Comparative characterization of soluble and membrane-bound PQQ-glucose dehydrogenases

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Two types of PQQ-glucose dehydrogenases were compared in solution and immobilized on ferrocene derivatives – modified carbon electrodes. The intracellular soluble enzyme from *Acinetobacter calcoaceticus* L.M.D. 79.41 (s-GDH) and the membrane-bound one (m-GDH) from *Erwinia* sp. 34-1 were purified and investigated. It has been shown that s-GDH strongly adsorbs and inactivates under immobilization. The immobilization procedure decreased the stability and substrate specificity of the enzyme. The main reason for inactivation of the enzyme was the loss of PQQ from the active center of the enzyme. Immobilization of m-GDH improves both the stability and substrate specificity of the enzyme. The anchor subunit of m-GDH is supposed to serve for the anchoring of the enzyme globule on the surface of the electrode.

Key words: quinoproteins, glucose dehydrogenase, bioelectrochemistry, biosensors

INTRODUCTION

More than twenty years ago was discovered a new enzyme, which catalyzed oxidation of substrates and contained a cofactor which was neither a NAD nor flavin [1]. Later this cofactor was identified [2], isolated [3, 4] and abbreviated as PQQ [4].

PQQ can be reduced by two electrons. PQQ has a distinctly high redox potential (+90 mV) as compared to NAD^+ (–320 mV) and FAD (–45 mV) [5]. PQQ is not covalently attached to the active center of the enzyme, but is complexed *via* Ca (or Mg) ions.

Natural electron acceptors of the soluble PQQ-dehydrogenases are not identified, while membrane-bound PQQ dehydrogenases transfer electrons to ubiquinones, but not to the oxygen [5–7]. This phenomenon was used for designing biosensors insensitive to oxygen, for example, for glucose determination in undiluted blood [8, 9]. A number of redox species can be used for the oxidation of the reduced form of PQQ. A significant number of systems have been developed that use synthetic redox couples to shuttle an electron between the active prosthetic moiety of the immobilized enzyme and the electrode. Various sensors based on ferrocene derivatives [8] and a number of biosensors based on quinone redox polymers [10, 11] and polypyrrole derivatives [12] have been designed. Electropolymerised fluorenone coatings [13] and a number of other heterocyclic compounds [14, 15] have been tested as potential mediators.

From the structural point of vew there are two types of quinoproteins: soluble and membrane-bound [6]. *Acinetobacter calcoaceticus* L. M. D. 79.41 produces two PQQ-depending D-glucose dehydrogenases: intracellular soluble and tightly bound to the outer surface of the cytoplasmic membrane [16]. They were shown to be different enzymes with different pH optimum, molecular weight, substrate selectivity, and kinetics [16]. The membrane-bound enzyme has a membrane anchoring protein part, and entrance to the active site is partially covered [6]. It explains why membrane-PQQ-GDH cannot catalyze oxidation of disaccharides, while a soluble enzyme with the open site does it.

The increasing importance of quinoproteins in biotechnological processes requires serious investigation of the action of these enzymes in immobilized form.

This communication describes investigations of two glucose dehydrogenases: membrane-bound from *Erwinia* sp. 34–1, (m-GDH) [17], and soluble one from *Acinetobacter calcoaceticus* L.M.D. 79.41 purified according to [18] (s-GDH). The parameters of both enzymes in solution (photometric data) and the electrochemical parameters of immobilized enzymes on different electroactive surfaces are compared and discussed.

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MATERIALS AND METHODS

Chemicals and enzymes

M-GDH was purified from *Erwinia* sp. 34-1 [17] (specific activity 12 U/mg). The enzyme was used as a solution in 0.02 mol/l potassium phosphate buffer (pH 7.0) containing 10% glycerol. S-GDH from *Acinetobacter calcoaceticus* L.M.D. 79.41 was purified according to the protocol [18]. The specific activity of partially purified s-GDH was 250 U/mg protein.

