ResearchGate

See discussions, stats, and author profiles for this publication at: https://www.researchgate.net/publication/12834480

Polypyrrole-Entrapped Quinohemoprotein Alcohol Dehydrogenase. Evidence for Direct Electron Transfer via Conducting-Polymer Chains

ARTICLE *in* ANALYTICAL CHEMISTRY · SEPTEMBER 1999

Impact Factor: 5.64 · DOI: 10.1021/ac981201c · Source: PubMed

CITATIONS
120

READS
53

5 AUTHORS, INCLUDING:

Arunas Ramanavicius
Vilnius University
183 PUBLICATIONS **3,910** CITATIONS

SEE PROFILE

Available from: Arunas Ramanavicius
Retrieved on: 29 January 2016

Anal. Chem. **1999,** *71,* 3581−3586

Polypyrrole-Entrapped Quinohemoprotein Alcohol Dehydrogenase. Evidence for Direct Electron Transfer via Conducting-Polymer Chains

Arunas Ramanavicius,[†,‡] Katja Habermüller,[‡] Elisabeth Csöregi,[§] Valdas Laurinavicius,[∥] and Wolfgang Schuhmann*[,‡]

Ruhr-Universität Bochum, Analytische Chemie, Elektroanalytik & Sensorik, Universitätsstrasse 150, D-44780 Bochum, Germany, University of Lund, Department of Biotechnology, P.O. Box 124, S-221 00 Lund, Sweden, and Institute of Biochemistry Vilnius, Mosklininku 12, Lt-2600 Vilnius, Lithuania

It is reported for the first time that direct electron-transfer processes between a polypyrrole (PPY) entrapped quinohemoprotein alcohol dehydrogenase from *Gluconobacter sp. 33* (QH-ADH) and a platinum electrode take place via the conducting-polymer network. The cooperative action of the enzyme-integrated prosthetic groups—pyrroloquinoline-quinone and hemes—is assumed to allow this electron-transfer pathway from the enzyme's active site to the conducting-polymer backbone. A hypothetical model of the electron transfer is proposed which is supported by the influence of various parameters, such as, e.g., ionic strength and nature of the buffer salts. This unusual electron-transfer pathway leads to an accentuated increase of the K_M^{app} value (102 mM) and hence to a significantly increased linear detection range of an ethanol sensor based on this enzyme.

The direct electron transfer (ET) between redox proteins and various electrode materials was intensively studied during the past 20 years. The state-of-the-art had been reviewed in 1992.[1] Increasing interest in the development of reagentless biosensors focused research on redox enzymes and thus intensified the search for enzymes and/or sensor designs exhibiting direct ET properties. The prerequisites for a direct ET have been derived from Marcus theory[2,3] showing the key importance of the ET distance in addition to the potential difference and the reorganization energy of the involved redox centers. Thus, an optimally designed electrode configuration has to ensure that the ET distance between an immobilized redox protein and a suitable electrode surface is made as short as possible. Hence, only those enzyme molecules which are immobilized in the first monolayer on an electrode surface are able to undergo a direct ET. This concomitantly limits important sensor characteristics such as sensitivity and stability.

Although a major breakthrough in protein electrochemistry was reported for cytochrome *c* in 1977[4,5] and laccase adsorbed on graphite in 1979,[6] the most intensively studied and best-characterized enzymes occasionally showing direct ET properties belong to the group of peroxidases, such as cytochrome *c* peroxidase,[7] horseradish peroxidase,[8,9] fungal peroxidase,[10] lactoperoxidase,[11] microperoxidase,[12,13] and chloroperoxidase,[14] immobilized mainly on carbonaceous materials and noble metals. Characteristics of these enzymes and various biosensor designs using them are summarized in a recently published review article.[15] In addition, direct ET was observed for diaphorase on carbon and metal electrodes,[16] and for oriented glucose oxidase;[17,18] however, the observed catalytic currents were, in general, very low.

Recently, multicofactor enzymes, mostly consisting of more than one subunit, such as pyrroloquinoline-quinone (PQQ) and heme-containing enzymes (D-fructose dehydrogenase,[19,20] alcohol

* Corresponding author: (fax) 49-234-709-4683; (e-mail) woschu@anaserv.anachem.ruhr-uni-bochum.de.

† On leave from the University of Vilnius, Department of Analytical Chemistry, Naugarduko 24, Lt-2006 Vilnius, Lithuania.

‡ Ruhr-Universität Bochum.

§ University of Lund.

∥ Institute of Biochemistry Vilnius.

