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Direct electrochemistry of heme multicofactor-containing enzymes on alkanethiol-modified gold electrodes

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Abstract

Direct electrochemistry of heme multicofactor-containing enzymes, e.g., microbial theophylline oxidase (ThOx) and D-fructose dehydrogenase (FDH) from *Gluconobacter industrius* was studied on alkanethiol-modified gold electrodes and was compared with that of some previously studied complex heme enzymes, specifically, cellobiose dehydrogenase (CDH) and sulphite oxidase (SOx). The formal redox potentials for enzymes in direct electronic communication varied for ThOx from −112 to −101 mV (vs. Ag|AgCl), at pH 7.0, and for FDH from −158 to −89 mV, at pH 5.0 and pH 4.0, respectively, on differently charged alkanethiol layers. Direct and mediated by cytochrome *c* electrochemistry of FDH correlated with the existence of two active centres in the protein structure, i.e., the heme and the pyrroloquinoline quinone (PQQ) prosthetic groups. The effect of the alkanethiols of different polarity and charge on the surface properties of the gold electrodes necessary for adsorption and orientation of ThOx, FDH, CDH and SOx, favourable for the efficient electrode–enzyme electron transfer reaction, is discussed.

Keywords: Theophylline oxidase; D-fructose dehydrogenase; Heme; Direct electron transfer; Alkanethiol self-assembled monolayers

1. Introduction

One of the intriguing phenomena known for the last few decades is bioelectrocatalysis, with the particular case of direct electron transfer (DET) between an electrode material and redox active proteins or protein clusters. The very first reports on DET with a redox active protein were published already in 1977 when Eddowes and Hill [1] and Yeh and Kuwana [2] independently showed that cytochrome *c* (cyt *c*) on bipyridyl-modified gold and tin-doped indium oxide electrodes, respectively, showed virtually reversible electrochemistry, as revealed by cyclic voltammetry (CV). After that, DET was established for peroxidases [3–5], laccases [6], ferredoxins [7,8], thus contributing essentially to the understanding ET mechanisms in these systems. Later, DET reactions of complex, multicofactor-containing enzymes and whole cell complexes were probed [7–15]. In parallel to a successful DET-based bioelectrocatalytic function of complex redox enzymes, DET for a number of enzymes at electrodes was shown to depend crucially on the "electrode environment". Thus, it was not attained or accompanied by a loss of enzymatic activity due to enzyme denaturation at the electrode surfaces, by nonspecific and unstable binding to the electrode, by random surface orientation or by impeded internal ET between the multiple redox sites present in the enzyme, in addition to the electrode–enzyme DET reaction.

Mimicking the biological environment of enzymes by the modified electrodes is then of particular interest to achieve DET between the electrode and the enzyme; knowledge of the redox potentials for the redox active sites present in the enzyme contributes essentially to the evaluation of the enzyme function and mechanism [10,11,15]. To provide a

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specific microenvironment at the electrode surface, self-assembled monolayers (SAMs) of synthetic terminally functionalised alkanethiols can be used [16–18]. The ability to manipulate the properties of the interface via the chemistry of the SAMs of alkanethiols attached onto the gold electrode surface is unique and unprecedented; these interfaces can be considered as ideal physiological membrane–mimetic systems for studies of the bioelectrochemical function of complex membrane and intermembrane redox enzymes [10,11,15,18,19]. Therewith, the electrostatic and hydrophilic/hydrophobic interactions between the enzyme and the modified electrode surface can be easily controlled by a proper choice of alkanethiols bearing head groups of different charge and polarity. As a result, a successful simulation of the molecular surfaces of the biological partners of the enzyme by the SAM of alkanethiols may be achieved, which in turn provides the necessary amount/orientation of the enzyme molecules for DET reaction with the electrode, as well as a conformation appropriate for its efficient bioelectrocatalytic function.

In this work, the DET reactions of the heme complex–cofactor-containing enzymes, specifically, microbial theophylline oxidase (ThOx) and D-fructose dehydrogenase (FDH) from *Gluconobacter industrius*, were studied on alkanethiol-modified gold electrodes to reveal the conditions for the DET reaction between the electrode and the heme active site of these enzymes, which may provide a bioelectrocatalytic function of the enzymes.

ThOx is a complex redox metalloenzyme involved in the metabolic oxidation of theophylline to form 1,3-dimethyluric acid. It requires no oxygen in its catalytic action. The reduced enzyme can then in turn be reoxidized by its natural redox partner cyt *c*. Microbial ThOx was isolated by GDS Technology, in 1988 [20,21]; however, no detailed kinetic characterization of ThOx, as well as no data on the crystal structure of ThOx and the number/nature of redox sites/domains present in the molecule, are still available. As a consequence, ET reactions of ThOx have not yet been studied, and just a few works on the electrochemistry of ThOx were focused on the development of ThOx biosensors for theophylline, based on spectrophotometrical [20–22] and electrochemical detection of theophylline oxidation catalysed by ThOx in the presence of cyt *c* [23] and some ferrocene mediators [24].