4-Ferrocenylphenol (FP), 4-(4-ferrocenfenylimino-methyl)phenol (FP1) and 4-ferrocenylnitrophenol ($FcNO_2$) were synthesized as described previously [13].

MINICO M 7000 G isolating and silver MINICO M 4200 pastes were obtained from Emerson & Cuming Specialty Polymers, (Westerio, Belgium). Carbon paste on a base of carbon black RAVEN-M, obtained from Columbian Chemicals Co (Atlanta, US), was designed at the Institute of Biochemistry (Vilnius, Lithuania) [19].

0.05 M acetate buffer (pH 6.0) containing 1 mM of Ca^{2+} was used as a default buffer. 0.05 M K-phosphate (pH 6.0–8.0) and 0.05 M Tris (pH 8.0–9.0) buffer solutions containing 1 mM of Ca^{2+} were used for pH dependence measurements. All reagents used in this work were of the highest purity available. Bidistilled water was obtained using a Purator Bi (Glas Keramik, Berlin, Germany).

Instrumentation

The screen-printed carbon biosensor was designed as described earlier [20]. The working electrode was coated with 4 μl FP or FP1 (1 mg/ml in acetone) and 3 μl of m-GDH or s-GDH. The electrodes were kept for 60 min (m-GDH) or 10 min (s-GDH) under 25% glutaraldehyde water solution and covered with semipermeable terylene film.

The enzyme electrode was installed into a home-made flow-through three-electrode amperometric cell made from polymethylmethacrylate [19]. The working electrode (0.04 cm^2) formed the bottom of the flow cell. Silver wire (1 mm in diameter and 2 cm long) in saturated KCl was used as a reference electrode. Titanium tube (diameter 2 mm and length on inlet2 cm) was used as a counter-electrode.

The flow cell was supplied with a peristaltic pump (type 315, ZALIMP, Warsaw, Poland). The rate of pumping was 5 ml/min. Steady-state currents of the biosensors were recorded on an OH-105 polarograph (Radelkis, Budapest, Hungary). Photometric data were obtained on a Specord UV VIS (Germany) spectrophotometer.

RESULTS AND DISCUSSION

Native s-GDH shows maximal activity at neutral pH (Fig. 1, curve 1), while m-GDH operates at more acidic pH 5.5 (Fig. 1, curve 2). The different dependence of activity on pH indicates that both enzymes have different roles in the cell. Immobilization of both enzymes onto a carbon electrode modified with ferrocenylphenol (FP) shifted maximal activities to the alkaline region, what can be expected due to the anionic nature of the oxidized carbon surface. Native s-GDH has a 5 times higher activity than membrane-bound, but the activity of both electrodes is almost equal (Fig. 1, curves 3 and 4). The S-GDH based electrode shows stabile activity in the pH region 6–8.5. Considering the high activity of the native enzyme, we can expect that diffusion restrictions are a rate-limiting factor in this case. This conclusion was confirmed and developed by data on substrate selectivity of immobilized enzymes presented in Fig. 2. Immobilization procedure of s-GDH decreases substrate selectivity. The immobilized enzyme catalyzes the oxidation of other substrates such as galactose, arabinose and even maltose and lactose with almost the same rate as glucose. On the basis of these data we can conclude that the rate-limiting step in this case is enzyme regeneration, but not substrate conversion. Immobilization of m-GDH leads to opposite results. Immobilized m-GDH improves their selectivity. Probably enzyme immobilization shapes the active center in such a manner that the binding of galactose or arabinose becomes more complicated as compared glucose. However, this shape was not enough to open the active center for disaccharides such as lactose