(1) Armstrong, F. A. *Adv. Inorg. Chem.* **1992**, *38*, 117−163.
(2) Marcus, R. A.; Sutin, N. *Biochim. Biophys. Acta* **1985**, *811*, 265−322.
(3) Marcus, R. A. *Angew. Chem., Int. Ed. Engl.* **1993**, *32*, 1111−1121.
(4) Yeh, P.; Kuwana, T. *Chem. Lett.* **1977**, 1145−1148.
(5) Eddowes, M. J.; Hill, H. A. O. *J. Chem. Soc., Chem. Commun.* **1977**, 770−772.
(6) Tarasevich, M. R.; Yarolopov, A. I.; Bogdanovskaya, V. A.; Varfolomeev, S. D. *Bioelectrochem. Bioenerg.* **1979**, *6*, 393−403.
(7) Armstrong, F. A.; Lannon, A. M. *J. Am. Chem. Soc.* **1987**, *109*, 7211−7212.
(8) Yaropolov, A. I.; Malovik, V.; Varfolomeev, S. D.; Berezin, I. V. *Dokl. Akad. Nauk.* **1979**, *249*, 1399−1401.
(9) Wollenberger, U.; Wang, J.; Ozsoz, M.; Gonzalez-Romero, E.; Scheller, F. *Bioelectrochem. Bioenerg.* **1991**, *26*, 287−296.
(10) Kulys, J.; Schmid, R. D. *Bioelectrochem. Bioenerg.* **1990**, *24*, 305−311.
(11) Csöregi, E.; Jönsson-Petterson, G.; Gorton, L. *J. Biotechnol.* **1993**, *30*, 315−317.
(12) Razumas, V.; Kazlauskaite, J.; Ruzgas, T.; Kulys, J. *Bioelectrochem. Bioenerg.* **1992**, *28*, 159−176.
(13) Lötzbeyer, T.; Schuhmann, W.; Schmidt, H.-L. *J. Electroanal. Chem.* **1994**, *377*, 291−294.
(14) Ruzgas, T.; Gorton, L.; Emnéus, J.; Csöregi, E.; Marko-Varga, G. *Anal. Proc.* **1995**, *32*, 207−208.
(15) Ghindilis, A. L.; Atanasov, P.; Wilkins, E. *Electroanalysis* **1997**, *9*, 661−675.
(16) Kobayashi, D.; Ozawa, S.; Mihara, T.; Ikeda, T. *Denki. Kagaku* **1992**, *60*, 1056−1062.
(17) Alvarez-Icaza, M.; Schmid, R. D. *Bioelectrochem. Bioenerg.* **1994**, *33*, 191−199.
(18) Jiang, L.; McNeil, C. J.; Cooper, J. M. *J. Chem. Soc., Chem. Commun.* **1995**, 1293−1295.
(19) Khan, G. F.; Shinohara, H.; Ikariyama, Y.; Aizawa, M. *J. Electroanal. Chem.* **1991**, *315*, 263.
(20) Ikeda, T.; Matsushita, M.; Senda, M. *Biosens. Bioelectron.* **1991**, *6*, 299−304.

10.1021/ac981201c CCC: $18.00 © 1999 American Chemical Society
Published on Web 07/10/1999

dehydrogenase[21]) or FAD and heme containing enzymes (*p*-cresolmethylhydroxylase,[22] fumarate reductase,[23] flavocytochrome c_{552},[24] D-gluconate dehydrogenase,[25] and cellobiose dehydrogenase[26]), were shown to display a direct ET mechanism[15,27] when immobilized on various electrode materials. The proposed mechanism assumes that the ET pathway between the enzyme's active center and an electrode consists of distinct ET steps between the individual electron-exchange sites, confirming the importance of the distance separating the active site from the electrode, well in agreement with Marcus theory.[2,3] Thus, a multiredox-center enzyme can be seen as a combination of a primary redox site and protein-integrated electron-transfer relays. Hence, the electron-transfer pathway in these proteins can be compared with modified redox enzymes obtained by covalent linkage of additional redox species to the inner[28,29] or outer protein shell.[30]

Common to the above-presented ET mechanisms is the direct immobilization of the enzymes on the electrode surface, either simply by adsorption or in a promoted way, favoring the right orientation of the enzymes and thus facilitating the direct exchange of electrons. Therefore, it is obvious that configurations integrating such enzymes into polymer films usually do not meet the mentioned requirements for a direct ET. It has been demonstrated that direct ET could take place even from glucose oxidase entrapped into polypyrrole films; however, special conditions were required, such as growth of the polymer within the pores of a membrane[31,32] or use of extremely dense polymer films.[33] In all mentioned cases, the rate of the direct ET was extremely low. Accordingly, a sensor configuration circumventing the limitation imposed by a low enzyme loading in a monolayer and simultaneously ensuring efficient direct ET, has to significantly augment the "virtual" electrode surface. Thus, it has to be assured that the number of enzyme molecules being immobilized in close distance to the electrode can be increased without losing the special prerequisites for direct ET processes. Since conducting polymers are significantly increasing the available electrode surface, possibilities which favor a direct ET using the ramified network of a conducting polymer, itself, as an active partner in the electron-transfer chain have to be investigated.

