Kinetics of glucose oxidase catalyzed electron transfer mediated by sulfur and selenium compounds

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Unusually high electron transfer rates in *Aspergillus niger* glucose oxidase catalyzed oxidation of glucose using 5,6:11,12-Bis(dithio)tetracene (TTT), 1,2-dimethyltetraselenafulvalene (DMTSF) and tetrathiafulvalene (TTF) were observed. At pH 7.0 oxidation rate constants (TN/K_m) in the range from $1.0 \cdot 10^7$ to $8.7 \cdot 10^7$ $M \cdot s^{-1}$ were deduced from experimental data. One of the investigated mediators, DMTSF, has been used for electrocatalytical glucose oxidation on graphite at a potential of 0.3 V vs. a standard calomel electrode (SCE). The prepared bioelectrodes have a sensitivity of 1.3 $\mu A/(cm^2 \cdot mM)$, a pH optimum at 6.5–7.0, and a linear range which covers the relevant range for monitoring physiological levels of glucose. The bioelectrodes are stable for more than one month.

Glucose oxidase; 5,6:11,12-Bis(dithio)tetracene; 1,2-Dimethyltetraselenafulvalene; Tetrathiafulvalene; Electron transfer; Aspergillus niger

1. INTRODUCTION

The electron transfer reaction catalyzed by glucose oxidase has found practical applications in biosensor preparations [1]. Screen-printed [2] and carbon paste [3] glucose biosensors represent successful utilizations of glucose oxidase catalysis. In these biosensors mediators are used to achieve a high electron transfer rate between the enzyme active center and a graphite electrode. The main requirement for mediators is a high electron transfer rate from the reduced glucose oxidase active center to the oxidizing mediator.

Organic salts [4] and organic salt composites such as 7,7,8,8-tetracyanoquinodimethane and *N*-methylphenazine [5] were the first mediators reported to mediate glucose oxidase electron transfer. In 1984 Cass et al. [6] introduced ferrocene as an effective glucose oxidase mediator and later Cardosi and Turner [7] proposed the use of tetrathiafulvalene. In 1983 and 1987 a series of quinones was reported for the investigation of the electron transfer rate dependence on substrate oxidation–reduction potential, and a hyperbolic dependence on the oxidation rate constant and the acceptor single-electron reduction potential was found for *Penicillium vitale* as well as for *Aspergillus niger* glucose oxidase [8,9].

In an attempt to find new glucose oxidase mediators we have studied different organic redox compounds, and unusually high electron transfer rates were observed for some sulfur and selenium containing heterocyclic compounds. The aim of this work was: (i) investi-

gation of the kinetics of glucose oxidation catalyzed by glucose oxidase from *Aspergillus niger* using 5,6:11,12-bis(dithio) tetracene (tetrathiatetracene, TTT), 1,2-dimethyl tetraselenafulvalene (DMTSF) and tetrathiafulvalene (TTF) as mediators; and (ii) preparation of graphite electrodes modified by adsorbed DMTSF and glucose oxidase and investigation of electrocatalytical glucose conversion.

2. EXPERIMENTAL

2.1. Reagents used

Glucose oxidase (GO) from *Aspergillus niger* (EC 1.1.3.4) (Sigma, USA) was used as supplied. The GO contained 91 ± 1% of catalytically active FAD as estimated spectrophotometrically under anaerobic conditions in the presence of 0.1 M of glucose at pH 7.0 in 0.1 M acetate buffer using an extinction coefficient of FAD $1.31 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$ at 459 nm [10].

Tetrathiafulvalene, graphite rods (8 mm diameter) and graphite rods (5.9 mm diameter) were used as supplied by Aldrich, Ringsdorff-Werke GmbH (Germany) and Reachim (Russia), respectively.

5,6:11,12-Bis(dithio)tetracene acetate and 2,6-dimethyl tetraselenafulvalene (DMTSF) were used as crystalline products [11].

Potassium phosphate (monobasic), potassium hydroxide, sodium chloride, sodium hydroxide, sodium acetate (analytical grade reagents) were obtained from Reachim, Russia. Acetonitrile (Reachim, Russia) was additionally purified by distillation from phosphorus pentoxide. Glucose (40% solution) was for medical use (Russia). For the solution deaeration chemically pure argon (Reachim, Russia) was used.

Measurements were carried out in 0.1 M sodium acetate buffer solutions (pH 7.0) containing 0.1 M NaCl.

