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Electrocatalytic oxidation of *L*-lactate by *Saccharomyces cerevisiae* flavocytochrome b_2 on redox mediator-modified glassy carbon electrodes

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In order to expand the variety of redox mediator-modified electrodes potentially used for the determination of *L*-lactate, in this work we investigated the reactions of *Saccharomyces cerevisiae* flavocytochrome b_2 (fcb_2) with quinones and its electrocatalytic reactions on glassy carbon electrodes, modified either by their electrochemical pretreatment, or by the products of the electrochemical reduction of dinitrobenzenes. The obtained electrochemically reversible surface redox groups were characterised by a midpoint potential of 0.06–0.10 V (vs Ag/AgCl, pH 7.0) and an electron transfer rate constant of 0.62–0.72 s^{-1}. In the presence of surface-entrapped fcb_2, the electrocatalytic oxidation of *L*-lactate started at ca. 0.10 V, and was characterised by a current density of 4.5–18 μA/cm^2 at 0.20 V. The linear part of the response was observed up to 0.3–0.4 mM *L*-lactate. However, the prepared electrodes were less stable than those using fcb_2 of other origin, most likely due to the modification of specific cysteine groups in the enzyme active center.

Keywords: flavocytochrome b_2, *L*-lactate, quinone, electrode

INTRODUCTION

L-lactate detection plays a significant role in healthcare and food industries. For that purpose, electrochemical, usually amperometric, biosensors based on various lactate oxidising enzymes are often used [1–3]. Among them, lactate electrodes based on flavocytochrome b_2 (*L*-lactate: cytochrome *c* reductase, fcb_2, EC 1.1.2.3) have attracted some interest. This enzyme consists of four 58 kD subunits which contain protoheme IX at the *N*-terminus and flavinmononucleotide (FMN) at the *C*-terminus [4, 5]. During the catalysis, *L*-lactate transfers two redox equivalents to FMN [6]. The maximal rates of the reaction on the two-electron basis are close to 200 s^{-1} (*Saccharomyces cerevisiae* fcb_2) or above 900 s^{-1} (*Hansenula anomala* fcb_2 [7–9]) at pH 7.0 and 25°C. Further, the electron is transferred from reduced FMN to heme with a rate of 1500 s^{-1} (on the one-electron basis) [8]. After the reoxidation of heme by cytochrome *c*, the second electron from FMN semiquinone ($FMN^{-}\cdot$) is transferred at a slower rate, 120 s^{-1} (*S. cerevisiae* fcb_2 [8]). One may note that cytochrome *c*, the physiological oxidant of fcb_2, may accept the electrons from heme only. However, the nonphysiological electron acceptors such as ferricyanide and quinones may accept electrons both from the reduced heme and the reduced forms of FMN [10].

For the development of biosensors, the efficient electrooxidation of reduced fcb_2 is required. Chemically modified electrodes with immobilised redox mediators were mostly used for that purpose: conductive *N*-methylphenazium salts (organic metals) [11], surface quinoidal groups on carbon black [12], cytochrome *c* [13], and

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ferricene derivative [14]. Electrodes containing yeast cells dispersed in carbon paste, using a soluble mediator [15], or entrapped into redox polymer, [16] worked on a similar principle. Direct (unmediated) electron transfer was observed only between fcb_2 and a nanoclustered Au electrode [17], and between a fcb_2-cytochrome *c* fusion protein and a nanotube-modified graphite electrode [18]. The obtained bioelectrocatalytic systems differed in their overvoltage, stability and range of measured *L*-lactate concentrations.

In order to expand the variety of mediator-modified electrodes potentially used for the determination of *L*-lactate, in this work we investigated the electrocatalytic reactions of *S. cerevisiae* fcb_2 on glassy carbon electrodes, modified either by their electrochemical pretreatment [19], or by the products of electrochemical reduction of dinitrobenzenes [20, 21].