In this work we report for the first time on an efficient, direct ET mechanism between a polypyrrole-entrapped quinohemoprotein alcohol dehydrogenase (QH-ADH) and a platinum electrode via the conducting-polymer chains.

EXPERIMENTAL SECTION

Enzymes and Chemicals. QH-ADH (E.C. 1.1.99.8) was purified from *Gluconobacter sp. 33* as previously described.[34] In short, *Gluconobacter sp. 33* was grown in A1 medium in a rotary shaker at 150 rpm at 30 °C for 20 h. The cell paste was obtained by centrifugation and washed with 0.9% NaCl. The cells were resuspended in phosphate buffer containing 1 mM Ca^{2+} ions to stabilize the PQQ in the enzyme's active site. The cells were disrupted by ultrasonic treatment, and the obtained liquid was separated from solid components by centrifugation. A 10% deoxycholate solution was added to a final concentration of 0.5%, and the mixture was incubated for 1 h at 4 °C. After removal of insoluble material, a 10% $CaCl_2$ solution was added to form a $Ca_3(PO_4)_2$ gel containing the adsorbed QH-ADH. The $Ca_3(PO_4)_2$ gel was collected by means of centrifugation, resuspended in phosphate buffer, and by addition of $(NH_4)_2SO_4$ the enzyme was precipitated, collected by centrifugation, and dissolved in 20 mM Tris-HCl buffer (pH 7.2) containing 1 mM Ca^{2+} and 1% sucrose. The activity of the obtained QH-ADH was increased by using a DEAE Toyo-pearl 650 M column, inducing precipitation with poly(ethylene glycol) 6000, using a CM-sepharose column, and dialyzing against high-viscosity carboxymethyl cellulose as the absorber. The used enzyme solution had an activity of 245 U mL^{-1} and a concentration of 7.6 mg of protein mL^{-1} and was stabilized using 1% Triton X-100.

Pyrrole, methanol, ethanol, *n*-propanol, propanol-2, *n*-butanol, isobutanol, isoamyl alcohol, acetone, NaOH, KCl, HCl, H_2SO_4, H_2PtCl_6, K_2HPO_4, *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid] (HEPES), and Na acetate were purchased from Merck (Darmstadt, Germany). Phenazine methosulfate (PMS), 2,6-dichlorophenolindophenol (DCPIP), sodium dodecylsulfate (SDS), and Tris were obtained from Sigma (Deisenhofen, Germany).

Solutions, if not otherwise stated, were prepared using HPLC-grade water produced in a MilliQ system from Millipore (Bedford, MA). Oxygen-free solutions were obtained by bubbling argon through the solution for at least 20 min or using a high vacuum/argon line.

Instrumentation. *Spectrophotometric Setup.* UV−vis measurements were done with a spectrophotometer (Perkin-Elmer, model 550, Friedrichshafen, Germany) or a diode array spectrophotometer (Analytik Jena, model SP100, Jena, Germany), using a standard cuvette with a 10-mm optical path length and a volume of 1 mL. All spectrophotometric measurements were carried out at 30 °C. The spectrophotometric assay for the determination of the activity of the QH-ADH consists of a solution of 0.033 mM PMS and 0.066 mM DCPIP in 50 mM potassium phosphate buffer (pH 7.3).

Electrochemical Equipment. (i) Cyclic voltammetry was performed using a potentiostat in connection with a PC for data acquisition (EG&G, model 263a, Munich, Germany). (ii) Pulse deposition was done by means of a polarograph in connection with a PC for pulse generation and data acquisition (EG&G, model 164a, Munich, Germany). The software program written in Microsoft Quick Basic 4.5 allows predefinition of the number of pulses, the potential steps, and the pulse width. The potential was applied to the potentiostat via a DA converter, and the current

(21) Ikeda, T.; Miyaoka, S.; Matsushita, F.; Kobayashi, D.; Senda, M. *Chem. Lett.* **1991**, 847−850.
(22) Guo, L. H.; Hill, H. A. O.; Lawrence, G. A.; Sanghera, G. S.; Hooper, D. J. *J. Electroanal. Chem.* **1989**, *266*, 379−396.
(23) Sucheta, A.; Cammack, R.; Weiner, J.; Armstrong, F. A. *Biochemistry* **1993**, *32*, 5455−5465.
(24) Guo, L. H.; Hill, H. A. O.; Hooper, D. J.; Lawrence, G. A.; Sanghera, G. S. *J. Biol. Chem.* **1990**, *265*, 1958−1963.
(25) Ikeda, T.; Miyaoka, S.; Miki, K. *J. Electroanal. Chem.* **1993**, *352*, 267−278.
(26) Larsson, T.; Elmgren, M.; Lindquist, S. E.; Tessema, M.; Gorton, L.; Henriksson, G. *Anal. Chim. Acta* **1996**, *331*, 207−215.
(27) Ikeda, T. In *Frontiers in Biosensorics*; *I Fundamental Aspects*; Scheller, F. W., Schubert F., Fedrowitz, J., Eds.; Birkhäuser: Basel, Switzerland, 1997; pp 243−267.
(28) Degani, Y.; Heller, A. *J. Phys. Chem.* **1987**, *91*, 1285−1289.
(29) Bartlett, P. N.; Whitaker, R. G.; Green, M. J.; Frew, J. E. *J. Chem. Soc., Chem. Commun.* **1987**, 1603−1604.
(30) Schuhmann, W.; Ohara, T. J.; Schmidt, H.-L.; Heller, A. *J. Am. Chem. Soc.* **1991**, *113*, 1394−1397.
(31) Koopal, C. G. J.; De Ruiter, B.; Nolte, R. J. M. *J. Chem. Soc., Chem. Commun.* **1991**, 1691−1692.
(32) Koopal, C. G. J.; Bos, A. A. C. M.; Nolte, R. J. M. *Sens. Actuators, B* **1994**, *18*, 166−170.
(33) Schuhmann, W. *Biosens. Bioelectron.* **1995**, *10*, 181−193.

