Utilization of adsorption effects for the continuous reduction of $NADP^+$ with molecular hydrogen by *Pyrococcus furiosus* hydrogenase

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Received 17th June 2003
First published as an Advance Article on the web 3rd October 2003

The use of hydrogenases for the direct reduction of cofactors with molecular hydrogen provides a means to utilize this clean and strong reducing agent for use in reductive biotransformations. The hydrogenase I from *Pyrococcus furiosus* is a promising enzyme for the production of NADPH from $NADP^+$ with molecular hydrogen. We observed adsorption of the active enzyme by using cyclic voltammetry. Our investigations revealed an electroactive enzyme species adsorbed onto the surface of the working electrode. This facile route for immobilisation of the active enzyme was subsequently utilized for the continuous reduction of $NADP^+$ to NADPH with molecular hydrogen in a continuously operated fluidized bed reactor with hydrogenase adsorbed on graphite beads. The reactor was operated at low conversion for more than 40 residence times (80 h). The apparent retention was found to be 98% on unmodified graphite carriers.

Introduction

Hydrogenases are enzymes capable of activating dihydrogen. They are found in numerous microorganisms. In the habitats of hyperthermophilic microorganisms where hydrogen is abundant, hydrogenases play an important role in hydrogen metabolism. The majority of these enzymes contain different metal clusters,[1] which are responsible for the redox capabilities.

The hydrogenase I from the hyperthermophilic archaeon *Pyrococcus furious* (*Pf* hydrogenase)[2] (EC 1.18.1.99) shows interesting redox properties, particularly the activation of molecular hydrogen. It is capable of catalyzing the direct reduction of $NADP^+$ to NADPH with molecular hydrogen.[3,4] Although the exact physiological role in the archeon is still unknown,[5] the enzyme shows *in vitro* activity towards the activation of dihydrogen, and has been utilized in biotransformations for dihydrogen production and reduction of dyes.[3,4,6]

We are interested in the *Pf* hydrogenase especially in its ability to reduce pyridine nucleotides and for utilization in biotransformations by means of cofactor regeneration.[7–10] For this purpose it is necessary to investigate the catalytic synthesis of the reduced cofactor as the first step of the reaction.[11] Utilization of hydrogen for reduction of $NADP^+$:

$$NADP^+ + H_2 \rightarrow NADPH + H^+$$

would provide a cheap and clean source for the generation and regeneration of the reduced phosphorylated nicotinamide cofactor.

Electrochemistry has proven to be a powerful technique for the investigation of these redox active enzymes. Electrochemical investigation of *Pf* hydrogenase has also been carried out in combination with other analytical techniques[12] and along with other metalloproteins of *Pf*.[13,14] Hydrogenases from other sources have been investigated electrochemically with respect to their redox or electrochemical properties[15] (*Alcaligenes eutrophus*,[16] *Thiocapsa roseopersiciana*,[17] *Thermococcus celer*,[18] *Desulfovibro vulgaris*,[19] *Chromatium vinosum*,[20] and *Megaspherae elsdinii*[21]).

Furthermore, the direct electrochemistry of immobilized enzymes has been demonstrated to provide means for the electrochemical generation and regeneration of NADH. This has been performed with isolated hydrogenase from *Alcaligenes eutrophus* by preparation of catalytic films,[22] and electrodes modified with whole cells of *Desulfovibro gigas*.[23] To yield an immobilized electroactive catalyst on the surface of the electrode, these approaches require the binding of the catalytically active species by laborious methods. Lately adsorption of the subcomplex Iα of mitochondrial NADH:ubiquinone oxidoreductase was demonstrated.[24]

We carried out cyclic voltammetric experiments to reveal whether the *Pf* hydrogenase shows adhesive properties. By means of cyclic voltammetry adsorption can be detected, since the observation of "line-crossing" phenomena can be ascribed to adsorption of electroactive species on the electrode surface.[25]

Methods and material

All chemicals were of the best available quality and obtained from Sigma (Steinheim, Germany), if not stated otherwise.