2.2. Spectrophotometric measurements

Spectrophotometric measurements were carried out at 25 ± 0.1°C, using a spectrophotometer Specord M-40 (Germany). Anaerobic reduction of the TTT was monitored spectrophotometrically by record-

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ing the spectrum change in time or an absorption increase at 680 nm (Fig. 1). The concentration of glucose oxidase in the cell was 1.2 nM whereas the glucose concentration was 0.1 M.

2.3. Spectroelectrochemical measurements

The reduction of cations of TTF and DMTSF was performed in a specially constructed optically transparent cell comprising a three-electrode system; two working graphite electrodes (diameter 8 or 5.9 mm) which were built into opposite cell walls perpendicular to the optical path, a titanium auxiliary electrode located at the bottom of the cell and a self made saturated Ag/AgCl reference electrode. The optical path of the cell was 1.35 cm, the distance between the graphite electrodes was 0.8 cm and the solution volume was 2 ml. The solution in the cell was stirred and deaereated by purging with argon.

Modification of the graphite electrodes was performed by adsorption from a 0.1 ml of a 1 mg/ml solution of TTF or DMTSF in acetone or acetonitrile, respectively. The solvent was allowed to evaporate for 12 h at room temperature.

Electrochemical oxidation of mediators adsorbed on graphite was carried out at 0.4 V and 0.5 V vs. a Ag/AgCl electrode for TTF and DMTSF, respectively, using a polarograph OH-105 (Radelkis, Hungary). The absorption spectra of cations of TTF and DMTSF are presented in Fig. 2. The reductions of the oxidized forms of TTF and DMTSF were monitored by recording the rate of absorbance decrease at 428 nm and 615 nm, respectively, in acetate buffer solutions containing 0.1 M glucose, 1.2 or 2 nM of GO and 5 μM bovine serum albumin to prevent cation adsorption on the electrode surfaces and on the cell walls.

The extinction coefficient of the oxidized TTF ($\varepsilon = (5.8 \pm 1.7)10^3$ $M^{-1} \cdot cm^{-1}$ at 428 nm) was calculated from the electrical charge used to generate cations. From the straight proportionality between electrolysis time and absorbance (Fig. 2), it was concluded that the adsorption of this cation on the electrodes was rather low. Due to strong DMTSF cation adsorption on the electrode surfaces, the extinction coefficient for this cation was not calculated.

2.4. Bioelectrode construction and electrochemical measurements

Bioelectrodes were prepared by glueing a copper wire to one end of the graphite rod (5.9 mm diameter and 50 mm long) by epoxy silver, and polishing the working surface of the electrode with emery paper. The electrodes were then washed with buffer solution, dried in air and the side surfaces were covered with isolating varnish. 20 μl of a saturated solution of DMTSF in acetonitrile was deposited onto the working surface of the electrode and subsequently air-dried. 20 μl of glucose oxidase solution in water (10 mg/ml) was dripped on the modified electrode followed by drying in a refrigerator.

The enzyme electrode current was measured in a thermostatted glass cell (5 ml) using a three-electrode circuit with a platinum plate as auxiliary electrode and a SCE as the reference.

To record the anodic current of glucose oxidation, the enzyme electrode was immersed into the buffer solution and a potential of 0.3 V vs. SCE was applied to the electrode until a constant residual current was established. After this pretreatment a solution of glucose was introduced into the cell and the catalytic current was estimated from the difference between the electrode current in the presence of glucose and the current which was established in a blank buffer solution.

3. RESULTS AND DISCUSSION

3.1. The rate of reduced glucose oxidase oxidation in homogenous media

The TTT cation is a highly hydrophobic compound, but due to positive charge it dissolves slightly in acetate buffer solutions (Fig. 1). Oxidized tetrathiafulvalene and dimethyl tetraselenafulvalene when prepared in situ dissolve in buffer solution, too (Fig. 2). In acetate buffer
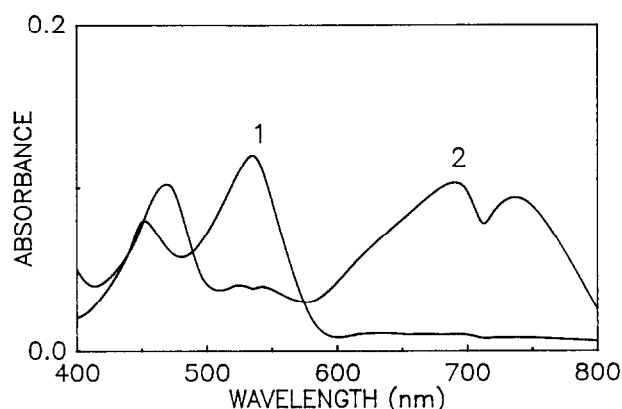

Fig. 1. Absorbance of tetrathiatetracene cation (1) and its reduced product (2) in 0.1 M acetate buffer solution, pH 7.0. Concentrations of tetrathiatetracene cation 9.3 μM (1,2), glucose oxidase 1.2 nM (2) and glucose 0.1 M (2).

solution the cations are rather stable. When glucose oxidase and glucose are introduced into a deaerated acetate solution the cations are reduced.