values were read into the computer using an AD converter. For this, a DCI Super 14 bit AD/DA card (J. Merz, Lienen, Germany) was used to interface the PC and the electrochemical equipment. (iii) Constant-potential amperometry has been performed using an electrochemical detector (Biometra, model EP20, Göttingen, Germany) in connection with a strip-chart recorder. These measurements were performed at a fixed potential of +300 mV in a 5-mL volume electrochemical cell containing 50 mM Na acetate buffer (pH 6.0) and 100 mM KCl. Changes of ethanol concentrations have been obtained by addition of defined aliquots of a 96% or 2 M ethanol stock solution.

All potentials are referred to an Ag/AgCl/3 M KCl reference electrode (+197 mV vs NHE) if not otherwise stated.

Electrode Preparation. *QH-ADH Entrapment in Polypyrrole.* A platinum wire was molten in soft glass exposing a disk electrode with a diameter of 1 mm. As cleaning and pretreatment procedures, the Pt electrodes were immersed in concentrated HNO_3 for 10 min in an ultrasonic bath, rinsed with water, and polished on a polishing cloth using alumina paste with 3, 1, and finally 0.3 μm grain size. After rinsing with water, 10 M NaOH, and 5 M H_2SO_4 in an ultrasonic bath, potential cycles with a scan rate of 100 mV s^{-1} in 0.1 M H_2SO_4 in chloride-ion-free electrolyte solution ($Hg/HgSO_4$ reference electrode; +650 mV vs NHE) were applied ((1) scan −610 to +1000 mV; (2) scan −810 to 1600 mV; subsequent scans from −610 to +1000 mV) until the cyclic voltammogram displayed the characteristic features of a bare platinum surface. Prior to its disconnection from the potentiostat, the electrode potential was held for 1 min at −210 mV vs $Hg/HgSO_4$.

To improve the adhesion of the conducting-polymer film and simultaneously increase the number of catalytic active sites on the electrode, the surface of the Pt electrode was platinized by cycling the electrode in 0.1 M KCl containing 0.8 mM H_2PtCl_6. The reductive deposition of Pt clusters which are homogeneously spread over the electrode surface was performed by 3 potential cycles between +500 and −400 mV vs Ag/AgCl with a scan rate of 10 mV s^{-1}.

Pyrrole was purified by passing 0.5-mL aliquots through a neutral Al_2O_3 column (5 cm × 0.4 cm) to remove any colored components.[35] A 100 mM solution of pyrrole containing 100 mM KCl was used for the electrochemical formation of polypyrrole films. Thirty microliters of the enzyme solution was added to 270 μL of this electrolyte/pyrrole mixture. The immobilization of QH-ADH within polypyrrole films was performed following the in-situ entrapment method during the electrochemical formation of the conducting-polymer film on the electrode surface. A potentiostatic pulse profile was applied to the working electrode with the height of the potential pulses defined by the oxidation potential of the used monomer and the duration determined by the diffusion properties of the monomers as previously described.[36] Thus, the electrochemical formation of the enzyme-containing polymer film was carried out by application of 30 potential pulses between 950 (1 s) and 350 mV (for 10 s), to allow the enzyme and the monomer to equilibrate in the neighborhood of the electrode. To decrease the necessary electrolyte volume, the polymer formation was performed using a previously described microcell[37] which allows work under controlled temperature and inert-gas atmosphere in an overall volume of 50−100 μL of electrolyte. As a control, polypyrrole-modified electrodes have been prepared using the same deposition conditions in the absence of the enzyme during the film-formation protocol.