Green Context

NADPH is a potentially very useful reductant which is formed by reduction of $NADP^+$. This paper demonstrates the utility of an enzyme to carry out this reduction, in a continuous manner, using molecular hydrogen. The enzyme is readily adsorbed onto an electrode where it is active in the reduction of $NADP^+$. This observation has potential in a number of applications. ***DJM***

$NADP^+$ (sodium salt) was obtained from Jülich Fine Chemicals, (Jülich, Germany). Deionized water was obtained by means of nanofiltration (Milli-Q académic, Millipore, Eschborn, Germany). Gases of 99.9990% purity were obtained from Messer (Krefeld, Germany). All buffers were degassed with helium and handled under an inert atmosphere of argon or nitrogen by standard Schlenk techniques. Protein content was determined by the method described by Sedmark *et al.*[31]

Concentrations of $NADP^+$ and NADPH were determined by capillary electrophoresis,[30] with uridine as the internal standard after dilution if appropriate. Standard conditions were: 40 mM potassium phosphate, 10 mM borate buffer, pH = 8.5, 30 kV, 40 °C, capillary: uncoated silica (50 cm total length, 43 cm to detection window, 50 μm inner diameter), typical migration times: uridine 2.9 min, $NADP^+$ 7.1 min, NADPH 9.9 min.

The enzyme preparation was derived following the procedure described by Haaker *et.al.*[3] The cell free extract was purified in one chromatographic step and the resulting crude enzyme preparation was concentrated *via* ultrafiltration to a protein content of 58 mg mL^{-1}. The enzyme preparation was used without further purification.

The cyclic voltammetric experiments were performed on a BAS 100 B/W Version 2.3 Electrochemical Workstation (Bioanalytical Systems, West Lafayette, Indiana, USA) using an airtight thermostated three-electrode electrochemical cell. The working electrode was a glassy carbon disk (3 mm diameter), while the counter electrode was a platinum wire. All potentials were measured and quoted *vs.* Ag|AgCl|3 M KCl (+0.21V *vs.* SHE) as the reference electrode. Before the addition of enzyme, the working electrode was polished mechanically to a mirror finish using 0.5 μm alumina powder and then rinsed with deionized water. The experiments were performed using a solution of 340 μg *Pf* hydrogenase preparation in 2 mL of 100 mM potassium phosphate buffer, pH = 8. The experiments were firstly carried out under an atmosphere of nitrogen (80 °C), which was subsequently replaced by hydrogen. During the experimental series the temperature was kept constant and the sweep rate varied from 8 to 100 mV s^{-1}.

The continuously operated fluidized bed reactor was adapted from a setup described by Biselli *et al.*[29] for cell culture, and slightly modified for use under oxygen-free conditions. The flow sheet is shown in Fig. 1. The total volume of the reactor

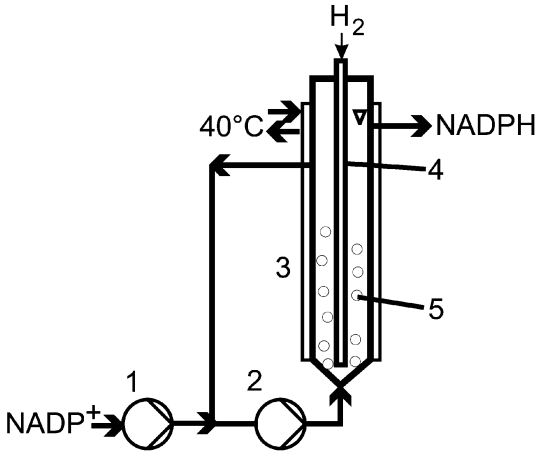

Fig. 1 Flow sheet of reactor setup: (1) dosing pump, (2) circulation pump, (3) glass cone with thermostating jacket, (4) tubular PTFE membrane, (5) graphite or glass beads.

was 25 mL. Hydrogen was supplied *via* a non-porous (dense) tubular PTFE membrane (**4**) (OD = 3.1 mm, ID = 2.2 mm) of 10 cm length in the center of the fluidized bed. The fluidized bed reactor (**3**) was constructed of glass. All connections and tubings were made of steel using viton sealings. A magnetic coupled gear pump (**2**) (Verder, Austria) was used to establish circulation, and a P-500 piston pump (**1**) (Amersham Pharmacia Biotech, Freiburg, Germany) equipped with a mass flow metering system (not shown) (Bronkhorst, Ruurlo, Netherlands) acted as a dosing pump as described previously.[32] The reactor was thermostated to 40 °C. The beads (**5**) were either graphite (Sigradur®, SGL Carbon, Bonn, Germany, 0.4–0.6 mm in diameter and used without further purification) or glass (unmodified porous SIRAN®, Schott, Germany, 0.7–1.0 mm in diameter, which was achieved by sieving as described previously[29]). The reactor was flushed with 10 volumes (250 mL) of degassed and deionized water prior to use. Subsequently, the feed was changed to 12 mM $NADP^+$ in 100 mM potassium phosphate buffer (pH = 8) for 5 volumes (125 mL) at a flow rate of 12.5 mL h^{-1}. The central tubular PTFE membrane was flushed with hydrogen; and pressurized to a inner pressure of 4 bar during the experiment. The outflow was collected in fractions and analyzed for yield and conversion by means of capillary electrophoresis, and for protein content.