Membrane-bound FDH refers to the pyrroloquinoline quinone (PQQ)-dependent dehydrogenases, which transfer electrons from substrates such as fructose to electron acceptors other than oxygen [25,26]. This multicofactor-containing enzyme is able to selectively oxidise D-fructose to 5-keto-D-fructose which is accompanied by the reduction of its PQQ-cofactor to $PQQH_2$ [27,28]. The natural electron acceptors of FDH are believed to be cyt *c* or ubiquinones [29]. However, both artificial $1e^-$- and $2e^-/H^+$-acceptors can be used as electron acceptors as well [28,30]. When purified from the membranes of *G. industrius*, FDH consists of three tightly bound units of 67 kDa (the PQQ-domain), 51 kDa (cyt *c*-type domain) and 20 kDa (peptide of unknown function, presumably "anchoring" the enzyme to the membrane) [28]. The integrity of the enzyme drastically depends upon the "conservation" of the membrane environment, i.e., the presence of detergents and other stabilisers. Due to its biotechnological importance, FDH was studied a lot for biosensor development ([31–34], and references therein), and there are only a few works on the kinetics of the catalysis [30]. The structural characteristics of the enzyme are still not available.

Both enzymes can communicate directly with the electrode surface [9,35,36]. However, despite the pronounced DET-based bioelectrocatalysis with FDH on carbon paste electrodes, no direct electrochemistry of the heme domain or the PQQ-domain of FDH was observed [9]. The achieved DET reactions of FDH adsorbed under controlled potential (0.5 V) on bare Pt and Au were correlated with the redox activity of its PQQ-cofactor [the reported values for the formal redox potentials of FDH were 80 mV (Ag|AgCl) at pH 4.5], but not with a redox transformation of the heme [35]. ThOx demonstrated similar DET-based bioelectrocatalytic activity on graphite, but no DET signal from the redox active sites of ThOx in the absence of the substrate was shown [36]. Spectroelectrochemical titration of ThOx in an aldrithiol-modified gold capillary electrode enabled determination of at least two redox active centres present in ThOx; however, sluggish electrochemistry of ThOx resulted in dispersion of the titration data, which deteriorated the significance of the obtained results [36]. To the best of our knowledge, no direct voltammetric data are still available on the redox transformations of the hemes in these two enzymes.

2. Experimental

2.1. Chemicals and materials

The kit containing stabilised dissolved microbial theophylline oxidase (the activity of 14 U/ml, ThOx) was obtained from STANBIO Laboratory (Boerne, TX, USA, Cat. No 2422) and was used as received. D-Fructose dehydrogenase (D-fructose:[acceptor]5-oxidoreductase; EC 1.1.99.11, FDH) from *G. industrius* was obtained as a lyophilised powder additionally containing salts and stabilizing agents (detergent, antioxidant and sugars) to prevent enzyme inactivation (Sigma, Cat. No F51520; the specific activity of 471 U/mg of protein) and was used without further purification. Removal of detergent facilitated the aggregation of FDH and caused its inactivation [28]. Cytochrome *c* from horse heart, theophylline and D(−) fructose were from Sigma (St. Louis, MO, USA) and were used as received. The buffer components were from Merck (Darmstadt, Germany). 2-Mercaptoethanol (98%, MC_2–OH), cysteamine (MC_2–NH_2) and 6-hydroxy-1-hexanethiol

(97%, MC_6–OH) were from Sigma-Aldrich (UK). 6-Amino-1-hexanthiol (MC_6–NH_2) was from Dojindo Laboratories (Japan). 18.2 MΩ Millipore water was used throughout the work.

2.2. Electrode modification with alkanethiols

Thiol films were prepared by 8 h adsorption from 5 mM solutions of alkanethiols in absolute ethanol. For mixed SAM, 5 mM MC_x–OH and 5 mM MC_x–NH_2 in proportion 3:1 v/v were used if not stated otherwise. Modified electrode surfaces were rinsed thoroughly with water to remove weakly adsorbed molecule.