MATERIALS AND METHODS

Recombinant *S. cerevisiae* flavocytochrome b_2 was a generous gift of Dr. Florence Lederer (Institut de Chimie Physique, Universite Paris-Saclay, France). It was obtained as described previously [9] and stored at –80°C in 0.1 M K-phosphate (pH 7.0, 1 mM EDTA) in the reduced state in the presence of 10 mM *L*-lactate. The concentration of the reduced form of fcb_2 was determined using ε_{423} = 183 mM^{-1} cm^{-1}. Further, all the experiments were performed in 0.1 M K-phosphate (pH 7.0, 1 mM EDTA) at 25°C. The enzyme maximal turnover rate, expressed as moles ferricyanide reduced per mole heme per second and determined at saturating concentrations of *L*-lactate and ferricyanide, 10.0 and 1.0 mM, respectively ($\Delta\varepsilon_{420}$ = 1.04 mM^{-1} cm^{-1}), was equal to 265 s^{-1}. *L*-lactate (Li-salt), ferricyanide and other compounds were obtained from Sigma-Aldrich.

The kinetics of the reduction of quinones by fcb_2 was measured by the rate of consumption of O_2 during the reaction in the presence of 10 mM *L*-lactate, fcb_2, and various quinone concentrations, using a Rank Brothers Ltd. (USA) oxygen electrode. It was assumed that at 25°C, $[O_2]$ = 250 μM. The bimolecular reduction rate constants (k_{cat}/K_m) were calculated using Wolfram Mathematica-10.

Electrochemical studies were performed using a Parstat 2273 (Princeton Applied Research) potentiostat. A rod of glassy carbon (Sigradur G, surface area 5.0 mm^2), sealed in PTFE tubing served as a working electrode. A saturated Ag/AgCl electrode (+0.22 V vs NHE) was used as a reference electrode. The potential values in this text are presented with respect to this electrode, unless stated otherwise. A platinum electrode (surface area 56 mm^2) was used as an auxiliary electrode. The anaerobic conditions were achieved by bubbling the working solution with Ar for 15 min.

The glassy carbon electrode was polished with silicon carbide abrasive and rinsed in a distilled water. Further, it was subjected to electrochemical pretreatment as described earlier [19]: after the establishment of anaerobiosis, the potential was swept for 6 cycles from 1.8 to –0.8 V at a rate of 10 mV/s, and, subsequently, it was swept for 10 cycles from 1.0 to –1.1 V at sweep rates of 500, 100 and 10 mV/s, keeping the electrode at 1.0 V for 1 min before each cycle. Alternatively, the working electrode was modified by the products of the electrochemical reduction of *o*- or *p*-dinitrobenzene [20, 21]. For this purpose, 1.0 mM of each dinitrobenzene in a CH_3CN stock solution (factor of dilution, 100) was introduced into an anaerobic working solution, and the electrode was subjected to 10 potential scans from 0.6 to –0.8 V at a rate of 10 mV/s without the stirring of solution.

Enzyme electrodes were prepared by applying a 10 μl fcb_2 solution (concentration, 90 μM) to the surface of modified electrodes covered with a nylon-6 grid (thickness 100 μm, 760 mesh). The electrode was then covered with a dialysis membrane (thickness 55 μm) held by a rubber ring.

RESULTS AND DISCUSSION

In addition to cytochrome *c* and ferricyanide, *H. anomala* fcb_2 reduces quinones and nitroaromatic compounds [10, 22], the former of which can be used as mediators in fcb_2-based *L*-lactate amperometric biosensors [12, 15]. The reduction proceeds in a one-electron way, with the formation of their radicals, which are rapidly reoxidised by O_2 [22]. In this way, the reaction rate can be monitored according to the O_2 consumption. In this work, we investigated the quinone reductase reactions of *S. cerevisiae* fcb_2. It should be noted that at high concentrations of partly substituted quinones, >100 μM, a decrease in the reaction