The experiments were started by adding 0.42 mL of the hydrogenase preparation (protein content 58 mg mL^{-1}), equivalent to 1.0 mg mL^{-1} protein content in the reactor. Prior to the addition the hydrogenase was activated following the published procedure protocol[3] by exposure to a hydrogen atmosphere at 80 °C. During the experiment the flow rate of the dosing pump was kept constant at 12.5 mL h^{-1}, resulting in a residence time of τ = 2 h. The flow rate in the circulation loop was adjusted to a level where no beads were driven out of the fluidized bed (1–2 L min^{-1}).

Results and discussion

The cyclic voltammetric investigations were carried out to reveal if the absorbed *Pf* hydrogenase absorbed onto surfaces is electrochemically active. Cyclic voltammetric experiments were performed first under an atmosphere of nitrogen, which was then replaced by hydrogen. An airtight cell was used with a standard three electrode setup, in which oxygen free conditions could be maintained. After introduction of hydrogen, the cyclic voltammetric experiments of the enzyme preparation of *Pf* hydrogenase showed a line-crossing phenomenon in the presence of hydrogen as depicted in Fig. 2. This line form is evidence for adsorption of an electrochemically active species.[25,26]

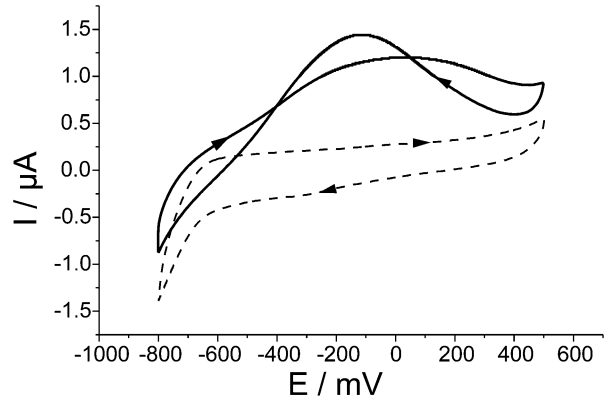

Fig. 2 Cyclic voltammograms of hydrogenase under nitrogen atmosphere (dashed line) and in the presence of hydrogen (solid line), sweep direction is indicated (80 °C, 8 mV s^{-1}, glassy carbon, *vs.* Ag|AgCl, other conditions see text, anodic current has positive sign).

We assign the adsorbed species to the $NADP^+$ reducing activity, as selective immobilization during the continuous experiments was observed (see below). It can then be deduced that hydrogenase is adsorbed and probably capable of direct electron transfer. The adsorption might be considered as

electrodeposition of a metalloprotein following the activation with hydrogen.

Further evidence for adsorption can be taken from the observation that the peak current of the backward scan increased, when the sweep rate is decreased (Fig. 3). The peak

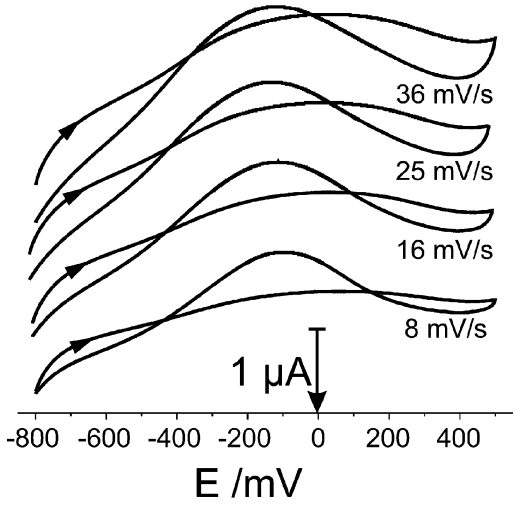

Fig. 3 Cyclic voltammograms (superimposed) of hydrogenase in the presence of hydrogen at different velocities (80 °C, 8–36 mV s^{-1}, glassy carbon, *vs.* Ag|AgCl, other conditions see text, anodic current has positive sign), forward sweep direction is indicated.

current corresponds to the amount of electroactive species deposited during the forward sweep due to the reaction with hydrogen. Hydrogen could be eliminated as the cause for the line-crossing by independent experiments, and by changing the sweep direction which essentially gave the same results (data not shown).

