ELSEVIER

Bioelectrochemistry 55 (2002) 21–23

Bioelectrochemistry

www.elsevier.com/locate/bioelechem

The design of dehydrogenase enzymes for use in a biofuel cell: the role of genetically introduced peptide tags in enzyme immobilization on electrodes

Catherine M. Halliwell [a], Evelyne Simon [b], Chee-Seng Toh [b], Anthony E.G. Cass [a,*], Philip N. Bartlett [b]

[a] *Department of Biochemistry, Imperial College of Science, Technology and Medicine, London SW7 2AY, UK*
[b] *Department of Chemistry, University of Southampton, Southampton SO15 5RR, UK*

Received 1 June 2001; received in revised form 7 July 2001; accepted 16 July 2001

Abstract

The immobilization of the mutants of L-lactate dehydrogenase (LDH) on poly(aniline) (PANi) composite films has been investigated. Mutants possessing peptide tags of varying charge and nucleophilicity were created to probe the nature of the interaction between the protein and PANi. These results are significant for the development of a 'generic' approach to the immobilization of enzymes and other proteins.

Keywords: Enzyme immobilization; Bioanode; Biofuel cell; Cofactor recycling; NAD^+

1. Introduction

We have investigated a number of mutants of L-lactate dehydrogenase (LDH) for their suitability as potential anodic biocatalysts in a biofuel cell [1,2]. LDH from *Bacillus stearothermophilus* is an NAD(H)-dependent oxidoreductase that catalyses the conversion of L-lactate to pyruvate with concommitant reduction of the cofactor NAD^+ to NADH [3,4]. The enzymes were genetically modified such that a number of different immobilization strategies compatible with a poly(aniline)–poly(vinyl sulfonate) (PANi–PVS) film could be investigated [5,6]. In particular, the nature of the attachment of the enzyme to the PANi-modified electrode was investigated through the introduction of peptide tags on LDH. The activities of the tagged LDH enzymes, once immobilised, were investigated using chronoamperometry.

The mutants that were constructed possessed either an N- or C-terminal (poly)histidine tag (LDH–NHis or LDH–CHis) to aid purification and immobilization [7]. Previously, it has been reported that PANi films can be readily modified covalently by thiol groups at the ortho-position with concommitant reduction to the leucoemeraldine state [8]. Therefore, a mutant possessing an exposed C-terminal cysteine (LDH–CCys) was created in order to facilitate the covalent attachment of LDH to PANi. The wild type and the N-terminal histidine-tagged LDH lack any solvent accessible cysteine residues [4]. These enzymes were then immobilised on PANi–PVS and their substrate-dependent currents were recorded. A clearer understanding of this interaction may lead to the development of more stable enzyme films on electrodes through the engineering of the protein to complement the film chemistry and to be particularly useful in the development of biosensors or other bioelectrodes.

2. Experimental

Sodium lactate, NAD^+ (98%) and poly(vinyl sulfonic acid sodium salt) were purchased from Aldrich. Mutagenesis, protein expression and purification were performed as described previously [9]. PANi–PVS composite films were deposited and conditioned on a glassy carbon electrode with an area of 0.38 cm^2 as described previously [5]. The PANi–PVS electrode was incubated in an enzyme solution (0.5

* Corresponding author. Tel.: +44-20-7594-5195; fax: +44-20-7594-5196.
E-mail address: t.cass@ic.ac.uk (A.E.G. Cass).

1567-5394/02/$ - see front matter
PII: S1567-5394(01)00172-4

mg/ml in Tris/HCl, 100 mM, pH 7.1) for 2 h at 4 °C. It was rinsed extensively before placing in the electrochemical cell. Chronoamperometric experiments were recorded using an EG&G 263A. The electrochemical cell was thermostatted at 35 °C and contained 10 ml Tris/HCl, 1.0 M, pH 7.1 and 30 mM NAD^+. A platinum gauze counter-electrode was used and all potentials are vs. a saturated calomel electrode.