rate was observed, so that close to the maximum reaction rates were not reached. Therefore, only the bimolecular reduction rate constants (k_{cat}/K_m, Table) were calculated based on the linear dependence of the reaction rate on the quinone concentration. A linear dependence of log k_{cat}/K_m on the single-electron reduction potential (E^1_7) of quinone is observed (data not shown), described by Eq. (1):

$$\log k_{cat}/K_m = (7.14 \pm 0.23) + (12.64 \pm 1.49)\, E^1_7\,(\mathrm{V, vs. NHE}),$$

$$r^2 = 0.9352. \qquad (1)$$

This suggests that the fcb_2 reaction can proceed according to an 'outer-sphere' electron transfer model [10], being little influenced by the structural features of the quinone. An analogous regularity is also characteristic of the reactivity of *H. anomala* fcb_2, and in both cases, the k_{cat}/K_m of quinones is quite close [22]. This shows that quinones would be suitable redox mediators for the electrochemical oxidation of *S. cerevisiae* fcb_2.

As in the case of *H. anomala* fcb_2 [12], using an unmodified glassy carbon electrode coated with the *S. cerevisiae* enzyme, no catalytic oxidation current was observed at a working potential of 0.0–0.4 V when *L*-lactate was introduced into the medium. This suggests that fcb_2 is electrochemically inactive on these electrodes, so mediator-modified electrodes were further used.

After the electrochemical pretreatment of a GC electrode [19], its cyclic voltammogram shows reversible redox peaks centred at 0.08 V (vs Ag/AgCl, pH 7.0) (Fig. 1). The electron transfer rate constant (k_s) of the electrogenerated quinone/hydroquinone redox pair, determined by the method of Laviron [23], is equal to 0.7 s^{-1}. The pH dependence

Table. **Single-electron reduction potentials of quinones (E^1_7) and their bimolecular reduction rate constants (k_{cat}/K_m) by fcb_2 of *Saccharomyces cerevisiae***

No.	Compound	E^1_7, V vs NHE	k_{cat}/K_m, $M^{-1}s^{-1}$ [a]
1	2,5-Dimethyl-1,4-benzoquinone	–0.08	2.3×10^6
2	5-Hydroxy-1,4-naphthoquinone	–0.09	8.9×10^5
3	5,8-Dihydroxy-1,4-naphthoquinone	–0.11	2.4×10^5
4	9,10-Phenanthrene quinone	–0.12	6.9×10^5
5	1,4-Naphthoquinone	–0.15	1.9×10^5
6	2-Methyl-1,4-naphthoquinone	–0.20	2.8×10^4
7	Tetramethyl-1,4-benzoquinone	–0.26	9.3×10^3

[a] Standard error of determination, ≤10%.

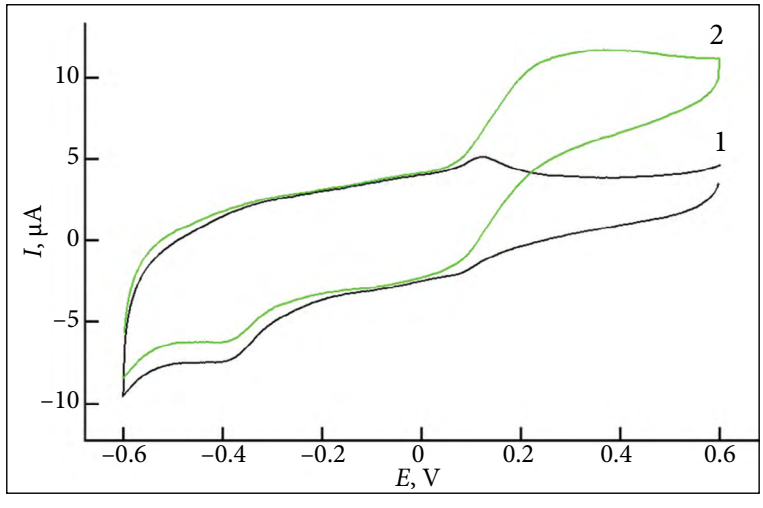

Fig. 1. Cyclic voltammograms of the electrochemically pretreated glassy carbon electrode in the absence (1) and in the presence of 1.0 mM L-lactate (2), $v = 10$ mV/s