Activation of the enzyme is most probably due to reduction by hydrogen at 80 °C[14,33] to form an electrochemically active enzyme species. A possible explanation might be a reorganization or reconstitution of the enzyme subunits as reported for other hydrogenases (*e.g. Alcaligenes eutrophus* H16,[34] *Rhodococcus opacus*[35]). An altered structure might also lead to the exposure of a hydrophobic surface on the enzyme, which would be a possible explanation for adsorption.

However, only the adsorption of an electrochemically active species on the surface of the electrode can be deduced from the cyclic voltammetric experiments alone, since the hydrogenase might not be the only electroactive species in the preparation used in investigations.[3]

Motivated by the observation of the adhesive properties of the enzyme preparation, attempts were made to utilize them for immobilization of the active enzyme. A setup was used with a fluidized bed where hydrogen was supplied *via* a dense PTFE (polytetrafluoroethylene) membrane (Fig. 1). The setup was chosen because conversion is directly correlated with enzyme activity in a continuously operated stirred tank reactor (CSTR). Therefore the input concentration of $NADP^+$ was set to 12 mM to operate the reactor in a region where the linear correlation of enzymatic activity holds true. This is opposed to operating at lower concentration with higher conversion where the amount of enzyme is not the limiting parameter. Thus conversion can be linearly correlated to the retention and stability of the enzyme. In order to synthesize under these conditions the unstable NADPH intermediate, a flow scheme closer to plug flow conditions would be superior, but then enzyme activity would not be correlated with conversion. The fluidized bed reactor was preferred to a fixed bed reactor for better mass transport of dissolved hydrogen, avoiding concentration gradients over the cross-section of the bed.[27–29]

Immobilization experiments were carried out with beads of graphite or glass. With graphite beads the reactor could be operated for more than 80 h (number of residence times more than 40) at a low conversion level, ensuring linear correlation of conversion and activity. A retention of 98% was obtained; whereas the retention on glass carriers was found to be too low for technical application. The decreased affinity to glass beads is probably due to surface specific adsorption. Overload of the immobilization matrix would lead to another curve form which cannot be described as exponential decay. Conversion and the relative protein content as a function of the number of residence times (time τ) for graphite beads as the immobilization matrix are shown in Figs 4 and 5 respectively. In Fig. 6 the conversion

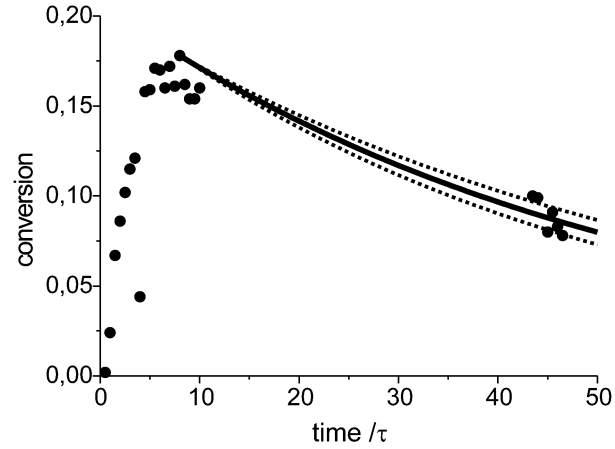

Fig. 4 Conversion of reactor outflow as a function of the number of residence times with immobilization on graphite beads. The straight line gives a relative retention value of 0.981, dotted lines represent the standard deviation of ±0.002. (τ = 2 h, 40 °C, pH = 8, [*Pf* hydrogenase]0 = 1 mg mL^{-1}, Inlet [$NADP^+$] = 12 mM).

