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Electrochemical and spectrophotometric investigations of electron-transfer pathways in biocatalysis by PQQ-ADH

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Aiming to investigate electron-transfer pathways in biocatalysis by pyrroloquinoline-quinone-dependent alcohol dehydrogenase (PQQ-ADH), the inhibition of hemes *c* of PQQ-ADH by CO was carried out. The inactivation kinetics was studied using a group of one- and two-electron acceptors: potassium ferricyanide (PF) and dichlorophenolindophenol (DCPIP). The inactivation constants (k_{in}), that characterize the PQQ-ADH inactivation rate due to inhibition of hemes *c* were calculated. The results showed that bioelectrocatalysis by PQQ-ADH proceeds via an energetically favourable way of internal mediation; however, an alternative electron transfer switches on when the usual pathway is blocked. A mechanism of the possible electron-transfer pathways in biocatalysis by PQQ-ADH is proposed.

Key words: PQQ-dependent alcohol dehydrogenase, electron transfer, biocatalysis, inactivation kinetics

INTRODUCTION

Understanding biocatalysis and bioelectrocatalysis at atomic resolution is not only an intellectual challenge but has already contributed greatly to improving the quality of life since it is the basis of a series of biosensing systems [1]. Obviously, the extending of these technologies will depend on determining the new properties of biocatalytical processes and their successful application. The study of direct and mediating electron transfer is of interest for the development of biocatalysts for various applications, including biosensor design [2].

Multi-cofactor enzymes, and PQQ-ADH among them, usually consist of more than one subunit integrating different cofactors, such as PQQ and hemes. These enzymes provide a unique possibility of studying both heterogeneous and internal electron transfer reactions. Some of these multi-cofactor enzymes have been shown to possess the ability to electrocatalyze reactions by a direct electron-transfer mechanism. To study the electron transfer mechanisms, the PQQ and heme-containing enzymes (such as D-fructose dehydrogenase [3–6] and alcohol dehydrogenase [7–9], the FAD or FMN and heme-containing enzymes (such as *p*-cresol methylhydroxylase [10], fumarate reductase [11], flavocytochrome *c*552 [12] and cellobiose dehydrogenase (CDH) [13]) and enzymes containing FAD-heme-Fe-S cluster, such as D-gluconate dehydrogenase [14]) were investigated and the reaction schemes for various quino- or flavo-enzymes have been suggested. The mechanism of bioelectrocatalysis for the mentioned above cases suggests that the catalytic transformation of the substrate occurs first on the PQQ or FAD site, then the electrons are transferred through the protein molecule to the heme redox site and further to the electrode, where the enzyme is re-oxidized either through a direct electron-transfer or by one-electron mediating stage [7, 9, 12, 14], though the two-electron mediating way when the electrons of CDH-$FADH_2$ are transferred to electrode with the help of a soluble mediator was presented as well [13].

In the current study, possible electron-transfer pathways in mediating and direct biocatalysis by PQQ-ADH have been investigated.

MATERIALS AND METHODS

PQQ-ADH was purified from *Gluconobacter* sp. 33 as described previously [15]. The specific activity of this enzyme was 7.12 U/mg. The PQQ-ADH solution (200 U/ml) was prepared for the experiments. Sodium acetate, acetic acid and $CaCl_2$ were obtained from J. T. Baker (Holland, NL). Ethanol and $K_3[Fe(CN)_6]$ (PF) were purchased from Riedel-de Haen (Germany). 2,6-Dichlorophenolindophenol (DCPIP) was from Sigma-Aldrich (Steinheim, Germany).

Electrochemical measurements were performed using a PARSTAT 2273 electrochemical system (Princeton Applied Research, USA) with a conventional three-electrode system consisting of a platinum plate electrode as the auxiliary electrode, a saturated Ag/AgCl electrode as the reference and screen-printed carbon (Ø 3 mm) as the working electrodes. The response of the prepared enzyme electrodes and the related control electrodes to concentrations of 2 mM of ethanol was investigated under potentiostatic conditions at +400 mV versus Ag/AgCl in a stirred 0.05 M acetate buffer, pH 6.0, containing 10 mM Ca^{2+}.

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Spectrophotometric measurements were performed on a UV 300, UV-Visible spectrometer (Thermo Spectronic, USA). Activity of the enzyme was measured in 50 mM acetate buffer, pH 6.0, in the presence of ethanol (2 mM) at 420 nm if $K_3[Fe(CN)_6]$ was used ($\varepsilon = 1000\ cm^{-1}\ M^{-1}$) or at 600 nm in the presence of DCPIP ($\varepsilon = 9300\ cm^{-1}\ M^{-1}$).

The synthesis of CO was carried out according to the published protocol [16]. The inactivation procedure was carried out by bubbling the solutions containing native PQQ-ADH or dipped PQQ-ADH-coated electrodes with CO for a different time.