The initial rate of reduction of the TTF cation, determined as absorbance changes, increases with increasing absorbance of the mediator, whereas in the case of DMTSF and TTT the initial rate of reduction saturates at high cation absorbance (Fig. 3).

The glucose oxidase oxidation constants were calculated following the ping-pong scheme of the enzyme action [8]. At high glucose and low mediator concentration, when the rate of oxidation of the enzyme is lower than the catalytic reaction rate, the cation reduction rate can be calculated using expression of bimolecular reaction rate:

$$A[M^+]/\Delta t = k_{ox}[E][M^+]$$

where [E] and [M$^+$] is the concentration of enzyme and oxidized mediator, respectively and k_{ox} is the oxidation constant which is equal to the turnover number (TN) divided by K_m.

Due to direct proportionality of mediators absorbance (A) and concentration the last equation can be re-written:

$$\Delta A/\Delta t = k_{ox}[E]_o A$$

This equation was used to calculate the TTF cation reduction rate. In Fig. 3 a linear anamorphosis–absorbance change, initial mediator absorbance is shown. The calculated oxidation rate constant was (1.3 ± 0.3) 10^7 $M^{-1} \cdot s^{-1}$.

Since the initial rate of the DMTSF cation reduction depends little on the initial mediator concentration (Fig. 3) the oxidation constant was calculated from an integral kinetic curve approximating the data of three kinetic curves according to [12]. For these calculations
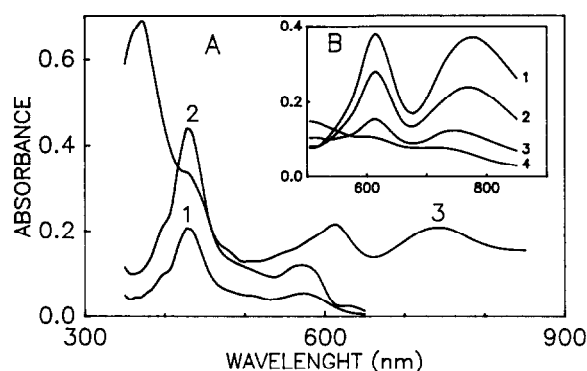
Fig. 2. (A) Absorbance of the cation of tetrathiafulvalene after 7 min (1) and 14 min (2) of electrolysis at an electrode potential 0.4 V vs. Ag/AgCl and of the cation of dimethyl tetraselenafulvalene (3) after 5 min of electrolysis at 0.5 V vs. Ag/AgCl. (B) Reduction of cation of dimethyl tetraselenafulvalene with glucose oxidase: initially (1), after 0.5 (2), after 1 (3) and after 1.5 (4) min. Enzyme concentration 2 nM, 0.1 M glucose, 0.1 M acetate buffer solution, pH 7.0.

only the data of low mediator concentration were used because the turbidity of the solution increases during the reaction due to insoluble product formation. Possibly, this causes the disappearance of an isosbestic point at 520 nm too (Fig. 2). From calculations it follows that the oxidation rate constant of cations of DMTSF was $(1.0 \pm 0.4) \ 10^7 \ M^{-1} \cdot s^{-1}$.

The reduction of TTT cations is similar to that of DMTSF cations (Fig. 3). The appearance of an isosbestic point at 575 nm (not shown in Fig. 1) at reduction of this cation shows that there is only a single reaction product. The spectrum of this product is comparable to that of TTT in benzene, which shows three absorption bands at 472, 642 and 701 nm [13]. The increase absorbance of the product at 680 nm was employed for rate constant calculation using the integral kinetic curve as described above. At 25°C the calculated rate constant of TTT cation reduction was $(8.7 \pm 1.2) \ 10^7 \ M^{-1} \cdot s^{-1}$.

