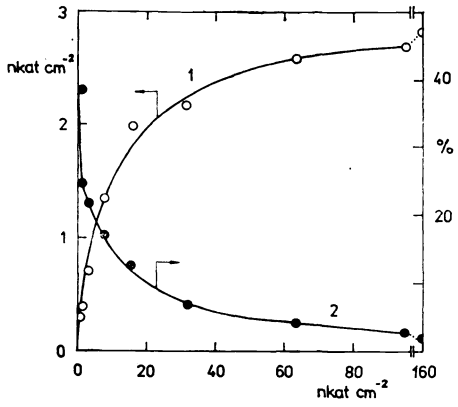
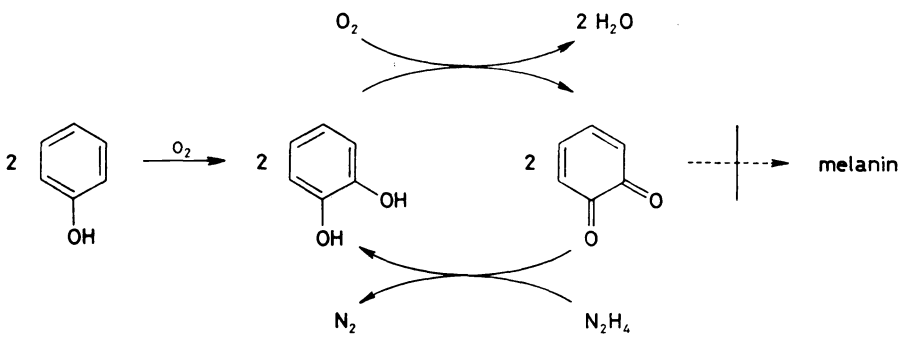


FIG. 1

Plot of quantity of mushroom phenol oxidase used for preparation of membrane (abscissa) versus apparent activity of enzyme membrane (ordinate). The activity of both the soluble enzyme and of enzyme membranes was measured at 30°C in 0·1M phosphate buffer at pH 7 in presence of 0·01M phenol. The activity is expressed in kat (1) and in relative % (2)

of the phenol activity inside the membrane is latent as a result of substrate transfer limitation by diffusion, even in case of substrate excess. The membranes loaded with a large enzyme content therefore show a higher operational viability yet are less sensitive to inhibitors. The slices of champignon tissue were used in this work as an adequate substitute of the membrane with low phenol oxidase content.

Limited lifetime is an inherent property of phenol oxidase reaction membranes. This phenomenon has been ascribed to inactivation of the enzyme by the reactive *o*-quinoid oxidation product of phenol whose spontaneous polymerization is also paralleled by a rapid darkening of the reaction membrane[1]. We determined empirically that sodium benzoate as reversible phenol oxidase inhibitor will stabilize both the dry membranes and also membranes immersed in a buffered benzoic acid solution for at least a year. We also made an effort to suppress the unfavorable influence of quinones by reducing agents. Ascorbate and ferrocyanide, used for quinone reduction during photometric phenol oxidase determination[14] were unsuitable: the first compound yields a current response by itself and the oxidation product of the second compound, i.e. ferricyanide, inhibited phenol oxidase; this resulted in nonlinear calibration curves for the phenolic substrate. We obtained positive results only when hydrazine was used: a several times higher increase of the sensitivity of the enzyme electrode was observed in its presence. We explain this phenomenon by postulating a chemical recycling of substrate in the reaction layer of the electrode (cf. scheme) paralleled by the liberation of nitrogen gas[15]. We were able to show in volumetric experiments with a model mixture of *p*-benzoquinone and hydrazine that both compounds react with each other in a few seconds in a stoichiometric ratio of 2 : 1 (2 mmol of benzoquinone liberated 22·5 ml (1 mmol) of nitrogen). Hydroxylamine reacted slower than hydrazine (see Scheme 1).