RESULTS AND DISCUSSION

Quinohemoprotein Alcohol Dehydrogenase. QH-ADH was previously isolated from various aerobic gram-negative bacteria, such as *Acetobacter*,[38] *Commamonas*,[39] and *Gluconobacter*.[40] The one used in the present work was isolated from *Gluconobacter sp. 33* and purified according to a previously published protocol[34] (see Experimental Section). The activity of the enzyme preparation was 32.2 units/mg of protein. To evaluate the possibilities for a direct ET mechanism between the enzyme and a conducting-polymer matrix, knowledge of the structural properties of the enzyme are of high importance. The membrane-bound enzyme consists of three subunits.[41,42] Subunit I (83 kDa) is considered to be the dehydrogenase unit containing one PQQ and one heme moiety; subunit II (52.1 kDa) contains three heme moieties; and subunit III (16.6 kDa), which is a peptide not containing any electrochemically active groups, is supposed to be essential for the expression of the active enzyme.[43] The absorption spectrum of the purified enzyme revealed three peaks, at 410−430, 523, and 553 nm, respectively, confirming the presence of the heme moieties in the enzyme.

QH-ADH catalyzes the oxidation of different alcohols to the corresponding aldehydes with ethanol being the main substrate. The conversion of methanol and higher aliphatic alcohols occurs with significant reduced activity.[34]

Evaluation of ET Pathways between QH-ADH and the Electrode Surface. PQQ-dependent enzymes were previously shown to be strongly influenced by Ca^{2+} concentration.[43] Since phosphate buffer may remove Ca^{2+} ions from the active center of the enzyme, simultaneously causing an inactivation of the enzyme, an improved stability was expected using a sodium acetate buffer. Hence, all experiments have been performed using acetate buffer.

Mediated ET. To evaluate the response of polypyrrole-entrapped QH-ADH (QH-ADH/PPy) to ethanol in order to ensure the entrapment of the active enzyme within the conducting-polymer film, constant-potential amperometry experiments were carried out in the presence of PMS acting as a free-diffusing redox mediator. Although the diffusion of the mediator into the QH-ADH/PPy film may be slow as a result of the properties and morphology of the conducting-polymer network, a current−concentration curve could be obtained saturating at about 5−6

(34) Marcinkeviciene, L.; Bachmatova, I.; Semenaite, R.; Rudomanskis, R.; Brazenas, G.; Meskiene, R.; Meskys, R. *Biologija* **1999**, *N2*, 109−112.

(35) Schuhmann, W. In *Methods in Biotechnology, Vol. 6: Enzyme and Microbial Biosensors: Techniques and Protocols*; Mulchandani, A., Rogers, K., Eds.; Humana Press: Totowa, NJ, 1998; pp 143−156.

(36) Schuhmann, W.; Kranz, C.; Wohlschläger, H.; Strohmeier, J. *Biosens. Bioelectron.* **1997**, *12*, 1157−1167.

(37) Habermüller, K.; Schuhmann, W. *Electroanalysis* **1998**, *10*, 1281−1284.

(38) Adachi, O.; Miyagawa, E.; Shinagawa, E. *Agric. Biol. Chem.* **1978**, *42*, 2331−2340.

(39) Groen, B.; Frank, J.; Duine, A. *Biochem. J.* **1984**, *223*, 921−924.

(40) Adachi, O.; Tayama, K.; Shinagawa, E. *Agric. Biol. Chem.* **1978**, *42*, 2045−2056.

(41) Matsushita, K.; Yakushi, T.; Takaki, Y. *J. Bacteriol.* **1995**, *177*, 6552−6559.

(42) Matsushita, K.; Yakushi, T.; Takaki, Y. *J. Biol. Chem.* **1996**, *271*, 4850−4857.

(43) Frébortova, J.; Matsushita, K.; Arata, H.; Adachi, O. *Biochim. Biophys. Acta* **1998**, *1363*, 24−34.

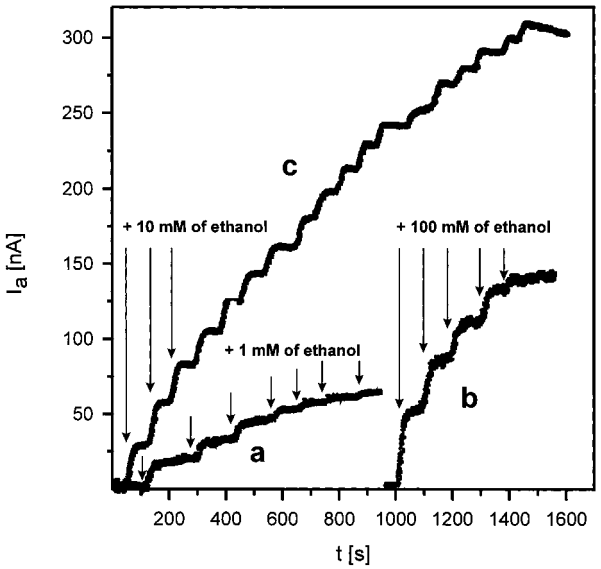

Figure 1. Typical current response of a QH-ADH/PPy electrode to the step-by-step addition of ethanol (+300 mV vs Ag/AgCl in 50 mM Na acetate buffer, pH 6.0, containing 100 mM KCl). (a) In the presence of 1 mM PMS (concentration increments of 1 mM). (b) In the absence of any free-diffusing mediator (concentration increments of 100 mM). (c) In the absence of the soluble mediator 1 day after the preparation of the biosensor (concentration increments of 10 mM).

