Technical Notes

Amperometric Sensing at High Temperature with a "Wired" Thermostable Glucose-6-phosphate Dehydrogenase from *Aquifex aeolicus*

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An amperometric enzyme sensor capable of operating at high temperatures was developed by utilizing a "wired" thermostable glucose-6-phosphate dehydrogenase (tG6PDH) from the hyperthermophilic bacterium *Aquifex aeolicus*. The response of the system was monitored through detection of the product of the enzymatic reaction, NADH, which was electrocatalytically reoxidized to NAD by a thermostable redox mediator, osmium (1,10-phenanthroline-5,6-dione)$_2$-poly(4-vinylpyridine), at $E_{app} = +150$ mV vs Ag/AgCl/KCl$_{sat}$. The enzyme was "wired" onto the surface of graphite electrodes by using an epoxy-based poly(ethylene glycol) diglycidyl ether cross-linker. The stability of the sensor at higher temperatures clearly surpassed the conventional system utilizing a mesophilic G6PDH (mG6PDH) from *Leuconostoc mesenteroides*. The mG6PDH-based system lost 26% of its response after 20 min at 50 °C. The response of the tG6PDH-based system remained unchanged under the same conditions. The tG6PDH-based system demonstrated excellent stability up to a temperature of 83 °C.

Enzymes isolated from thermophilic microorganisms, termed thermostable or thermophilic enzymes, have received a lot of attention because of their ability to catalyze biological reactions at high temperature.[1−3] Thermostable enzymes not only withstand heat but also typically resist denaturation in the presence of organic solvents and detergents.[4] From an application perspective, such thermostable enzymes are attractive because of their remarkable stability, thereby increasing the operational and storage stability of the devices that incorporate them. One of the most important advances in biotechnology during the last two decades is the development of the polymerase chain reaction

(PCR).[5,6] *Thermus aquaticus* DNA polymerase (*Taq* DNA polymerase) was the first thermostable DNA polymerase characterized[7,8] and facilitated the automation of PCR. Biosensors is another area that could greatly benefit from the availability of a stable biological component. For example, thermostable enzymes have been employed in biosensing systems for the determination of compounds such as phenol and asparagine using thermostable phenol hydroxylase and thermostable asparaginase, respectively,[9,10] and for assaying analytes such as glucose and lactate by a coupled-enzyme system utilizing a thermostable soybean peroxidase.[11]

Dehydrogenases are used in electrochemical biosensing systems for a wide variety of analytes. The detection of analytes in amperometric systems employing dehydrogenases is based on the signal generated by oxidation of the cofactor NADH or NADPH at the electrode surface. However, the direct electrochemical oxidation of NAD(P)H on conventional electrodes requires high overpotentials[12,13] necessitating the use of "mediators" to facilitate the hydride transfer. This becomes more challenging with dehydrogenases from thermophilic sources, as they require a mediator capable of operating at elevated temperatures.

Thermostable glutamate dehydrogenase and a polymeric toluidine blue O mediator have been used for the amperometric detection of glutamate at temperatures up to 60 °C.[14] The system was optimized using carbon paste electrodes in batch mode and further developed for flow injection analysis.[15] The oxygen-

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independent thermostable diaphorase from *Bacillus stearothermophilus* and thermostable NADH oxidase from *Thermus aquaticus* were used for amperometric NADH determination[16] or were coupled with dehydrogenases in the design of sensing systems for a variety of analytes.[17,18] The mediation was carried out by ferrocene-based compounds, but the sensors were not evaluated at temperatures of >25 °C. Although, there has been a considerable amount of research on mediators that can effectively recycle NADH/NADPH at the electrode surface,[19] most currently available mediators have poor stability at elevated temperatures. Therefore, mediator stability is, presumably, one of the obstacles in the development of thermostable dehydrogenase based biosensor.

Herein, we report an amperometric enzyme electrode utilizing a "wired" thermostable glucose-6-phosphate dehydrogenase (tG6PDH) from *Aquifex aeolicus*, for the determination of glucose 6-phosphate. To address mediator stability, we have used a novel redox mediator, osmium (1,10-phenanthroline-5,6-dione)$_2$−poly-(4-vinylpyridine) (Osphendione−PVP), for the electrocatalytic oxidation of NADH. The mediator was immobilized on oxidized graphite electrodes where the phendione moiety allows for shuttling electrons between the graphite electrode and NADH. Enzyme immobilization was achieved by cross-linking the amine groups of lysine residues with an epoxy-based poly(ethylene glycol) diglycidyl ether (PEGDGE) cross-linker. The performance of the system was compared to a system utilizing the glucose-6-phosphate dehydrogenase from the mesophilic microorganism *Leuconostoc mesenteroides*.