2.3. Instrumental procedure

All measurements were performed at ambient temperature: 22±1 °C. Spectroscopic measurements were done using an UVIKON 930 spectrophotometer (Kontron Instruments, NorthStar Scientific, Leeds, UK). Cyclic voltammetry (CV) and differential pulse voltammetry (DPV) experiments were done in anaerobic solutions using a potentiostat AUTOLAB PGSTAT 30 (Eco Chemie, Netherlands) equipped with GPES 4.9 software. An Ag|AgCl (KCl_{sat}) was the reference and a Pt plate was the auxiliary electrode. For voltammetry, gold disk electrodes (CHI, USA, A=0.031 cm^2) were abraded on emery paper, successively mechanically polished to a mirror luster in an alumna slurry, and further electrochemically polished by cycling in 0.1 M H_2SO_4 between −0.3 and 1.7 V. Modification of these electrodes with alkanethiol layers was done as described above. After modification, a Teflon cap was put on the top part of the electrode, thus forming a 5-μl volume well-like microcell, with the bottom representing the electrode surface [15]. Then, a 5-μl droplet of an enzyme stock solution was dropped into the micro-cell, and a dialysis membrane was pressed onto the electrode Teflon cap and fitted tight to the cap surface with a rubber O-ring. In such a manner, the modified electrodes were kept for 30 min in working buffer solutions under a nitrogen flux. Further experiments were done both with the enzymes entrapped under the membrane and without it. CV and DPV measurements with ThOx were performed in deaerated 0.01 M phosphate buffer solution, containing 0.15 M NaCl and 0.1 mM EDTA (PBS), pH 7.0, and with FDH in 0.1 M citrate–phosphate buffer system containing 0.1% Triton X-100 (citrate-PBS), in the pH range from 4.0 to 6.0.

3. Results and discussion

Both ThOx and FDH share one and the same property: they have a heme domain in their molecular structures. Spectral studies demonstrated that the absorption features of the heme dominate in the UV/vis spectra of ThOx (Fig. 1),

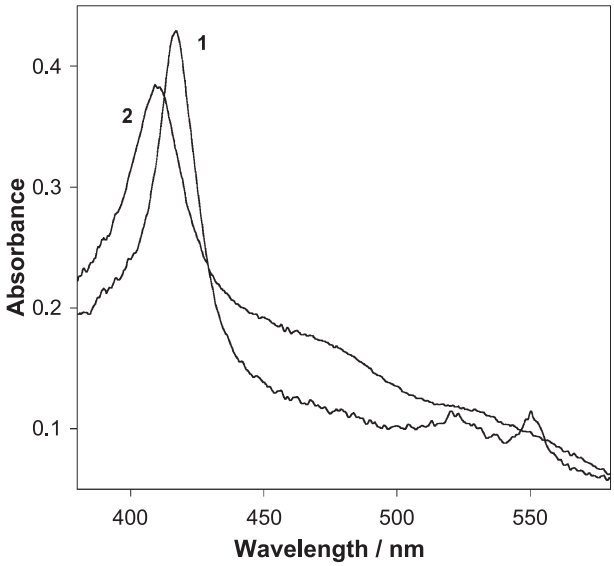

Fig. 1. Absorption spectra of ThOx, (1) totally reduced state upon addition of a saturated solution of theophylline and (2) totally oxidised state upon addition of 0.5 mM ferricyanide.

similarly to those observed with other heme-containing complex enzymes, such as FDH (cyt *c*-type heme domain) [28,30], cellobiose dehydrogenase (CDH, cyt *b*-type heme domain) [37,38] or sulphite oxidase (SOx, cyt b_5-type heme domain) [39–42]. The reaction of ThOx with an excess of theophylline results in spectral changes characteristic of the heme, from the oxidized state to the fully reduced one (Fig. 1, curve 1). Furthermore, ThOx is able to communicate with cyt *c* acting as a natural electron acceptor and completing the biocatalytic cycle [21,23], or with other electron acceptors such as ferricyanide. This reaction results in the full oxidation of the heme of ThOx which can be followed from the heme spectral changes as well (Fig. 1, curve 2). Similar spectral variations characteristic for the heme are shown for FDH upon interaction with fructose and ferricyanide [30].

To determine the redox potentials for the heme redox sites of ThOx and FDH, a direct electron exchange between the electrode and the enzymes should be established. No direct electrochemistry of the heme domains of these two enzymes or DET-based bioelectrocatalysis with ThOx or FDH was attained with bare gold electrodes in the present work. It is reasonable to suggest that communications of ThOx and FDH with their biological electron acceptors should occur through their heme domains, while the substrate redox conversion takes place in another domain. In this case, the heme domain serves as a “built-in mediator” [9], wiring the electrons between the electron accepting site and the enzyme biological redox partner [30,38–40,43]. Studies with heme-containing complex enzymes have demonstrated that their physiological electron acceptor, specifically, cyt *c*, can be replaced directly by the electrode modified with SAMs of properly chosen, terminally functionalised alkanethiols [4,11,15,18,45]. The same meth-