In non-water solutions TTF, DMTSF and TTT show two reversible one-electron step oxidation–reduction reactions [11,13,14]:

$$M - e \rightarrow M^+$$

$$M^+ - e \rightarrow M^{++}$$
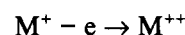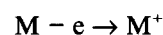
The redox potential of the first step of TTF oxidation in acetonitrile was determined as 0.35 V vs. SCE (0.59 V vs. NHE) [14]. The redox potential of DMTSF oxidation (first step) was close to that of TTF ((0.34 ± 0.05) V vs. SCE [11]). The redox potential of TTT in benzonitrile is 0.22 V vs. Ag/AgCl [13]. The difference between the second and first oxidation step potentials achieves 0.4 V. This indicates that at moderate potentials the single oxidized mediators (cations of mediators) are involved in the mediation.

The mechanism of glucose oxidase catalysis of the oxidation of β-D-glucose and the concomitant reduction of artificial electron acceptors, i.e. quinones, ferricenes etc. [6,8,9,15,16] includes reduction of the enzyme active centre following its oxidation by the cation of mediator:

$$E\text{-}FAD + Glucose \rightarrow E\text{-}FADH_2 + glucono\text{-}\delta\text{-lactone}$$

Since the E-FADH$_2$/E-FAD couples are 2 e and 2 H$^+$ systems, 2 equivalents of cations of mediators should be involved in oxidative process:

$$E\text{-}FADH_2 + 2 \ M^+ \rightarrow E\text{-}FAD + 2 \ M + 2 \ H^+$$
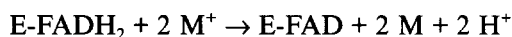
The rate constant of this multistep reaction should be half of k_{ox} if the rates of first and second electron transfer from the reduced enzyme are equal.

As it follows from experimental data the oxidation of reduced glucose oxidase by cations of TTF and DMTSF with glucose oxidase proceeds at a similar rate $(1.3 \cdot 10^7 \ M^{-1} \cdot s^{-1} \text{ and } 1.0 \cdot 10^7 \ M^{-1} \cdot s^{-1}$, respectively). The redox potentials of these mediators are comparable, too. However, the reactivity of TTT cations is near 10 times higher than cations of TTF and DMTSF. Furthermore the redox potential of TTT is about 0.15 V lower than the redox potential of TTF and DMTSF. When these data are compared to data of previously investigated mediators, great differences in reactivity are noticed. The rate constants of investigated mediators are close to positively charged oxidized ferrocenes, i.e. ferrocene methanol, (dimethylaminomethyl) ferrocene, and the promazine dication radical [15]. However, the reactivity of the presently investigated cations are one to two orders of magnitude higher than the reactivity of quinones [8,9,16]. The reactivity of TTT is nearly three orders of magnitude higher than that of
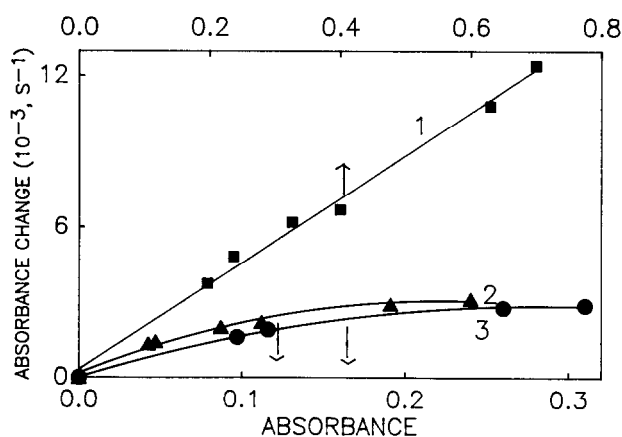

Fig. 3. The dependence of absorbance changes of cations of tetrathiafulvalene (1), tetrathiatetracene (2) and dimethyl tetraselenafulvalene (3) on the initial absorbance of mediators. Glucose concentration 0.1 M (1–3), glucose oxidase 1.2 nM (1,2) and 2 nM (3), 0.1 M acetate buffer solution, pH 7.0.

other ferrocene derivatives in spite of their higher potential [6,15,17,18].

A possible explanation of the high TTT as well as TTF and DMTSF cation reactivities can be accomplished by assuming uncommon self-exchange constants. It is known that the maximum electron transfer rate depends on the reagents (both enzyme and mediator) self-exchange constant values [19]. Unusually high values of these constants for DMTSF, TTF and TTT, may be responsible for the observed large oxidation rate constants. However, it is difficult to explain such large self-exchange constants, which calls for further investigation.