SCHEME 1

The highest increase in electrode sensitivity was observed when 3 – 5 mM hydrazine was used; the amplification degree (chemical gaining) was dependent both on the

substrate type and also on the quantity of the enzyme contained in the membrane (Table I); the effect of pH over the range 4–7 was not pronounced. Hydrazine markedly prevented the phenol oxidase membranes from blackening yet they became more fragile and their operational stability was lower compared to control membranes, contrary to our expectation. Hydrazine most likely reacts also with bound glutaraldehyde. It should be also noted that the addition of hydrazine was without effect on the sensitivity of the enzyme electrodes based on other immobilized cuproteins, such as ascorbate oxidase[16] and ceruloplasmin[17].

Quantification of Inhibiting Compounds

Mushroom phenol oxidase is inhibited by certain aromatic acids and thio compounds. The phenol-sensitive electrode is thus sensitive to these compounds and can therefore be used for their quantification: when the biosensor is placed in thermostated and stirred buffer containing a suitable quantity of phenol then the electrode current of oxygen reduction, corresponding to accordingly decreased pO_2 in the biocatalytic membrane, will again increase after the addition of the inhibitor since oxygen is supplied via diffusion from the bulk of the ambient phenol solution.

As shown in Fig. 2 the electrode current levels off practically at the same value no matter whether the given inhibitor quantity was added after the addition of phenol or together with phenol from the very beginning. The plot of electrode current change at steady state (Δi) versus inhibitor concentration (c_i) was hyperbolic (Fig. 3*a*). For purposes of calibration the curve can be linearized in the usual manner, e.g. according to Lineweaver and Burk by the double reciprocal plot of $1/\Delta i$ versus

TABLE I

Sensitivity increase of phenol oxidase pO_2 electrode with biocatalytic membrane of various composition. The mean value of the amplification factor was calculated from three successive electrode responses for phenol and catechol measured in the absence and presence of 5 mM hydrazine

Composition of enzyme membrane			Amplification factor for	
phenol oxidase nkat cm^{-2}	serum albumin mg cm^{-2}	glutaraldehyde mg cm^{-2}	phenol	catechol
0·95	0·20	0·08	3·6	2·5
0·95	0·50	0·20	1·5	1·5
1·90	0·20	0·08	3·0	5·0
1·90	0·50	0·20	1·6	2·0

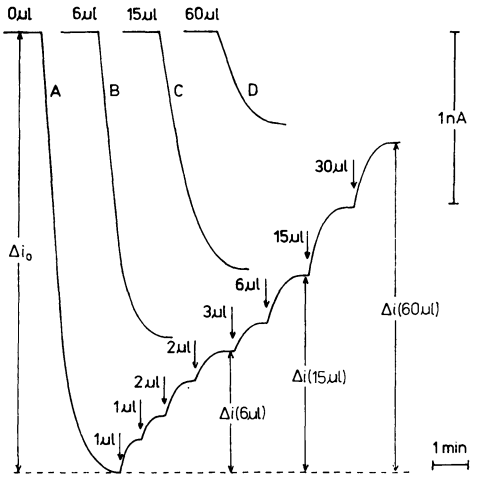


FIG. 2

Typical profile of decrease of sensitivity of phenol oxidase membrane electrode as a result of action of benzoate. The measurement of the current of cathodic oxygen reduction was started always by the addition of 12 μl of $5 . 10^{-2}$M phenol (final concentration 0·2 mmol l^{-1}) either in absence of benzoate (A) or in presence of various quantities of benzoate (B—D) in 3 ml of 0·1M phosphate at pH 7. The volume of the 0·3M benzoate solution added during experiment is designated by arrows