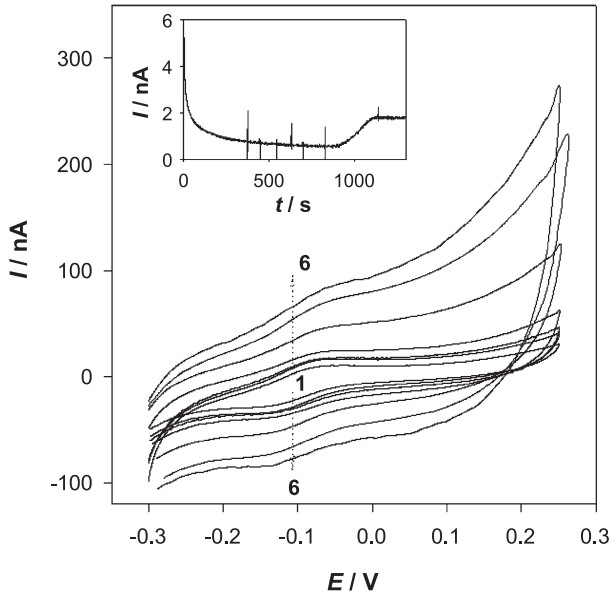

Fig. 2. CVs of ThOx on a gold electrode modified with a mixture of MC_2–OH/MC_2–NH_2; scan rates, (1) 10, (2) 20, (3) 30, (4) 50, (5) 100 and (6) 200 mV s^{-1}; pH 7.0. Inset: Amperometric response to 1.68×10^{-6} M theophylline at 150 mV. Vertical lines reflect additions of first 0.03×10^{-6} M and further stepwise addition of 0.33×10^{-6} M of theophylline.

odology was used in our studies of the redox properties of ThOx and FDH to provide the surface properties of the electrode appropriate for DET between the electrode and the heme domain of the enzymes.

CV of ThOx at electrodes modified with MC_2–OH, cysteamine and mixed MC_2–OH/cysteamine layers revealed a strong dependence of the electrochemistry of ThOx on the nature of the alkanethiol head groups. In fact, only on the mixed SAMs of MC_2–OH and cysteamine that a pronounced direct electrochemistry of ThOx was observed. In the absence of the substrate, a single pair of redox peaks with a mean value for the redox potential of −101 mV, at pH 7.0, was registered (Fig. 2, Table 1). The intensity of the peak currents depended linearly on the potential scan rate [46], thus designating the surface electrochemistry of ThOx on the studied layers. The integration of the peaks gave a surface coverage of the enzyme, Γ, close to 1.7 pmol cm^{-2} of the electrochemically active enzyme, assuming a $1e^-$ transfer process. No redox activity of ThOx was detected on cysteamine layers, and a significantly decreased amount of the adsorbed enzyme as well as a negative shift in the redox potential values were registered on MC_2–OH SAMs (Table 1). Thus, a slightly polar/positive character of the hydroxyl-terminated or mixed hydroxyl-/amine-terminated alkanethiols provided adsorption/orientation of ThOx, which is favourable for DET between the modified gold electrode and ThOx, similarly to the data obtained previously with another heme-containing complex enzyme, SOx [15].

On both alkanethiol layers, (–OH)- and mixed (–OH)/($–NH_2$)-substituted, ThOx was bioelectrocatalytically active due to the achieved DET reaction (Fig. 1, inset). With ThOx, the conversion of theophylline should occur at the active centre other than the heme, and the heme just wires electrons to the external electron acceptor, as supported by spectral studies. Thus, bioelectrocatalytic activity of ThOx allows us to assume the heme domain of ThOx as being in DET contact with the modified electrode. The obtained values for the redox potentials of the heme in ThOx are expected to correspond to the biological values because similar experimental conditions provided close values of the redox potentials of the heme in SOx determined both by direct electrochemistry and by titration with mediators (Table 1).

With FDH, a positively charged cysteamine SAM was required for electrostatic binding of the enzyme to the electrode surface to achieve pronounced DET (Fig. 3). Experiments were performed both with FDH entrapped under the membrane and upon its stripping, with FDH physically adsorbed onto the electrode surface. In both cases, a pair of well-defined peaks was observed in DPVs (Fig. 3a). However, the height of the anodic peak was, in average, 1.5 times higher than that of the cathodic one; as well as the peak potential for the reduction process was 9–15 mV more positive than that for the anodic process, similarly to the DPV data of Khan et al. [35], obtained with bare gold electrodes. Upon membrane stripping, the amount of FDH oxidised in DET did not principally change, contrary to the amount of the re-reduced enzyme, which further decreased and constituted around 50% of the oxidised species. On further scanning, the DPV peaks

Table 1
Direct electron transfer characteristics of some heme multicofactor-containing enzymes