of the redox peaks corresponds to the $2e^-$, $2H^+$ transfer at pH 5.5–9.0. In the presence of 30–100 μM fcb_2 entrapped on the electrode surface, *L*-lactate oxidation started at 0.05 V (Fig. 1). The linear part of the response was observed up to 0.4 mM *L*-lactate, and the maximum catalytic current obtained at 0.2 V was 4.5 μA/cm².

Glassy carbon electrodes can also be modified with the products of the reduction and reoxidation of nitroaromatic compounds ($ArNO_2$), most likely polymers containing azoxy (Ar-N=N(→O)-Ar) groups [20, 21]. We modified the electrodes by the potential scanning in the presence of 1.0 mM *p*- or *o*-dinitrobenzene. This resulted in the formation of stable surface electroactive compounds with redox peaks centred at 0.06 V (*p*-dinitrobenzene) and 0.1 V (*o*-dinitrobenzene) with k_s values of 0.62 and 0.72 s^{-1}, respectively. Like in the case of electrochemically pretreated GC, the pH dependence of the redox peaks was characterised by a slope of the –0.06 V/pH unit. In the presence of entrapped fcb_2, in both cases *L*-lactate oxidation started at 0.1 V (Figs. 2, 3), the linear part of the response reached 0.3–0.4 mM *L*-lactate, and the maximal catalytic current at 0.2 V was 12–18 μA/cm².

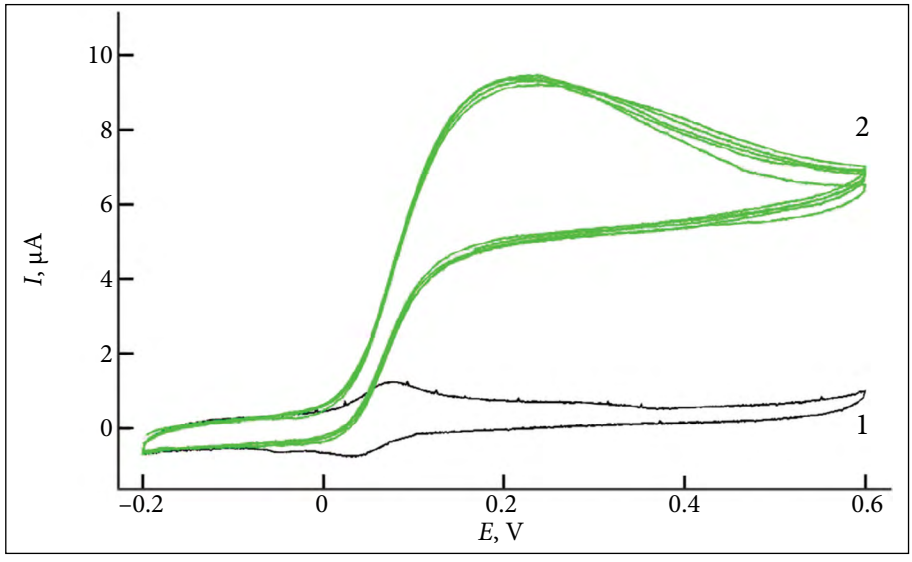

Fig. 2. Cyclic voltammograms of the electrode modified by the products of electroreduction/reoxidation of *p*-dinitrobenzene in the absence (1) and in the presence of 1.0 mM *L*-lactate (2), $v = 10$ mV/s