EXPERIMENTAL SECTION

Chemicals. Glucose-6-phosphate dehydrogenase from the mesophile, *L. mesenteroides* (mG6PDH), glucose 6-phosphate, and β-nicotinamide adenine dinucleotide (NAD) were purchased from Sigma (St. Louis, MO). PEGDGE and poly(4-vinylpyridine) (MW 40 000) were purchased from Polysciences (Warrington, PA). Graphite rods (3 mm in diameter) were purchased from Carbone of America (Bay City, MI). All other reagents were of analytical grade. Deionized distilled water was obtained from a Milli-Q system (Millipore, Milford, MA). The isolation and purification of tG6PDH from *A. aeolicus* is described elsewhere.[20] The synthesis of the bromoethylamine-modified poly(4-vinylpyridine) (binder polymer) has been published.[21]

Synthesis of Osmium (1,10-Phenanthroline-5,6-dione)$_2$−Poly(4-vinylpyridine) Mediator. The mediator was synthesized by refluxing a mixture of 80 mg of [Os(1,10-phenanthroline-5,6-dione)$_2$Cl$_2$] and 50 mg of poly(4-vinylpyridine) in 3 mL of ethylene glycol under argon in darkness for 1 h at 220 °C. The reaction mixture was cooled to room temperature and was added to 100 mL of aqueous 1 M NaCl over a period of 15 min. After cooling the mixture overnight at 4 °C, the resulting precipitate was filtered, washed with water, and air-dried. The UV spectrum of Osphen-
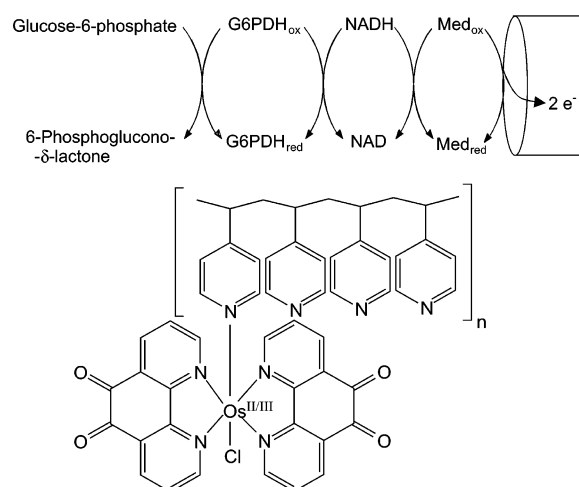

Figure 1. Reaction scheme for the mediated electrodes and chemical structure of the Osphendione−PVP mediator. The counterions necessary for charge balance are not shown (presumably, there is an additional one or two chlorine counterions depending on whether osmium is in the +2 or +3 oxidation state, respectively).

dione−PVP in ethylene glycol showed peaks due to the osmium complex at 361 and 467 nm and the pyridine backbone-dependent peaks at 250 and 298 nm. The proposed structure of the resulting redox polymer is shown in Figure 1. Anal. Calcd: C, 61.52%; H, 1.76%; N, 10.99%. Found: C, 61.71%; H, 1.70%; N, 10.7%.

Preparation of Electrodes. A graphite rod was wet polished on fine (grit 400 and 600) emery paper. The bars were cleaned by sonication for 5 min in water and oxidized by applying a potential of 1 V versus Ag/AgCl/KCl$_{sat}$ during 30 s in 0.100 M NaH$_2$PO$_4$/HCl, 0.500 M NaCl, pH 7.00. The electrodes were washed with water and dried at 60 °C for 10 min. A volume of 1.0 μL of 1.8 mg/mL Osphendione−PVP in ethylene glycol was deposited per electrode and washed with 30 μL of ethylene glycol and plenty of water. The sensing layer was prepared by placing on each graphite electrode 5.0 μL of the mixture composed of 50 μL of 10 mg/mL binder polymer solution, 4.0 mg of NAD, 16.6 μL of 6.75 mg/mL PEGDGE aqueous solution, and 68 μL of tG6PDH (20 units/mL at 70 °C) or 83 μL of mesophilic G6PDH (40 units/mL at 25 °C) in 0.100 M NaH$_2$PO$_4$/HCl, 0.500 M NaCl, pH 7.00. The electrode was dried at room temperature under vacuum for 24 h.