Enzyme	Electrode modification	E_0' mV, electrode DET (Ag|AgCl)	Γ, pmol cm^{-2}	E_0' mV, redox titration (Ag|AgCl)
CDH *Phanerochaete chrysosporium*	Cysteamine or MC_3–COOH	−28; −42 and −81 at pH 3.1, 4.2 and 5.6; −42, pH 5.1 [29,37]		−44, pH 6.0 [36]
CDH *Humicola insolens*	Cysteamine	−78, pH 5.0 [38]		Not determined
SOx chicken liver	Cysteamine, MC_2–OH	−119, pH 7.4 [15]		−113, pH 7.0 [33]
FDH *Gluconobacter industrius*	MC_2–OH	−89±1, pH 4.0	5.7±0.51	Not determined
FDH *Gluconobacter industrius*	Cysteamine	−142.5±4, pH 5.0	4.04±0.34	Not determined
Microbial ThOx	MC_2–OH	−112±2, pH 7.0	0.05±0.03	Not determined
Microbial ThOx	MC_2–OH/MC_2–NH_2	−101±2, pH 7.0	1.65±0.12	Not determined

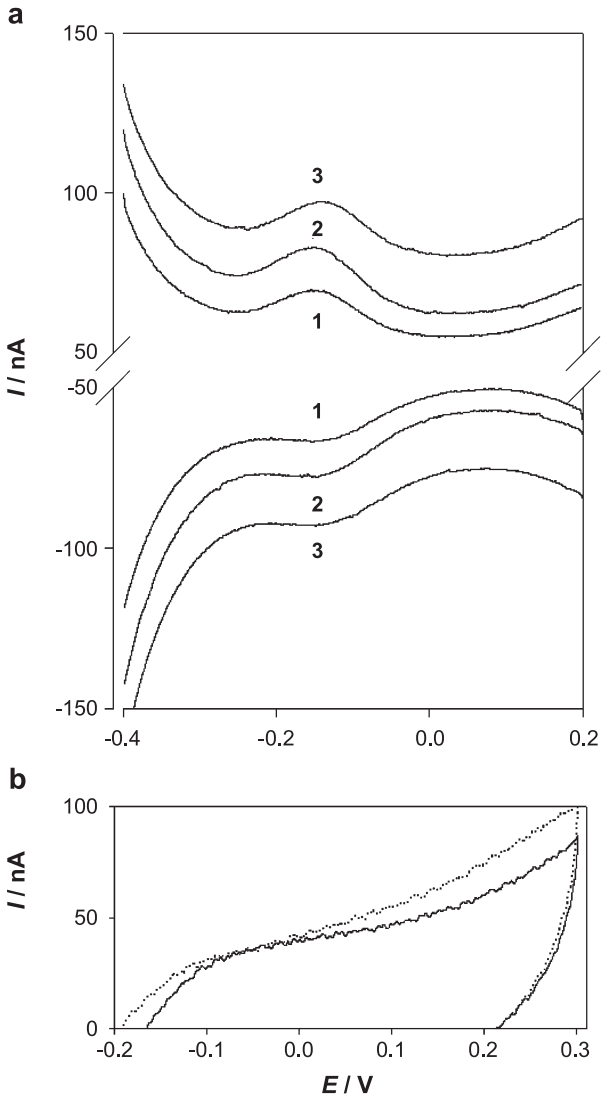

Fig. 3. DPV of FDH on a gold electrode modified with cysteamine; modulation amplitude, 25 mV; modulation time, (1) 50, (2) 70 and (3) 100 ms; step potential, 2 mV; effective scan rate, 10 mV s^{-1}; pH 5.0. Inset: CVs; scan rate, 30 mV s^{-1}; solid line, FDH itself; dotted line, on addition of 3 mM fructose. All the rest conditions as in the main figure.

successively degraded. It is known that FDH is stable in its fully reduced state, [$PQQH_2$–Fe^{2+}]: the stabilised state that is present in the commercial samples. FDH is stable at pH 4.5–6.0; the stability of purified FDH being much enhanced by the presence of detergent in the enzyme solution [28]. FDH is readily oxidised, producing the following states [$PQQH_2$–Fe^{3+}] (oxidation of the heme by external electron acceptor) and [PQQ–Fe^{2+}] (oxidation of the reduced PQQ-cofactor, directly or due to internal ET). All these forms can be reversibly converted into each other during the catalytic cycle; however, the formation of the fully oxidised state [PQQ–Fe^{3+}] leads to a "dead-end" of biocatalysis, when the totally oxidised enzyme is irreversibly inactivated [30]. The shift of the overall DET reaction in the oxidative direction, as well as the "inverse" difference of the peak potentials in DPV, may be interpreted within the terms of a proceeding internal ET reaction. Then, during the oxidation process, the heme is oxidised and "wires" oxidation equivalents further to the PQQ-cofactor, which is in the reduced form in the used commercial sample. This process implies a stepwise transfer of $2e^-$. Upon the reverse re-reduction process, the internal ET may be impeded, and in this case, only the oxidised heme is readily reduced, which is just a $1e^-$ transfer reaction, which roughly corresponds to a twofold decrease in the peak currents. Therewith, the internal ET can contribute to the re-reduction process as well. The formal redox potential estimated as a mean value of the potential of the redox peaks, both from CV and DPV, was close to −143 mV, at pH 5.0, whereas the potential for the PQQ-cofactor redox transformations was shown to be 80 mV at pH 4.5 [35,47]. This allowed us to consider that it was the heme of FDH that in the initial turn underwent the DET reaction at the cysteamine-modified gold electrodes. The surface concentration of adsorbed FDH, calculated from the anodic peaks in the DPVs assuming a $1e^-$ reaction [15], was around 4 pmol cm^{-2}, and decreased twofold if a $2e^-$ transfer was assumed. Upon addition of fructose, FDH, adsorbed on the cysteamine SAM, was bioelectrocatalytically active due to DET between the electrode and the heme domain of the enzyme; however, the efficiency of catalysis was sufficiently low (Fig. 3b) compared to the amount of the electrochemically active enzyme.