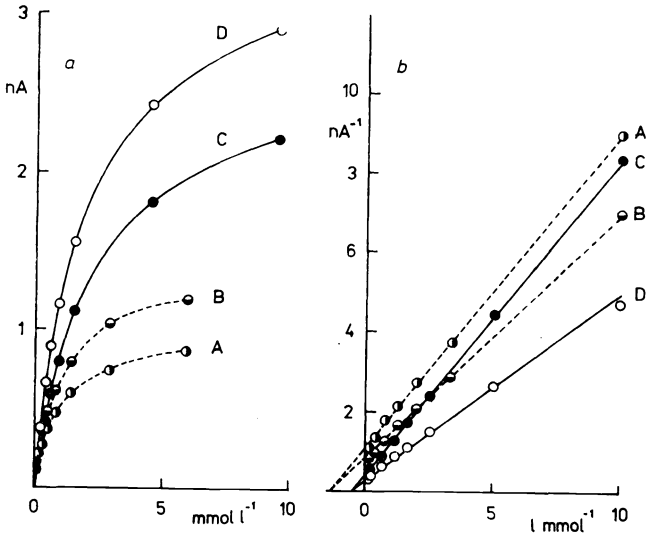


FIG. 3

Calibration curves for two aromatic acids at different starting levels of oxygen in phenol oxidase membrane. The different starting levels of oxygen were adjusted by the addition of an appropriate volume of 0·05M phenol. The following saturation values (in relative % with respect to the reaction mixture saturated with air at 30°C) were achieved: 69% (A), 63% (B), 40% (C), and 21% (D). The reaction layer of the electrode consisted either of crosslinked phenol oxidase (full line) or of a tissue slice of champignon fruit-body (dashed line). *a* Relation of steady-state electrode response versus 4-aminobenzoate concentration (A, B) or benzoate concentration (C, D); *b* linearization of curves by double reciprocal plot of data

$1/c_i$, or according to Woolf–Augustinsson and Hofstee, i.e. by plotting Δi versus $\Delta i/c_i$ since the change in current measured is proportional to the rate of the enzymatic substrate oxidation. The slope of the calibration graph depends on the starting oxygen level in the reaction membrane (i.e. on the concentration of phenol used), as shown in Fig. 3. A considerable role plays also the enzyme content in the membrane. The higher the latent activity of the membrane the less is the membrane sensitive to the inhibitor (in accordance with Fig. 1). As a result of the successive decrease of the activity of the electrode membrane the measuring system has to be calibrated for each individual experiment by the method of internal standard. The relative slope values measured by the electrode with the champignon slice were, e.g. in three successive calibration experiments with 4-aminobenzoic acid 100, 77 and 72%. It is not necessary to test the whole series of standard concentrations in order to construct the calibration curve and mostly two points only are sufficient: the first marginal point $(1/\Delta i_0)$, corresponding to zero inhibitor concentration is known from the beginning of the analysis – it represents the reciprocal current

TABLE II

Some inhibitory compounds measurable amperometrically with phenol oxidase membrane electrode. The solutions of aromatic acids were neutralized with sodium hydroxide

Analyzed inhibitor	Approximate linear range[a] mol l^{-1}
3-Aminobenzoic acid	$1 . 10^{-5}$—$3 . 10^{-3}$
4-Aminobenzoic acid	$2 . 10^{-5}$—$3 . 10^{-3}$
	$3 . 10^{-5}$—$6 . 10^{-3}$[b]
4-Aminosalicylic acid	$2 . 10^{-4}$—$1 . 10^{-2}$
Benzoic acid	$1 . 10^{-5}$—$6 . 10^{-3}$
	$5 . 10^{-6}$—$7 . 10^{-3}$[b]
Nicotinic acid	$5 . 10^{-5}$—$2 . 10^{-2}$
Salicylic acid	$1 . 10^{-4}$—$1 . 10^{-2}$
	$6 . 10^{-5}$—$9 . 10^{-3}$[b]
1-Naphthol	$1 . 10^{-2}$—$5 . 10^{-2}$
4-Nitrophenol	$2 . 10^{-3}$—$5 . 10^{-2}$
Cysteine[c]	$5 . 10^{-6}$—$5 . 10^{-4}$
Glutathione reduced[c]	$1 . 10^{-5}$—$1 . 10^{-3}$
Phenylthiourea[c]	$3 . 10^{-8}$—$5 . 10^{-6}$
Thioacetamide[c]	$2 . 10^{-5}$—$1 . 10^{-3}$

[a] Double reciprocal plot; [b] electrode with mushroom fruit-body slice; [c] partially irreversible inhibition.

value of the starting oxygen concentration after the addition of the corresponding quantity of phenol; the second marginal point is obtained after the first inhibitor addition. The addition of the analyzed sample to the same reaction medium may follow afterwards. The values of 0·2 mM 4-aminobenzoic acid measured in a series of six experiments varied by 11% on the average.