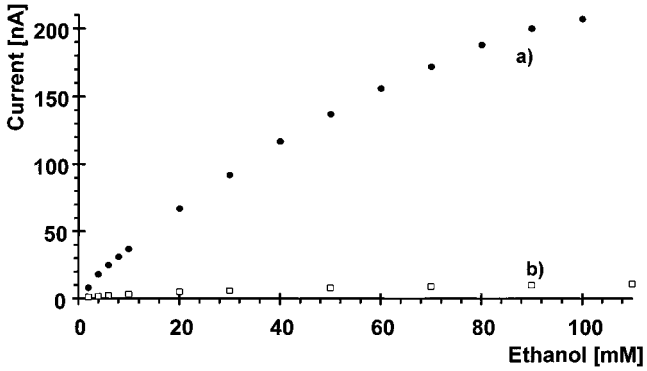

Figure 2. (a) Ethanol calibration curve obtained with a QH-ADH/PPy electrode (50 mM Na acetate buffer, pH 6.0, containing 100 mM KCl (+300 mV vs Ag/AgCl). The control experiment (b) shows the response of a platinized Pt electrode with adsorbed QH-ADH to increasing ethanol concentrations.

mM of ethanol (Figure 1, curve a). Surprisingly, after addition of ethanol to concentrations of 100 mM, an unexpected increase of the steady-state current was observed (Figure 1, curve b). At the used small concentration of the free-diffusing redox mediator, this effect is very unlikely to occur because of a mediated ET process. Moreover, repetition of the experiment in the absence of any free-diffusing mediator clearly demonstrated that the assumption of a direct ET process might be responsible for the observed relation between the current and the ethanol concentration. A deliberation of PQQ from the active site of the enzyme, which may be active as free-diffusing redox mediator, can be excluded from the high stability of the enzyme in flow systems.

Direct ET. Since no direct ET could be observed from a polypyrrole-entrapped, PQQ-dependent glucose dehydrogenase via the conducting-polymer chains (results not shown), the above-mentioned currents (Figure 1b) were assumed to be caused by a possible internal ET pathway involving the different enzyme-integrated redox sites (PQQ and hemes) located in the different subunits of the enzyme. It was therefore supposed that the alcohol is primarily oxidized via the PQQ site which might be regenerated in a subsequent step by reduction of the heme units located in subunits I and II (see Figure 6). Assuming that the heme sites are located closer to the protein surface than the PQQ unit, a direct ET pathway can be proposed from these heme sites to the electrode surface via the conducting polypyrrole chains. This process can be compared with the model of artificially modified enzymes obtained by, e.g., integration of ferrocene units into glucose oxidase (GOx)[28−30] or after attaching ET mediators to the FAD cofactor of GOx prior to the formation of the holoenzyme.[44,45] In all these cases the ET distance was obviously significantly decreased, resulting in improved ET kinetics and, hence, a measurable current.

After a period of storage of the QH-ADH/PPy electrode at 20 °C for 24 h, an increased sensitivity and a long linear measuring range were observed (Figure 1, curve c). The accommodation of the proteins in the immobilization matrix combined with the swelling of the film during the storage period might have caused a better accessibility of the enzyme's active centers and, hence, the observed increase in sensitivity. A steady-state calibration curve recorded for increasing ethanol concentrations is shown in Figure 2, characterizing the described ethanol sensor by a linear range of up to about 100 mM, a sensitivity of 2.07 nA/mM, and an apparent K_M^{app} of 102.3 mM (calculated following an algorithm proposed by Eisenthal and Cornish). The control experiment with the enzyme adsorbed at a platinized Pt surface demonstrates clearly the efficient electron-transfer pathway via the conducting-polymer chains.

Thus, the obtained results demonstrate that, at low substrate concentrations in the presence of PMS, a competition occurs between the reoxidation of the PQQ moiety either directly by the free-diffusing PMS, by PMS via the enzyme-integrated heme groups or by a direct electrical communication through one or several heme groups, and finally via the conducting polypyrrole chains. At higher substrate concentrations the regeneration of PMS at the electrode surface becomes rate limiting because of its slow diffusion through the polymer network. Considering this fact together with the low PMS concentration, a direct ET pathway is assumed which is dominating the electrode response in this concentration range. The use of the different possible ET pathways might be advantageous for the development of related biosensors for practical applications.