The data obtained with FDH on the cysteamine-modified electrodes correlate well with those previously reported for the heme domain of flavocytochrome CDH, for which direct redox activity was achieved with positively charged amine- and carboxyl-terminated alkanethiols in acidic solutions (Table 1) [37,44,45]. The similarity between the heme redox potentials obtained from direct electrochemistry of CDH and that from its redox titration (Table 1) suggests that the redox potential for the heme active site in FDH estimated from voltammograms can be considered as adequate to that in the naturally existing state of FDH.

On the (–OH)-terminated alkanethiol SAM, FDH, entrapped under the membrane, was oxidised in the first scan. However, no re-reduction of the enzyme and no pronounced DET signals were observed on the following potential scans (Fig. 4). Upon addition of fructose, its catalytic oxidation was observed starting from −120 mV, at pH 5.0 (Fig. 4, curve 3). "Solution" electrochemistry of FDH was supposed in this case. Variation of the buffer solution pH provided a variation of the surface charge of FDH (p*I* of 4.55 [48]). That resulted at pH 4.0 in a pronounced direct electrochemistry of FDH on the (–OH)-terminated SAM (Fig. 4, curve 4), but not in DET-based bioelectrocatalysis when the substrate was added. The obtained discrepancy between the redox potentials of FDH on the cysteamine and MC_2–OH SAMs may be due both to the variation in the pH and the different orientations of the enzyme at the electrode surface, which correlated with the

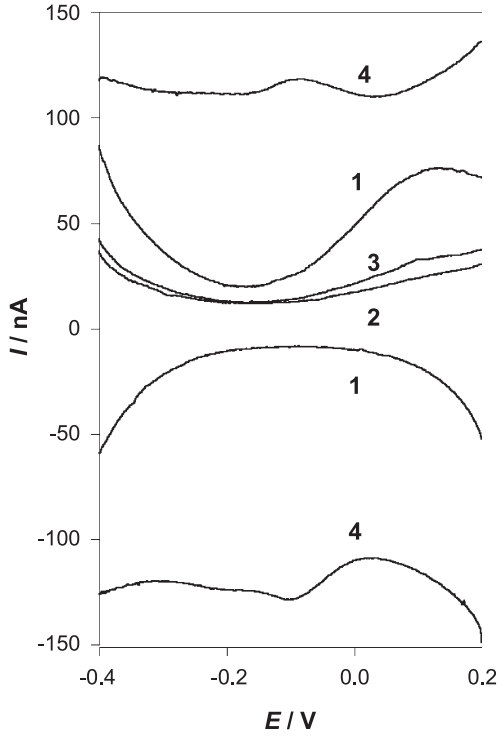

Fig. 4. DPV of 0.02 mg ml^{-1} FDH on a gold electrode modified with MC_2–OH; modulation amplitude, 10 mV; modulation time, 70 ms; step potential, 2 mV; effective scan rate, 10 mV s^{-1}. (1–3) pH 5.0 and (4) 4.0. (1) First successive anodic and reverse cathodic scans, (2) second anodic scan and (3) third anodic scan upon addition of 3 mM fructose.

absence of catalytic activity of FDH on the latter layer, at pH 4.0. In the latter case, the solution pH is likely to affect not only the SAM-FDH interactions but also the value of the redox potential: these effects being difficult to separate. The redox behaviour of FDH on the mixed (MC_2–OH)/(MC_2–NH_2) SAM was similar to that on MC_2–OH, except that weakly developed broad peaks due to DET in the absence of the substrate were detected at pH 5.0 (Table 2). The peak potential values were between the values obtained on pure (–OH)- and (–NH_2)-terminated layers and corresponded to some "intermediate" state of adsorption. A similar dependence of the heme redox potentials on the nature of the alkanethiol head groups was observed with alkanethiols of longer chain length, i.e., containing six methylene groups (Table 2).