RESULTS AND DISCUSSION

Membrane-bound pyrroloquinoline-quinone-dependent alcohol dehydrogenase PQQ-ADH from *Gluconobacter* sp. 33 is a quinohemoprotein having prosthetic groups: PQQ and hemes c. The enzyme structure and assumed one- (A) or two-electron (B) transfer pathways during bioelectrocatalysis are schematically shown in Fig. 1.

As was previously reported, the oxidized cofactor transforms during bioelectrocatalysis to quinol ($PQQH_2$) by accepting two electrons and two protons, and re-oxidation proceeds easily by releasing two electrons [17]. Afterwards the electrons might be transferred to the electrode either via the heme *c* groups, which thereby behave as internal mediators (A pathway), or via the B pathway (Fig. 1). Aiming to examine the proposed electron-transfer ways (Fig. 1), the internal A pathway was blocked by inhibiting the hemes *c*. The heme group containing an iron ion is the oxygen-binding site (for example, in hemoglobin). It is known that the binding of oxygen can be affected by a CO molecule because hemoglobin affinity to CO is 200 times greater than its affinity to oxygen. Analogically to hemoglobin, it was supposed that PQQ-ADH might be inactivated by carbon monoxide. The inactivation process of PQQ-ADH was investigated spectrophotometrically or electrochemically, using one- and two-electron acceptors. PQQ-ADH activity during the inactivation procedure was evaluated by analysis of responses to ethanol. The inactivation kinetics of PQQ-ADH during biocatalysis was found to be significantly different for one- and two-electron mediating processes (Fig. 2). Beside that, the same inactivation procedure was performed on PQQ-dependent glucose dehydrogenase (PQQ-GDH) which has no hemes *c*, and no inactivation was observed (data not shown).

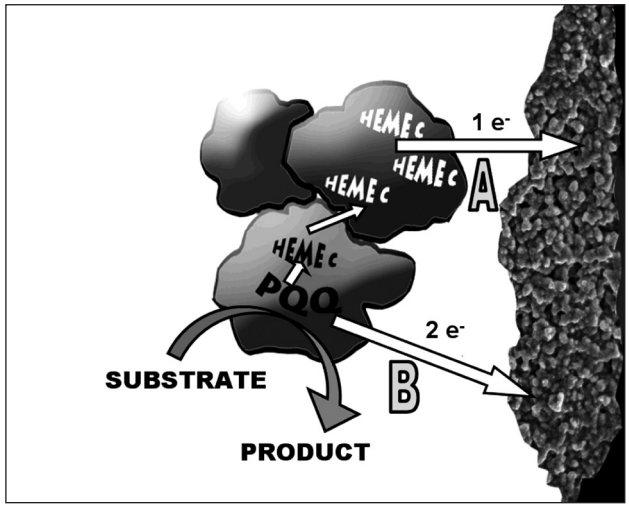

Fig. 1. Proposed electron-transfer pathways for bioelectrocatalysis by PQQ-ADH. One-electron mediating way: from PQQ via heme c to the electrode (A), and two-electron mediating way: from PQQ to the electrode (B)

The calculated inactivation constant (k_{in}) for native PQQ-ADH using the one-electron mediator PF (k_{in} 0.344 min^{-1}) differed sufficiently from the k_{in} of the control sample (0.002 min^{-1} in the absence of CO). This indicates that hemes *c* were successfully inhibited and the electron-transfer mechanism via the internal mediators is hardly possible, whereas the mediating way by using the two-electron acceptor DCPIP (Fig. 1, B) exhibits a significantly lower inactivation (k_{in} 0.027 min^{-1}) in comparison with the one-electron mediating way (Fig. 1, A). This fact implies that an alternative electron transfer way – shunting the hemes pathway – may be switched on in the presence of two-electron mediators even if the hemes are blocked.

Aiming to shed light on the proposed alternative electron-transfer pathway during biocatalysis by PQQ-ADH, electrochemical measurements were carried out as well. For this purpose, the enzyme was adsorbed on carbon electrodes (CE), and inactivation kinetics using CO was studied. Inactivation constants for direct (mediatorless) bioelectrocatalysis or one- or two-electron-mediated processes were calculated. The inactivation data are presented in Fig. 3.

Calculated from electrochemical measurements, the inactivation constant (k_{in}) of the control sample in the absence of CO (0.004 min^{-1}) correlated with the k_{in} of the control sample of na-

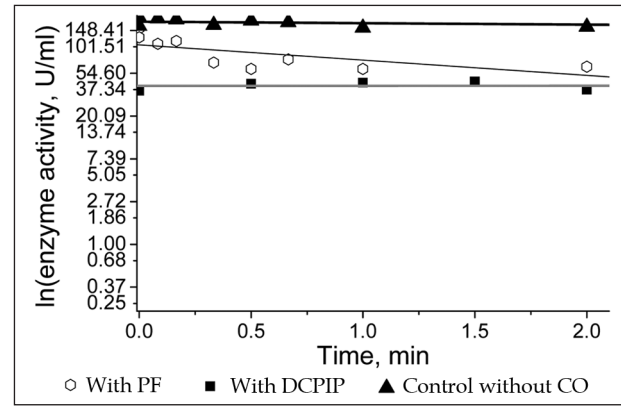

Fig. 2. Spectrophotometric data on inactivation kinetics of biocatalysis by PQQ-ADH