Evidence for Direct ET. Cyclic Voltammetry. The definition of a direct ET implies that the redox reaction should occur close to the formal potential of the involved active site. To support the hypothesis of a direct ET from the enzyme-integrated heme moieties to the conducting polypyrrole chains, cyclic voltammograms have been recorded in oxygen-free 50 mM acetate buffer containing different ethanol concentrations (Figure 3). Obviously, ethanol addition caused an increase of the heme oxidation peak at +190 mV. This observation is in good agreement with the reported potentiometric titration of the redox sites of QH-ADH

(44) Ramanavicius, A.; Meskys, R.; Laurinavicius, V. *Biologija* **1995**, *1*, 53−56.
(45) Riklin, A.; Katz, E.; Willner, I.; Stocker, A.; Bückmann, A. F. *Nature* **1995**, *376*, 672−675.

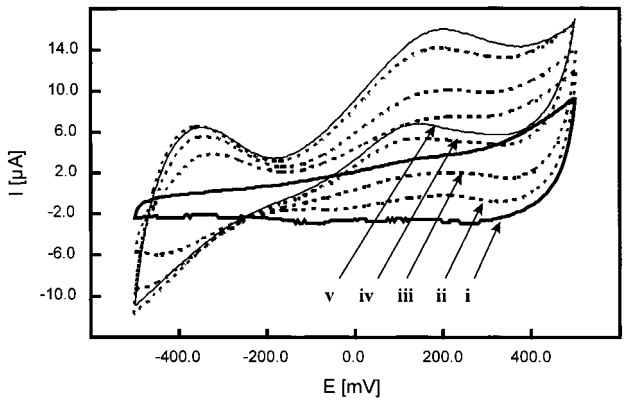

Figure 3. Cyclic voltammograms of a QH-ADH/PPy electrode with increasing concentrations of ethanol: (i) 0 mM, (ii) 50 mM, (iii) 100 mM, (iv) 300 mM, (v) 500 mM (sweep rate, 100 mV s^{-1}; 50 mM Na acetate buffer, pH 6.0).

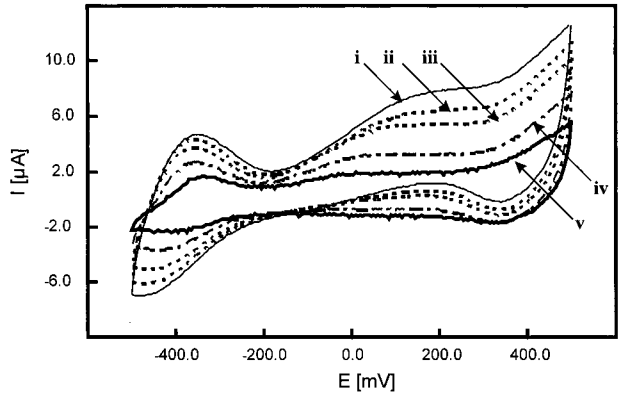

Figure 4. Cyclic voltammograms of a QH-ADH/PPy electrode in the presence of 100 mM ethanol and increasing concentrations of KCl: (i) 0 mM, (ii) 50 mM, (iii) 100 mM, (iv) 300 mM, (v) 500 mM (sweep rate, 100 mV s^{-1}; 50 mM Na acetate buffer, pH 6.0).

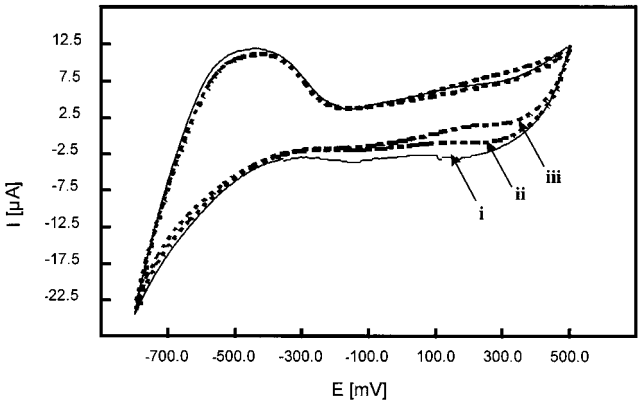

Figure 5. Cyclic voltammograms of a QH-ADH/PPy electrode in 50 mM HEPES buffer, pH 7.4, in the presence of different concentrations of ethanol: (i) 0 mM, (ii) 200 mM, (iii) 500 mM (sweep rate, 100 mV s^{-1}).