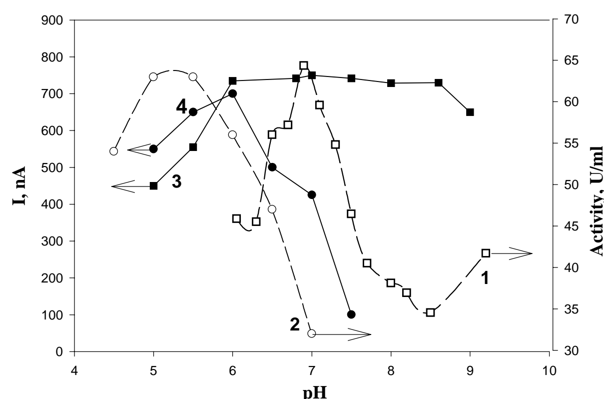

Fig. 1. pH dependence of native and immobilized PQQ-glucose dehydrogenases: *1* – specific activity of s-GDH (reduced 5 times); *2* – specific activity of m-GDH; *3* – response of carbon electrode modified with FP and immobilized s-GDH; *4* – response of the carbon electrode modified with FP and immobilized m-GDH. Concentration of substrates 0.5 mM. Electrodes potential 0.4 V

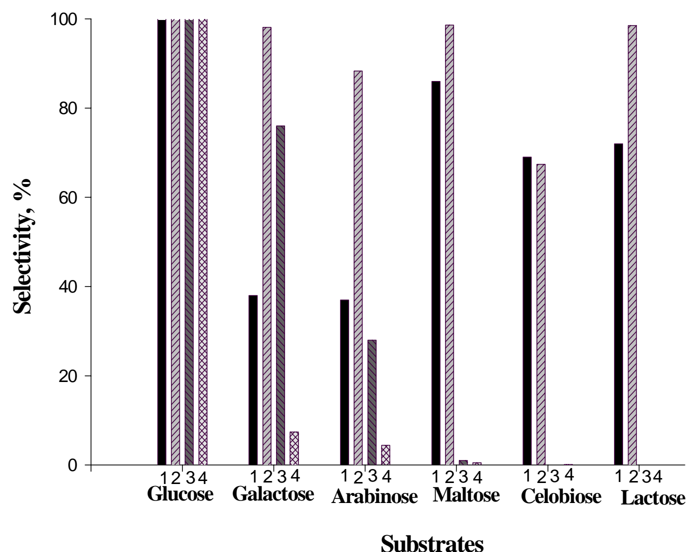

Fig. 2. Substrate selectivity of native and immobilized PQQ-glucose dehydrogenases. Column *1* of each group – native s-GDH; column *2* – s-GDH immobilized on FP modified carbon electrode; column *3* – native m-GDH; column *4* – m-GDH immobilized on FP modified carbon electrode. Electrodes potential 0.4 V. Concentration of substrates 1 mM

or maltose. Substrate conversion rate is limiting the rate of the overall process in this case.

Kinetic data on the native and immobilized enzymes are presented in Table. A certain increase in $K_{M(app.)}$ values for immobilized m-GDH indicates substrate diffusion restrictions, but they are not very high, showing a complicated mechanism of the action of the immobilized enzyme: a substrate diffusion limiting stage at low concentrations of the substrate and an enzyme regeneration limiting step at high substrate concentrations. For s-GDH we found no reliable $K_{M(app.)}$ data, probably because of the rate-limiting step of enzyme regeneration. This means that the nature of the electron acceptor plays an important role in the rate of enzyme regeneration. In both cases introduction of nitro-group into the mediating layer (Table, $FcNO_2$ line) increases the redox potential of the mediator, reduces the reaction rate of the regeneration of the reduced PQQ and leads to a low sensitivity of the biosensor. But the difference between the two other mediators is quite small from the charge density point of view, and the response of the biosensor is rather weak in the case of s-GDH and significant in the case of m-GDH. On the grounds of the data we can conclude that s-GDH adsorbs on the electrode surface quite strongly, leading to a high inactivation of the protein globule. It operates only the enzyme molecules, in which the active center (PQQ) is deposited at a close distance to the ferrocene moiety. M-GDH has a different mechanism of immobilization. The enzyme is anchored on the surface of the electrode *via* a hydrophobic anchoring subunit, and the enzyme globule operates at a certain distance from the surface. Inactivation of the enzyme globule is low, and some freedom in the movement of the enzyme globule allows exposing a more pronounced sensitivity to the different modifiers of the surface.