3. Results and discussion

The three mutants, LDH–NHis, LDH–CHis and LDH–CCys, as well as the wild-type LDH were immobilised on poly(aniline)–poly(vinyl sulfonate) films and placed in a solution of the cofactor NAD^+. Aliquots of lactate were then added and the resulting currents were measured. These currents were approximately proportional to the lactate concentration of up to 0.4 M, which is consistent with those arising from enzymatically generated NADH. The catalytic current densities for each LDH variant are shown in Fig. 1. The current obtained for LDH–CCys was the highest and that of the wild type was the lowest, which is opposite to the trend observed for the solution kinetics. Previously, we have shown that the k_{cat} values for LDH–NHis and wild-type LDH are similar (140 and 135 s^{-1}, respectively), while the introduction of a C-terminal hexahistidine tag significantly reduced the specific activity to 90 s^{-1} [9]. The specific activity of LDH–CCys was lower still at 50 s^{-1}.

These amperometric results indicate that the peptide tags play a role in the enzyme immobilization on PANi–PVS composite films. The NADH-dependent current was much higher for LDH–CHis than for the wild-type LDH despite the latter's (~33%) higher specific activity. This is consistent with more LDH–CHis than wild-type LDH being immobilised. It would be reasonable to assume that the same immobilization chemistry would occur for both LDH–NHis and LDH–CHis, and therefore, equal quantities of these two would be immobilised on PANi–PVS. However, the current for LDH–NHis was not as high as that predicted from the differences in the activity between LDH–NHis and LDH–CHis, assuming equal masses of the two were loaded. The currents might indicate that relatively more LDH–CHis than LDH–NHis was immobilised. Despite the considerable lowering of the specific activity of the LDH–CCys, this variant produced the highest catalytic current on immobilization. This is consistent with the greatest mass load of LDH onto the PANi–PVS being achieved with this mutant.

The greater mass loading of all three mutants compared to the wild-type LDH may be due to the increased interactions between the mutant LDH and the PANi–PVS composite film. These interactions could be electrostatic or covalent in nature. Scanning electron microscopy of PANi–PVS reveals that the film is dense and fibrous [10]. The PANi polymers are extensively protonated when the film is in the conducting emeraldine state, and the long PVS counter-anions associated with PANi maintain the overall electroneutrality across the film. However, regions of localised charges exist throughout the film. Therefore, it is reasonable to assume that the electrostatic interactions between the LDH and the film play an important role in immobilization. At pH 7.1, approximately 25% of the histidine residues are protonated [11], corresponding to a charge difference of +1.5 between each hexahistidine-tagged LDH monomer and the wild-type monomer. The active form of LDH is a tetramer [4], so this charge difference is significant (+6) and consistent with more of the histidine-tagged mutant than the wild-type LDH being immobilised due to the increased charge on the protein.

The LDH–CCys also possesses an N-terminal hexahistidine tag (to aid purification), and some of its increased loading on the PANi–PVS film may be derived from this.

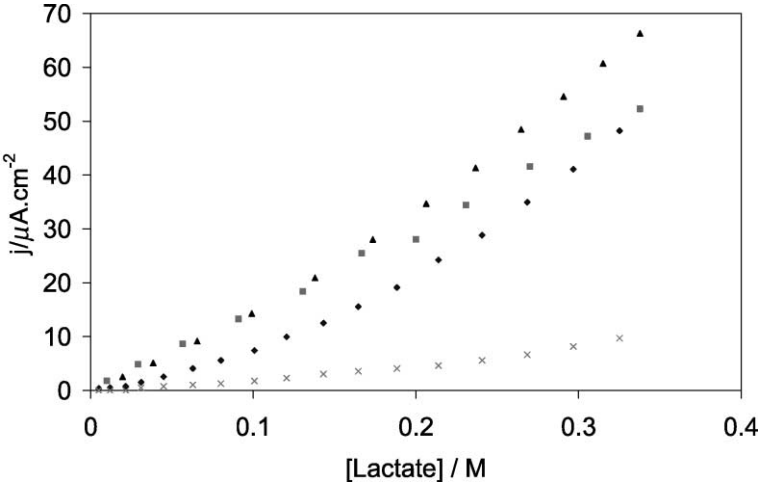

Fig. 1. Current recorded at +0.1 V vs. SCE for PANi–PVS-coated glassy carbon electrodes (deposition charge ~200 mC in all cases, area = 0.38 cm^2) at 9 Hz in 1 M Tris/HCl buffer, pH 7.1, 35 °C. The enzymes immobilised on the electrodes are LDH–CCys ▲, LDH–NHis ■, LDH–CHis ♦ and wild–type LDH ×.