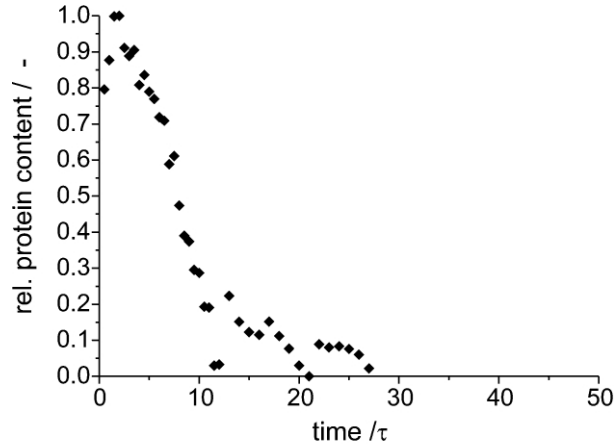

Fig. 5 Relative protein content of reactor outflow as function of the number of residence times. After t/τ = 30 protein content was below the detection limit (τ = 2 h, 40 °C, pH = 8, [*Pf* hydrogenase]0 = 1 mg mL^{-1}, Inlet [$NADP^+$] = 12 mM).

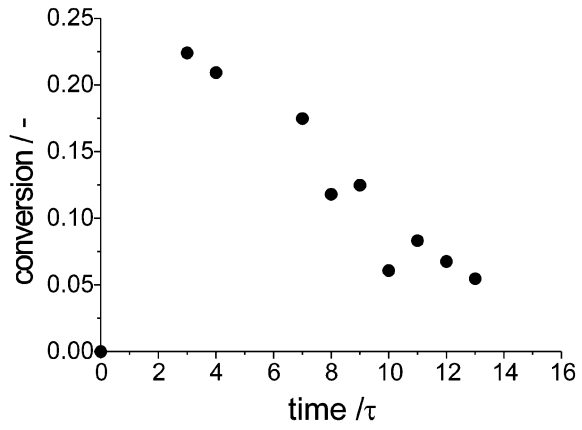

Fig. 6 Conversion of reactor outflow as a function of the number of residence times with immobilization on glass beads (τ = 2 h, 40 °C, pH = 8, [*Pf* hydrogenase]0 = 1 mg mL^{-1}, Inlet [$NADP^+$] = 12 mM).

for the immobilization on glass beads is shown. Generally, the obtained yield is lower than the conversion (data not shown) due to instability of NADPH under the reaction conditions.[36] It is noteworthy that the maximum conversion level is equal to a NADPH concentration of about 2 mM in both cases. So, presumably, operating at lower inlet concentrations of $NADP^+$ would have given higher conversion levels. However, the drastic loss of activity, as observed in the case of the glass beads, would not have been observable, because enzyme activity would not have been limiting. The decrease in conversion for the graphite beads can be described by exponential decay, with a retention value of $r = 0.981 \pm 0.002$, as depicted in Fig. 4. However, it should be noted that this description is weak due to a lack of measurements. This is mirrored by the relative large confidence bands. The protein content in the outflow of the reactor does not correlate with the remaining activity in the reactor. Consequently the $NADP^+$ reducing species is bound to the surface. Also the apparent loss of activity of 2% per residence time (1% per hour) is in the order of magnitude of deactivation for the hydrogenase (data not shown). It is therefore plausible, that the true retention value due to adsorption is greater. For conditions optimized for enzyme stability, longer run times are possible.[4]

Conclusions

By conformation of the adsorption of hydrogenase activity by immobilization and continuous application in a fluidized bed, the adsorbed electrochemical active species can be correlated with hydrogenase activity. Adsorption onto surfaces provides a method for the immobilization of the active enzyme. However, the adsorption as a tool for immobilization has to be investigated further. It may provide means for applications in bio fuel cells without the need of an additional immobilization matrix, since the cyclic voltammetric experiments show that the adsorbed species is electroactive. Thus further experiments have to be conducted to reveal whether the adsorption might be considered as electrodeposition.

The operating stability of the hydrogenase was demonstrated by the continuous application. Furthermore, the results show that hydrogenase I from *Pyrococcus furiosus* can be used for the continuous synthesis of NADPH by reduction of $NADP^+$ by molecular hydrogen. Therefore the reactor has to be optimized for yield in a true attempt for the synthesis of NADPH. Also other modes of recycling for the promising enzymatic catalyst are the subject of current research.[4]

Acknowledgements

We wish to thank Rita Mertens (Forschungszentrum Jülich, Germany), Eyke van den Ban, and Hans Wassink (University of Wageningen, Netherlands) for the kind gift of *Pf* hydrogenase preparation and discussion; Huub Haaker and Colja Laane (University of Wageningen) for discussion; Christian Reimers and Lilia Härter (Forschungszentrum Jülich) for skillful assistance; and Christian Wandrey (Forschungszentrum Jülich) for support of this project.

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