Another explanation could be based on suggesting complexation of the investigated cations with the glucose oxidase active center. The precursor complex formation would increase the electron transfer rate proportional to the complexation constant value [19]. This assumption finds some support from the analysis of the kinetics of TTT and DMTSF cation reduction. For both cations extremely low apparent Michaelis constants were observed (Fig. 3), and their solubilities even in acetate buffer solution were low due to their high hydrophobicities. This makes a complexation of the cations with the glucose oxidase active center plausible. The possibility of complex formation of glucose oxidase with oxidized ferrocenes was also hypothesized in a very recent publication by Bourdillon et al.; a histidine residue or thiol group located in close vicinity of the flavin would be a likely candidate for the binding site [15].

3.2. Electrocatalytical glucose oxidation on DMTSF-modified graphite electrodes

To show that the investigated mediators are useful for electrochemical glucose oxidation, one of the most effective mediators (DMTSF) was adsorbed together with glucose oxidase on a graphite electrode. After adding 10 mM glucose the highest electrocatalytical current was

Fig. 5. The dependence of the bioelectrode current on solution pH. Buffer solutions used: acetate (1), phosphate (2), Tris-HCl (3); glucose concentration 10 mM (1–3), electrode potential 0.3 V vs. SCE.

found when a potential of 0.3 V vs. SCE was applied to the electrode. The sensitivity of the electrode at this potential was 1.3 $\mu A/(cm^2 \cdot mM)$. A linear relationship between electrode current and glucose concentration ($R = 0.9985$, slope 1.38 $\mu A/(cm^2 \cdot mM)$ and intercept $-0.98 \mu A$) was observed in the physiologically relevant range from 1 to 22 mM (Fig. 4). When bubbling the solution with argon the electrocatalytical current increased about 30% (Fig. 4). Increasing the temperature from 17 to 40°C also resulted in an increase in current. An activation energy was calculated for this temperature dependence using the Arrhenius equation and found to be 36.2 kJ/mol. The enzyme electrode, modified with DMTSF, had a pH optimum in the range of 6.5–7.0 (Fig. 5). Lowering or increasing the pH resulted in a decrease of the biocatalytic current. The electrode current also depends on the buffer composition, as would be expected from the differences of cation solubilities and reactivities in different buffer solutions [1].

It should be noticed that the bioelectride response was rather stable over a period of one month when it was kept in a refrigerator in buffer solution. In this period the sensitivity of bioelectrode decreased by about 10% as determined by periodical operation at room temperature using 10 mM glucose.

In summary, it has been demonstrated that some aromatic sulfur and selenium compounds exhibit exceedingly high electron transfer rates in glucose oxidation catalyzed by glucose oxidase. The new mediators discovered can be used for electrocatalytical glucose oxidation on graphite and they may find broad application in connection with other oxidoreductases, providing a range of useful bioelectrodes.
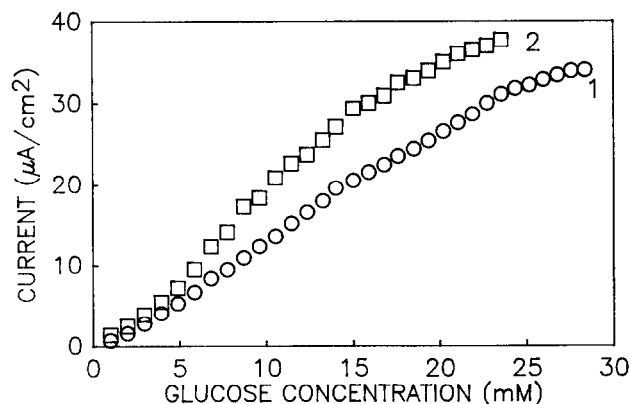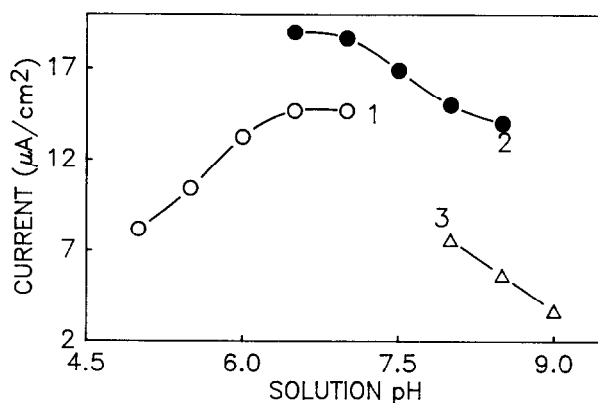

Fig. 4. The dependence of the bioelectrode current on the glucose concentration under aerobic (1) and anaerobic (2) solutions. Electrode potential 0.3 V vs. SCE, 0.1 M acetate buffer solution, pH 7.0.

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