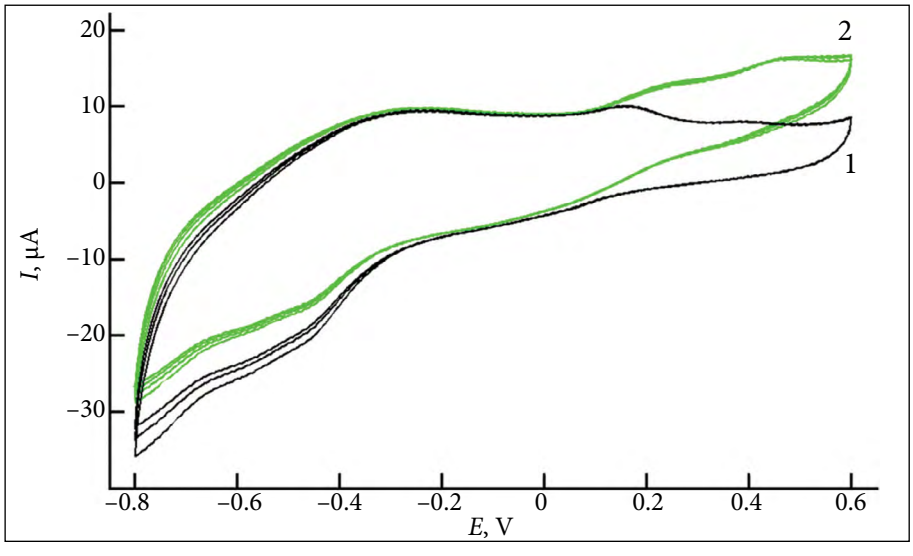

Fig. 3. Cyclic voltammograms of the electrode modified by the products of electroreduction/reoxidation of o-dinitrobenzene in the absence (1) and in the presence (2) of 1.0 mM L-lactate, $v = 10$ mV/s

Although those electrodes were good in terms of their catalytic currents and linear part of the response, they were not sufficiently stable. Their activity dropped by 50% after 24 h storage at pH 7.0 and 4°C. In comparison, previously studied electrodes based on fcb_2 from *Hansenula anomala* and carbon black [12], and fcb_2 from *Ogataea polymorpha* and gold nanoclusters [17] were at least one order more stable. The reason for the rapid inactivation may be the reactions of Cys200,216,233 with quinones and other electrophiles at the electrode surface. These cysteines are at a 7–10 Å distance from the FMN isoalloxazine ring, and are not conserved in fcb_2 from *H. anomala* [4, 5].

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ELEKTROKATALITINĖ *L*-LAKTATO OKSIDACIJA *SACCHAROMYCES CEREVISIAE* FLAVOCITOCHROMU b_2 ANT REDOKSO MEDIATORIAIS MODIFIKUOTŲ STIKLO ANGLIES ELEKTRODŲ

S a n t r a u k a

Siekiant išplėsti redokso mediatoriumi modifikuotų elektrodų, galimai naudojamų *L*-laktatui nustatyti, įvairovę, šiame darbe ištyrėme *Saccharomyces cerevisiae* flavocitochromo b_2 (fcb_2) reakcijas su chinonais ir jo elektrokatalitines reakcijas ant stiklo anglies elektrodų, elektrochemiškai apdorotų arba modifikuotų dinitrobenzenų elektrocheminės redukcijos produktais. Gautoms elektrochemiškai grįžtamoms paviršiaus redokso grupėms buvo charakteringas 0,06–0,10 V standartinis potencialas (Ag/AgCl atžvilgiu, pH 7,0) ir elektronų pernešimo greičio konstantos, lygios 0,62–0,72 s^{-1}. Esant paviršiuje imobilizuotam fcb_2, elektrokatalitinė *L*-laktato oksidacija prasidėjo maždaug nuo 0,10 V, o srovės tankis buvo 4,5–18 μA/cm^2 esant 0,20 V potencialui. Linijinė atsako dalis buvo stebima iki 0,3–0,4 mM *L*-laktato. Tačiau paruošti elektrodai buvo mažiau stabilūs nei naudojant kitos kilmės fcb_2, greičiausiai dėl specifinių cisteino grupių modifikacijos fermento aktyviajame centre.