However, the cysteine tag may also provide a means of covalently attaching LDH–CCys to PANi via its nucleophilic thiol group. The wild-type LDH and the histidine-tagged variants lack any highly nucleophilic surface residues. This introduced thiol group is readily oxidised (causing the protein to form oligomers that readily precipitate) in the absence of high salt concentrations. Indeed, if LDH–CCys is oxidised prior to immobilization on the electrode, the resulting current is low (data not shown). In addition, PANi composite films have been shown to be modified at the ortho-position of the diiminoquinoid ring [8] by organic thiol groups such as mercaptoproprionic acid. Therefore, an additional covalent interaction between the LDH–CCys and PANi–PVS may account for the increased current (through increased protein loading) for this mutant.

4. Conclusions

We have demonstrated that mutants of LDH can be readily immobilised on PANi–PVS and retain their specific activities towards the substrate and NAD^+. Furthermore, enzymatic loading can be dramatically improved by the addition of either a polyhistidine or cysteine residue despite the changes in the specific activity these mutations cause. These PANi–enzyme electrodes are potentially of great use in biosensor and biofuel cell applications, in particular, where the efficient coupling of cofactor oxidation and recycling are required at the electrode, for example, to achieve high current densities and transport-limited currents.

Acknowledgements

This study was funded by the US Office of Naval Research. CMH wishes to thank The Royal Academy of Engineering, UK, for an International Travel Grant in order to attend BEB2001.

References

[1] I. Willner, G. Arad, E. Katz, A biofuel cell based on pyroloquinoline quinone and microperoxidase-11 monolayer-functionalized electrodes, Bioelectrochem. Bioenerg. 44 (1998) 209–214.
[2] G.T.R. Palmore, H. Bertschy, S.H. Bergens, G.M. Whitesides, A methanol/dioxygen biofuel cell that uses NAD^+-dependent dehydrogenases as catalysts: application of an electro-enzymatic method to regenerate nicotinamide adenine dinucleotide at low overpotentials, J. Electroanal. Chem. 443 (1998) 155–161.
[3] D.A. Barstow, A.R. Clarke, W.N. Chia, D. Wigley, A.F. Sharman, J.J. Holbrook, T. Atkinson, N.P. Minton, Cloning, expression and complete nucleotide sequence of the *Bacillus stearothermophilus* L-lactate dehydrogenase gene, Gene 46 (1986) 47–55.
[4] D.B. Wigley, S.J. Gamblin, J.P. Turkenburg, E.J. Dodson, K. Piontek, H. Muirhead, J.J. Holbrook, Structure of a ternary complex of an allosteric lactate dehydrogenase from *Bacillus stearothermophilus* at 2.5a resolution, J. Mol. Biol. 223 (1992) 317–335.
[5] P.N. Bartlett, P.R. Birkin, E.N.K. Wallace, Oxidation of beta-nicotinamide adenine dinucleotide (NADH) at poly(aniline)-coated electrodes, J. Chem. Soc., Faraday Trans. 93 (1997) 1951–1960.
[6] P.N. Bartlett, E. Simon, Poly(aniline)–poly(acrylate) composite films as modified electrodes for the oxidation of NADH, Phys. Chem. Chem. Phys. 2 (2000) 2599–2606.
[7] M. Mejare, G. Lilius, L. Bulow, Evaluation of genetically attached histidine affinity tails for purification of lactate dehydrogenase, Plant Sci. 134 (1998) 103–114.
[8] C.-C. Han, W.-D. Hseih, J.-Y. Yeh, H. Shih-Ping, Combination of electrochemistry with concurrent reduction and substitution chemistry to provide a facile and versatile tool for preparing highly functionalized polyanilines, Chem. Mater. 11 (1999) 480–486.
[9] C.M. Halliwell, G. Morgan, C.P. Ou, A.E.G. Cass, The introduction of a (poly)histidine tag in L-lactate dehydrogenase produces a mixture of active and inactive molecules, Anal. Biochem. 295 (2001) 257–261.
[10] P.N. Bartlett, E.N.K. Wallace, The oxidation of beta-nicotinamide adenine dinucleotide at poly(aniline)-coated electrodes: Part II. Kinetics of reaction at poly(aniline)–poly(styrenesulfonate) composites, J. Electroanal. Chem. 486 (2000) 23–31.
[11] D. Voet, J.G. Voet, Biochemistry, 2nd edn., Wiley, New York, 1995, pp. 56–62.