Electron transfer reactions of FDH were studied in the presence of its physiological electron acceptor cyt *c* as well. To avoid both the adsorption and inactivation of cyt *c* at the electrode surface, a sufficiently long-chained MC_6–OH was used [15,19,49]. As followed from the previously discussed experiments with MC_2–OH, FDH was also expected not to be adsorbed on this SAM at pH higher than 5.0. The neutral (–OH)-head group of the alkanethiol and high ionic strength of the buffer solutions enabled observation of the "solution" electrochemistry of cyt *c* (linear square-root scan rate dependence of the peak currents [46]), which provided the possibility for free interactions between FDH and cyt *c* in the solution. CVs, measured at pH 7.0 (maximal catalytic response compared to pH 5.0 and 6.0) in solutions containing both cyt *c* and FDH, exhibited some scan-rate-dependent peculiarities. At low scan rates, the typical cyt *c*-mediated bioelectrocatalysis of fructose oxidation was observed starting from the potentials of the cyt *c* redox transformations (Fig. 5a). With increasing the scan rate, a second additional oxidation process appeared at more positive potentials, specifically, starting from 80 mV (Fig 5b–c). Therewith, less of cyt *c* became involved in the catalytic process, as can be followed from the relation between the anodic and cathodic peaks for the cyt *c* redox transformation.

Two types of reactions of FDH with ferricyanide are known from homogeneous kinetics: a "fast" one, at low concentrations of the electron acceptor, and a "slow" one, at high concentrations [30]. The specificity of interactions between the external electron acceptor and the two redox-active domains of FDH predetermines both reactions. In the "fast" reaction, the electron acceptor reacts directly with $PQQH_2$; and the observed catalysis was shown not to involve a redox conversion of the heme [30]. The "slow" catalysis involves interactions of the electron acceptor with the heme-domain of FDH, and the overall catalytic process implies an internal ET from $PQQH_2$ to the heme of FDH, and further to ferricyanide (or another electron acceptor). Although FDH catalysis with cyt *c* has never been studied in detail, the first analysis of the CVs in Fig. 5 allowed suggesting the same catalytic scheme for a cyt *c*-assisted catalytic function of FDH. At high scan rates, the catalytic route may be preconditioned first of all by the interactions of cyt *c* with the PQQ-domain of FDH; while at low scan rates, catalysis occurs via the commonly known cyt *c*–heme–PQQ-pathway. Recently, a coupling of cytochromes with a PQQ-domain of another PQQ-dependent enzyme, glucose dehydrogenase, was shown to provide its efficient bioelectrocatalytic function as well [50,51]. It is interesting that in the absence of cyt *c*, catalysis of fructose oxidation by FDH in solution, at scan rates higher than 5 mV s^{-1}, started at potentials close to that of the heme of FDH; however, an essential enhancement of the catalytic function

Table 2
Direct ET characteristics of FDH from *Gluconobacter industrius* on differently modified gold electrodes

Modifying alkanethiols	Redox potential E'_0, mV, DET	pH	Start potential for DET-based catalysis of fructose oxidation
MC_2–NH_2	−142.5±4	5.0	−80 mV
MC_2–NH_2/ MC_2–OH	−116±2	5.0	−80 mV
MC_2–OH	−89±1	4.0	−120 mV
MC_6–NH_2	−158±5	5.0	−80 mV
MC_6–NH_2/ MC_6–OH	−111.5±4	5.0	−80 mV
MC_6–OH	Not detectable	4.0	−110 mV

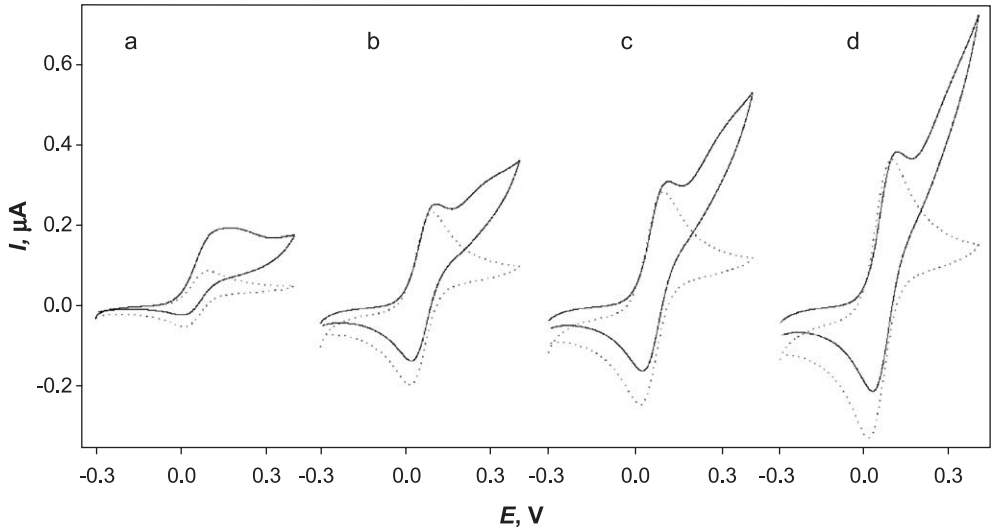

Fig. 5. CVs of cyt *c* (6 mg ml^{-1}) and FDH (0.02 mg ml^{-1}) on (MC_6–OH)-modified gold electrodes (dotted lines) upon addition of 3 mM fructose (solid lines), pH 7.0. Scan rate, (a) 2, (b) 20, (c) 30 and (d) 50 mV s^{-1}. Integration of the anodic branches of the voltammograms (solid lines) gives (a) 32.9, (b) 5.3, (c) 4.6 and (d) 3.5 μC.