Measurements. Electrochemical experiments were performed using a PAR 273 potentiostat and model 270 software (EG&G Princeton Applied Research, Princeton, NJ). All measurements were performed using a water-jacketed electrochemical cell in 2.0 mL of 0.100 M NaH$_2$PO$_4$/HCl, 0.500 M NaCl, pH 7.00. Cyclic voltammetry (CV) measurements were carried out in a conventional three-electrode electrochemical cell. Ag/AgCl/KCl$_{sat}$ and a platinum wire served as reference and counter electrode, respectively. Chronoamperometry experiments were performed at an applied potential of +150 mV versus Ag/AgCl/KCl$_{sat}$. To evaluate the effect of temperature on biosensor response, the electrodes were heated from room temperature to 83 °C, while current densities were monitored at fixed intervals. All pH values are at room temperature, unless otherwise stated.

RESULTS AND DISCUSSION

Dehydrogenases are a class of enzymes that catalyze a wide variety of reactions that are useful in biosensors/diagnostics and

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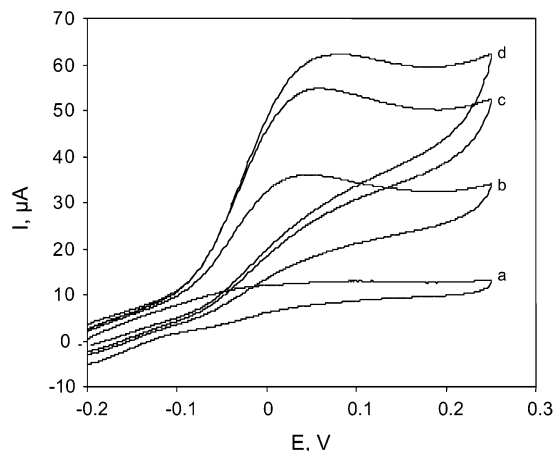(21) Katakis, I. Ph.D. Dissertation, The University of Texas, Austin, 1994.

Figure 2. Cyclic voltammograms of immobilized Osphendione−PVP at 70 °C in 0.100 M Na_2HPO_4/HCl, 0.500 M NaCl, pH 7.00 containing (a) no NADH (b) 2.4 mM NADH (c) 4.7 mM NADH, and (d) 6.9 mM NADH. Scan rate 30 mV/s. Potentials vs Ag/AgCl/KCl_{sat}.

Figure 3. Calibration curve for tG6PDH-based biosensing system in 0.100 M Na_2HPO_4/HCl, 0.500 M NaCl containing 30 mM NAD, pH 6.80 at 70 °C. E_{app} = +150 mV vs Ag/AgCl/KCl_{sat}. *Y*-axis values represent current density, *J*.

Figure 4. Response curve of tG6PDH (filled circles) and mG6PDH (open circles) biosensors as a function of temperature.

biotransformation applications. Industrial processes that require high temperature could benefit from the use of on-line sensors based on thermostable enzymes, which could potentially not only operate at elevated temperatures but also be resistant to organic solvents, detergents, and other denaturants. To establish the feasibility of using dehydrogenases from extremophiles in sensors, an amperometric biosensing system utilizing a thermostable glucose-6-phosphate dehydrogenase was employed. The system is based on the mediated oxidation of NADH, produced by G6PDH, by a thermostable mediator, osmium (1,10-phenanthroline-5,6-dione)$_2$−poly(4-vinlypyridine) immobilized on the electrode surface (Figure 1). For the purposes of comparison, parallel experiments were performed using a G6PDH from a mesophilic microorganism, *L. mesenteroides*.[22]

The *gsdA* gene encoding for glucose-6-phosphate dehydrogenase from the hyperthermophilic bacterium, *A. aeolicus* was cloned and expressed as a fusion protein in *Escherichia coli*. The fusion protein contained a polyhistidine tail to facilitate purification to homogeneity by affinity chromatography. The characteristics of the recombinant enzyme have been previously described.[20] In brief, the enzyme has been found to be a dimer with a subunit molecular weight of 55 000. The enzyme exhibited dual coenzyme specificity, though it showed a preference in terms of k_{cat}/K_M of 20-fold for NADP over NAD at 40 °C and 5.7-fold at 70 °C. *A. aeolicus* G6PDH had an optimum temperature for catalytic activity at 90 °C.

Cyclic voltammograms of the mediator were pH dependent, with a formal standard potential of +38.7 mV versus Ag/AgCl/KCl_{sat} at pH 6.00. The peak-to-peak potential separation (ΔE_p) was 57.5 mV for the scan rate of 50 mV/s. The response of the mediator to increasing concentrations of NADH was studied at 70 °C (Figure 2). Cyclic voltammograms obtained showed a dramatic increase of the anodic peak currents in the presence of NADH at 70 °C, demonstrating the thermostability and strong electrocatalytic effect of the Osphendione complex.