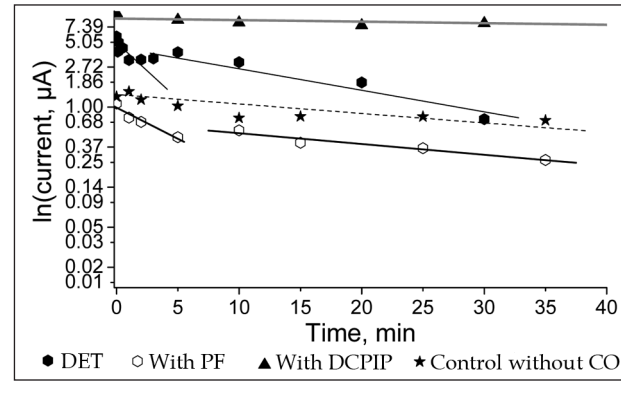

Fig. 3. Electrochemical data on inactivation kinetics of bioelectrocatalysis by PQQ-ADH

Table. **Inactivation constants calculated for inactivation of biocatalytic processes shown in Figs. 2 and 3**

Investigations	k_{in} describing one-electron mediating pathway	k_{in} describing two-electron mediating pathway
Spectrophotometric	**0.344** min^{-1}, using PF	**0.027** min^{-1}, using DCPIP
	0.002 min^{-1} (k_{in} of biocatalysis without inhibition by CO)	
Electrochemical	**0.442** min^{-1} (faster stage of inactivation of direct bioelectrocatalysis by PQQ-ADH) **0.155** min^{-1} (faster stage of inactivation of bioelectrocatalysis by PQQ-ADH using PF)	**0.053** min^{-1} (slower stage of inactivation of direct bioelectrocatalysis by PQQ-ADH) **0.026** min^{-1} (slower stage of inactivation of bioelectrocatalysis by PQQ-ADH using PF) **0.024** min^{-1} (inactivation of bioelectrocatalysis by PQQ-ADH using DCPIP)
	0.004 min^{-1} (k_{in} of bioelectrocatalysis without inhibition)	

tive PQQ-ADH (0.002 min^{-1}). Furthermore, in all cases of inhibition demonstrated in Fig. 3 (with the exception of two-electron mediating by DCPIP), the inactivation process of PQQ-ADH-dependent bioelectrocatalysis can be divided into the faster and the slower steps characterized by the different inactivation rates. The calculated k_{in} for both stages corresponded to k_{in} obtained spectrophotometrically of the native enzyme in one-electron (the faster stage) and two-electron (the slower stage) mediating pathways, respectively. Evidently, the k_{in} values of the faster and the slower inactivation processes in both cases of bioelectrocatalysis: mediatorless (Fig. 3, DET) or mediated using one-electron mediators (Fig. 3, with PF) differed at least 10 times. All k_{in} values are summarized in Table.

It can be concluded that the electrochemical analysis of inactivation kinetics justifies the fact that the bioelectrocatalysis by PQQ-ADH in the case of no additional two-electron mediators proceeds via an energetically favourable way of the internal mediation (Fig. 1, A). However, an alternative two-electron transfer pathway from PQQ to the electrode (Fig. 1, B) is switched on when hemes are inactivated. This electron transfer pathway can be realized due to function groups of the carbon electrode material. Furthermore, two-electron mediators promote this alternative way of bioelectrocatalysis by PQQ-ADH independently of the inhibition of hemes.

The obtained results are important not only in terms of a fundamental knowledge of electron pathways in quinohemoproteins, but also for producing new CO sensing systems.

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ELEKTRONŲ PERNAŠOS KELIŲ BIOKATALIZĖJE SU PQQ-ADH ELEKTROCHEMINIAI IR SPEKTROFOTOMETRINIAI TYRIMAI

Santrauka

Inhibuojant anglies monoksidu *c* hemus, esančius nuo pirolochinolinchinono priklausomoje alkoholio dehidrogenazėje (PQQ-ADH) iš *Gluconobacter* sp. 33, ištirti du galimi elektronų pernašos keliai. Tam tikslui buvo tirta PQQ-ADH inaktyvacijos kinetika biokatalizėje dalyvaujant vienelektroniniams (VM) ir dvielektroniniams (DM) elektronų akceptoriams (mediatoriams), taip pat bemediatorinė bioelektrokatalizė su PQQ-ADH. Remiantis elektrocheminiais ir spektrofotometriniais duomenimis buvo apskaičiuotos inaktyvacijos konstantos. Iš jų matyti, kad, be anksčiau nustatyto energetiškai palankiausio elektronų pernašos kelio nuo PQQ *c* hemų grandine, galimas ir kitas kelias, kai elektronai nuo PQQ perduodami tiesiai elektrodui bemediatorinės bioelektrokatalizės metu. Gauti duomenys naudingi tiek fundamentaliu požiūriu, tiek kuriant biojutiklių sistemas.