is observed at potentials of PQQ-redox transformations, i.e., more positive than 70 mV (Fig. 6). These are the first results which suggest an extremely interesting bioelectrocatalytic scheme for the function of FDH, and further work is in progress.

To summarise, some parallels can be followed between the pH optimum for the biological catalytic activity of the studied enzymes and the properties of the modifying layers. The lower the pH optimum, a more acidic SAM is necessary to provide electrostatic interactions between the electrode and the enzyme, which would favour the DET reaction between the electrode surface and the heme domain of the enzyme. For enzymes with pH optima around pH 7.0–7.4, DET-based bioelectrocatalysis on "protonated" cysteamine layers is impeded. No DET was observed with ThOx on cysteamine SAMs, which may result in further efficient bioelectrocatalytic function of the enzyme. For the previously studied SOx on cysteamine layers [15], clear DET was shown, but the desired orientation of SOx on this SAM was not provided, which enabled DET-based bioelectrocatalysis [15].

Concerning the enzymes with acidic pH optima, e.g., FDH and CDH (Table 1), pronounced redox chemistry was observed from both of them, first of all on the positively charged SAM of cysteamine, providing the DET-based bioelectrocatalytic activity of the enzymes at acidic pHs as well.

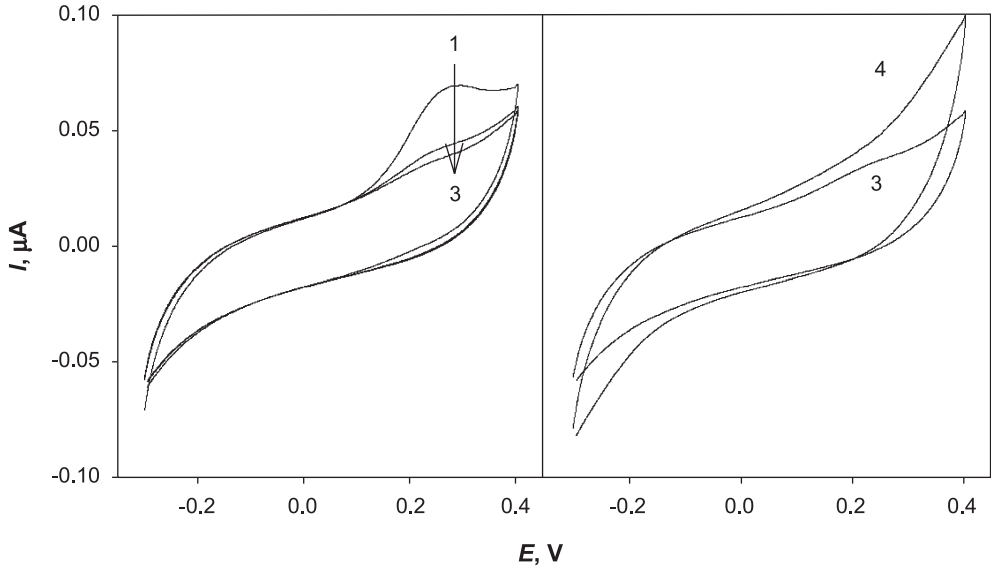

Fig. 6. CVs of 0.02 mg ml^{-1} FDH on (MC_6–OH)-modified gold electrodes (1–3) upon addition of 3 mM fructose (4), pH 4.0; scan rate, 50 mV s^{-1}. (1) First, (2) second and (3) third scans.

4. Conclusions

Complex cofactor-containing enzymes and enzyme complexes embedded in biological membranes play an important role in the living cell. However, membrane-bound enzymes need a hydrophobic reaction environment for their catalytic activity and stability. This impedes electrochemical research of their function, and special approaches should be used for their studies. For this purpose, modification of the electrode surface by a properly charged amine- or hydroxyl-terminated alkanethiol SAM enables to achieve both adsorption and orientation of ThOx and FDH favourable for DET between the electrode and the heme active sites of the enzymes, thus enabling the determination of the redox potentials of the hemes and providing DET-bioelectrocatalytic function of the enzymes. The physiological pH optimum for the catalytic function of the enzymes seems to predetermine to some extent the surface charge of the modifying layer required for an efficient DET reaction.

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