The steady-state kinetics of the immobilized tG6PDH was measured amperometrically at a working potential of +150 mV versus Ag/AgCl/KCl_{sat} at 70 °C (Figure 3). The immobilized
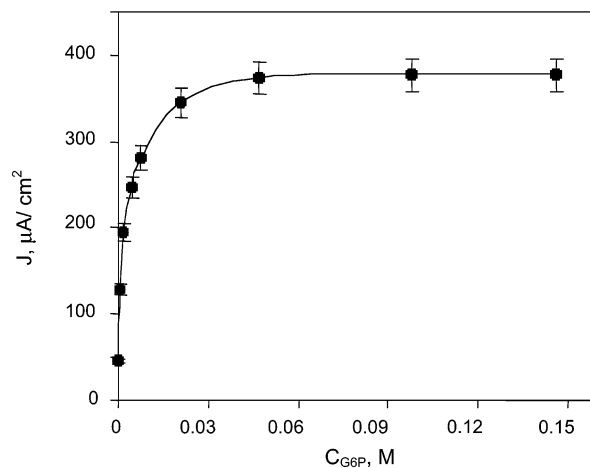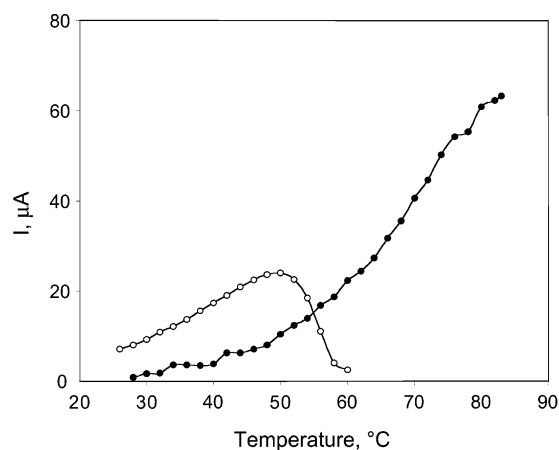
tG6PDH showed classic Michaelis−Menten kinetics with a K_M of 2.9 mM for glucose 6-phosphate (K_M = 0.18 mM in homogeneous solution).[20] The current densities, *J*, at 0.05 M glucose 6-phosphate reached a plateau of 390 μA/cm^2 at 70 °C. The mG6PDH-based system had an apparent K_M of 6.1 mM (K_M = 0.069 mM in homogeneous solution at 25 °C).[22] Biosensors based on tG6PDH exhibited a detection limit of 2×10^{-4} M (S/N = 3) and had a working range of 6×10^{-4}−2×10^{-2} M. Since the system showed maximum current densities at 0.05 M G6P concentration, this concentration was used for further stability studies.

The most important characteristic of thermostable enzymes is their ability to operate at higher temperatures for extended periods of time. Figure 4 shows the effect of temperature on the response for both electrodes. The biosensing system based on tG6PDH showed clear catalytic activity even at 83 °C. An Arrhenius plot of the data presented in Figure 4 for the immobilized enzyme gave an activation energy of 63.7 kJ/mol (40−80 °C). The system based on mG6PDH showed an optimum activity at 50 °C, after which there was a steady drop in current densities due to enzyme denaturation. The operational stability of the tG6PDH and mG6PDH systems was measured at 50 °C. The mG6PDH-based system lost 26% of response after 20 min at

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50 °C, whereas the tG6PDH system retained 100% activity under the same conditions.

Although the osmium (1,10-phenanthroline-5,6-dione)$_2$−poly-(4-vinlypyridine) mediator appears to have higher than usual stability at elevated temperatures compared to other polymeric mediators, repetitive CVs at 60 °C demonstrated that a small amount of the osmium (less than 10%) was lost after repetitive scanning at this temperature. However, when CVs were taken before and after the sensor was exposed to NAD(H), the loss of the phendione peak was higher. Therefore, it appears that loss in redox mediation is somehow linked to the presence of NAD(H) catalysis, probably due to side reactions with free radicals.[23] Despite this, when assembled as a biosensor, the tG6PDH system exhibited an operational half-life of 2 h at 60 °C, whereas the mG6PDH-based system showed no activity at this temperature.

In conclusion, an amperometric biosensor for G6P based on a recombinant tG6PDH from *A. aeolicus* was developed. The sensor was based on the electrocatalytic oxidation of NADH at the electrode transducer using a thermostable Osphendione−PVP mediator. The system showed excellent mediation at temperatures as high as 83 °C at a working potential of +150 mV versus Ag/AgCl/KCl$_{sat}$. The scheme of mediation reported here should be applicable to over 250 dehydrogenases that use the cofactors β-nicotinamide adenine dinucleotide and β-nicotinamide adenine dinucleotide phosphate (NADP).

Note Added after ASAP. The axis lettering on Figures 2−4 was misaligned. The figures were corrected, and the article was replaced on the Web on June 17, 2003.

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