The stability of the protein globule was investigated at room temperature (25 °C). The rate constants of both the decrease of sensitivity to native enzymes and the decrease of biosensor response (k_i) have been calculated and used as a measure of enzyme stability. The stability of s-GDH was quite low ($k_i = 2.7 \times 10^{-3}$ min^{-1}), and the stability of m-GDH was much higher ($k_i = 0.8 \times 10^{-3}$ min^{-1} [17]). Partially purified m-GDH shows an even higher stability ($k_i = 0.23 \times 10^{-3}$ min^{-1}) [20]. Immobilization of s-GDH onto a modified carbon electrode surface decreases both the activity and the stability of the enzyme ($k_i = 3.8 \times 10^{-3}$ min^{-1}). Such a rapid inactivation of s-GDH can be explained by the loss of PQQ from the active center of the enzyme, because the presence of soluble PQQ under the terylene membrane improves stability of immobilized s-GDH ($k_i = 1.7 \times 10^{-3}$ min^{-1}). Probably deformation of the protein globule under immobilization decreases the binding of PQQ.

Our previous data showed that immobilization of m-GDH into a polyquinonic redox polymer network increased the stability of the enzyme 3.8 times [20]. Immobilization of m-GDH onto FP-modified carbon electrode surfaces stabilizes the enzyme essentially. In the first three days the sensitivity of

Table. **Kinetic parameters of the enzymes studied**

Enzyme	Electrone acceptors	K_M and $K_{M(app.)}$, mM	I_{max}, nA	r
m-GDH	FMS/DCPIP (in solution)	0.185		
m-GDH	FP1	2.43	3089	0.9956
m-GDH	FP	0.83	766	0.9986
m-GDH	$FcNO_2$	2.73	248.7	0.9969
s-GDH	FMS/DCPIP (In solution)	0.5		
s-GDH	FP1	0.73	1989	0.9903
s-GDH	FP	1.0	1968	0.9961
s-GDH	$FcNO_2$	0.89	352	0.9898

the biosensor increases almost twice due to matrix reorganization and improved contact between the enzyme globule and the matrix. Stabile activity of the biosensor is observed during the next 15 days. After 20 days of exploitation continuous degradation of enzyme activity is observed. It is a typical behavior of diffusion-controlled biosensors.

CONCLUSIONS

Soluble and membrane-bound PQQ-glucose dehydrogenases exhibit different behavior in immobilization process on the modified carbon electrodes. S-GDH strongly adsorbs on the matrix with a high inactivation of enzyme. Immobilization leads to a decrease of both enzyme stability and substrate specificity. Immobilization of m-GDH leads to stabilization of the enzyme and improvement of the substrate selectivity. Probably m-GDH is anchoring to the surface of the electrode through the anchor-subunit similar to natural binding of this enzyme into the cell membrane.

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TIRPIOS IR MEMBRANINĖS PQQ-GLIUKOZĖS DEHIDROGENAZIŲ LYGINAMOJI CHARAKTERISTIKA

S a n t r a u k a

Viduląstelinė tirpi PQQ-gliukozės dehidrogenazė, išskirta iš *Acinetobacter calcoaceticusi* L.M.D.79.41, ir membraninė PQQ-gliukozės dehidrogenazė, išskirta iš *Erwinia* sp. 34-1 kamienų, buvo palygintos tarpusavyje tirpale ir imobilizuotos ant ferocenais modifikuotų anglinių elektrodų paviršių. Abiejų fermentų elgesys imobilizacijos metu yra skirtingas. Tirpi gliukozės dehidrogenazė imobilizacijos metu stipriai inaktyvavosi, jos stabilumas ir substratinis specifiškumas sumažėjo. Manoma, kad to dalinė priežastis – PQQ difuzija iš aktyvaus centro. Tuo tarpu membraninės gliukozės dehidrogenazės imobilizacija padidino fermento stabilumą ir substratinį specifiškumą. Manoma, kad membraninė gliukozės dehidrogenazė immobilizavosi per inkarinį subvienetą, panašiai kaip ji yra įjungta į natūralias membranas.