When reversible inhibitors are measured, which can be washed off the electrode membrane, then the phenol-sensitive electrode can be employed several times for analysis. This is the case, e.g. of certain aromatic acids, 1-naphthol and 4-nitrophenol. Sulfur-containing organic compounds irreversibly inhibit phenol oxidase and the enzyme membrane can be used once only in the extreme case. The preparation of stock membranes, however, is simple and the replacement of the inactive membrane by a new one is the matter of several minutes.

A survey of analytically relevant inhibitors together with the corresponding concentration range is given in Table II; compounds important as preservatives, vitamins and pharmaceuticals are included. Some of the other acids chosen at random, such as, e.g. o-phthalic acid, mandelic and oxalic acid were electrode inactive. Of the thiocompounds were tested L-cysteine, reduced glutathione, thioacetamide, in orienting experiments also 2-mercaptoethanol, 2,3-dimercaptopropanol and thiourea. Oxidized glutathione and cystine were inactive. Phenylthiourea, from which certain drugs such as, the antileprosy drug thiambutosin are derived, was the strongest irreversible inhibitor (Table II). In view of the low specificity of the inhibition we can conclude that it is necessary to know the inhibitor kind before its concentration can be measured by a phenol-sensitive electrode.

I wish to acknowledge the skillful technical assistance of Miss J. Haubrová.

REFERENCES

1. Macholán L., Schánĕl L.: Collect. Czech. Chem. Commun. *42*, 3667 (1977).
2. Schiller J. G., Liu C. C.: Biotechnol. Bioeng. *18*, 1405 (1976).
3. Pfeiffer D., Risinger L., Wollenberger U., Scheller F., Johansson G.: Presented at *5th Internat. Symp. Dec. 4—8. 1988, Berlin.*
4. Macholán L., Londýn P., Fischer J.: Collect. Czech. Chem. Commun. *46*, 2871 (1981).
5. Macholán L.: Collect. Czech. Chem. Commun. *44*, 3033 (1979).
6. Kyzlink J., Macholán L.: Vodní hospodářství, B *9*, 240 (1979).
7. Hall G. F., Best D. J., Turner A. P. F.: Anal. Chim. Acta *213*, 113 (1988).
8. Macholán L., Schánĕl L.: Biologia (Bratislava) *39*, 1191 (1984).
9. Schubert F., Wollenberger U., Scheller F.: Biotechnol. Lett. *5*, 239 (1983).
10. Sidwell J. S., Rechnitz G. A.: Biotechnol. Lett. *7*, 419 (1985).
11. Macholán L., Boháčková I.: Biologia (Bratislava) *43*, 1121 (1988).
12. Kertesz D., Zito R.: Biochim. Biophys. Acta *96*, 447 (1965).
13. Broun G., Thomas D., Gellf G., Domurado D., Berjonneau A. M., Guillon C.: Biotechnol. Bioeng. *15*, 359 (1973).
14. Sussman A. S.: Arch. Biochem. Biophys. *95*, 407 (1961).

15. Houben–Weyl in: *Methoden der Organischen Chemie*, Vol. 7 (3a), 4. ed. *Chinone* I (E. Müller, Ed.), p. 650. G. Thieme, Stuttgart 1977.
16. Posádka P., Macholán L.: Collect. Czech. Chem. Commun. *44*, 3395 (1979).
17. Macholán L., Jílek M.: Collect. Czech. Chem. Commun. *49*, 752 (1984).

Translated by V. Kostka.