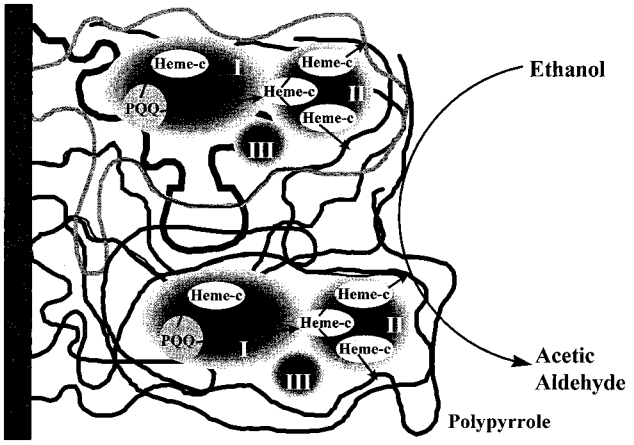

Figure 6. Proposed ET pathway for a QH-ADH/PPy electrode from PQQ via the enzyme-integrated heme groups to the conducting polypyrrole chains and finally to the electrode.

from *Acetobacter methanolicus* and *Gluconobacter suboxidans*, in which the potential of at least one of the heme groups is reported to be 188 mV.[43] Additionally, the redox-switching of the polypyrrole, itself, to its nonconducting state can be seen at potentials of about −300 mV. Both redox waves are increasing up to an ethanol concentration of 500 mM (Figure 3, curves ii−v).

Influence of the Ionic Strength. The pH optimum of the QH-ADH/PPy electrode shifted by about 2 units, to 7.5, as compared with the enzyme in solution in the presence of PMS/DCPIP as free-diffusing redox mediators. However, it is not yet clear if the pH optimum of the dissolved enzyme is governed by the used redox mediator. If the proposed ET model is right, assuming that a direct ET is only possible when the (positively charged) polypyrrole chains are in close proximity to the heme sites of the enzyme, then a local compensation of charges should weaken the interaction between the enzyme and the polypyrrole chains. As a consequence, the amperometric signal of the QH-ADH/PPy electrode should decrease with increasing ionic strength. Therefore, cyclic voltammograms have been recorded in the presence of a constant concentration of ethanol with a stepwise increase in the ionic strength of the electrolyte by KCl addition (Figure 4). The heme-attributed current peak at about 190 mV gradually decreases with the increase in the ionic strength, vanishing totally at a KCl concentration of about 500 mM. Diluting the electrolyte reestablished the ethanol-related current response. This result is in good agreement with a similar phenomenon observed for cytochrome *c* adsorbed on a 4,4′-dithiodipyridine-modified gold electrode which has been attributed to the blocking of surface charges.[46] In addition, the current peak at −300 mV (supposed to be connected with the reduction of the polypyrrole chains) also decreased with increasing ionic strength.

To support these findings, cyclic voltammograms of QH-ADH/PPy have been recorded in oxygen-free 50 mM HEPES buffer (pH 7.5) (Figure 5). Obviously, the influence of increasing concentrations of ethanol on the heme redox wave is much less pronounced than in the case of acetate buffer. Probably, the large anions of HEPES buffer are isolating the heme site from the polypyrrole chains and thus preventing a direct ET process.

CONCLUSIONS AND FUTURE ASPECTS

On the basis of above-stated considerations, a schematic representation of the described QH-ADH/PPy electrode has been made showing the proposed direct ET pathway from the poly-

(46) Willner, I.; Helegshabtai, V.; Blonder, R.; Katz, E.; Tao, G. L. *J. Am. Chem. Soc.* **1996**, *118*, 10321−10322.

(47) Scheller, F. W., Schubert, F., Fedrowitz, J., Eds. *Frontiers in Biosensorics*; *I Fundamental Aspects*; Birkhäuser: Basel, Switzerland, 1997; pp 220−221.

pyrrole-entrapped QH-ADH to the electrode via the conducting polypyrrole chains (Figure 6). Ethanol diffuses to the PQQ site of the entrapped enzyme where it is oxidized to the respective aldehyde. The PQQ center is subsequently regenerated by the heme sites of the enzyme, leading to a localization of the transferred electrons close to the outer surface of the enzyme. In the case of moderated ionic strength, the enzyme or at least its heme sites are tightly wrapped into polypyrrole chains, and the direct ET between the heme sites and the polypyrrole chains becomes feasible with reasonable ET kinetics. The observed ET process occurs at the formal potential of at least one of the heme sites.

The obtained results strongly encourage further investigations concerning entrapment of other quinohemoproteins in polypyrrole films in order to establish the possibility of direct ET processes. Additionally, the similarity of the ET mechanisms observed for enzymes with integrated multiple redox sites and redox-mediator modified enzymes should motivate future work focusing on site-specific modification of enzymes to allow direct ET processes.

ACKNOWLEDGMENT

This work was supported by the EU in the framework of an INCO-COPERNICUS program, Contracts ERB IC15CT96-1008 and ERB IC15CT98-0907. A.R. is grateful to A. Hengstenberg, H. Zimmermann, B. Ballesteros Katemann, and T. Erichsen for the help given during his stay at the Ruhr-Universität Bochum. The authors thank Dr. R. Meskys for valuable advice concerning the properties of QH-ADH and I. Bachmatova and L. Marcinkeviciene for the preparation of the enzyme.

Received for review November 4, 1998. Accepted March 25, 1999.

AC981201C