COMMUNICATION TO THE EDITOR

Stability and Sulfur-Reduction Activity in Non-Aqueous Phase Liquids of the Hydrogenase from the Hyperthermophile *Pyrococcus furiosus*

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Abstract: Hydrogenase from the hyperthermophilic archaeon, *Pyrococcus furiosus,* catalyzes the reversible activation of H_2 gas and the reduction of elemental sulfur (S°) at 90°C and above. The pure enzyme, modified with polyethylene glycol (PEG), was soluble (> 5 mg/mL) in toluene and benzene with $t_{1/2}$ values of more than 6 h at 25°C. At 100°C the PEG-modified enzyme was less stable in aqueous solution ($t_{1/2}$ ~ 10 min) than the native (unmodified) enzyme ($t_{1/2}$ ~ 1 h), but they exhibited comparable H_2 evolution, H_2 oxidation, and S° reduction activities at 80°C. The H_2 evolution activity of the modified enzyme was twice that of the unmodified enzyme at 25°C. The PEG-modified enzyme did not catalyze S° reduction (at 80°C) in pure toluene unless H_2O was added. The mechanism by which hydrogenase produces H_2S appears to involve H_2O as the proton source and H_2 as the electron source. The inability of the modified hydrogenase to catalyze S° reduction in a homogeneous non-aqueous phase complicates potential applications of this enzyme. © 1999 John Wiley & Sons, Inc. *Biotechnol Bioeng* **65:** 108–113, 1999.

Keywords: *Pyrococcus furiosus;* hydrogenase; S° reduction; hyperthermophile; non-aqueous solvents

INTRODUCTION

While biocatalysis in organic solvents is expected to greatly expand the role of bioprocessing in the chemical, fuel processing, and bioremediation industries (e.g., see Kilbane and Bielaga, 1990, and Monticello, 1994), naturally occurring enzymes are not soluble in non-aqueous solvents and usually spontaneously denature. However, certain enzymes when suspended in organic solvents maintain both their structural integrity and the ability to catalyze chemical reactions. Under such conditions, they exhibit unusual properties, including the ability to catalyze additional reactions (Almarsson and Klibanov, 1995; Zaks and Klibanov, 1984), and their activity in organic solvents can also be enhanced by various additives such as certain salts, buffers, or ligands (Dabulis and Klibanov, 1993; Khmelnitsky et al., 1994; Skrika-Alexopoulos and Freeman, 1993; Zaks, 1991; Ottolina et al., 1992). Unfortunately, enzymes that do retain their catalytic activities when suspended in organic solvents usually do so at greatly reduced rates, often by several orders of magnitude compared to the activity in aqueous solution. In addition, heterogeneous suspensions of proteins, rather than homogeneous ones, greatly limit fundamental studies on their structure and biochemical properties. Alternative approaches include genetically engineering enzymes so that they are more solvent resistant (Chen and Arnold, 1993) and, the subject of this report, chemically modifying enzymes. The attachment of hydrophobic groups such as polyethylene glycol (PEG) can render enzymes not only active, but also completely soluble in organic solvents (Hernaiz et al., 1997; Inada and Matsushida, 1990; Inada et al., 1986; Inoue et al., 1997; Kaufman et al., 1995; Pina et al., 1989; Takahashi et al., 1985; Veronese et al., 1985; Woodward and Kaufman, 1996).

The object of the present study was to investigate the properties of a chemically modified form of the enzyme hydrogenase in organic solvents. The enzyme was obtained from *Pyrococcus furiosus* (Pf), a hyperthermophilic microorganism that grows optimally at 100°C (Bryant and Adams, 1989; Fiala and Stetter, 1986). *Pyrococcus furiosus* hydrogenase is a heterotetramer (165,000 Da), and catalyzes at temperatures above 90°C both the reversible activation of

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H_2 gas (Bryant and Adams, 1989) and the reduction of elemental sulfur to hydrogen sulfide (Ma et al., 1993). Partially purified preparations of Pf hydrogenase modified by polyethylene glycol (PEG) were reported to be soluble in pure toluene and to catalyze the H_2-dependent reduction of S° to H_2S, a reaction of potential use in the petroleum and coal industries (Woodward and Kaufman, 1996). The aim of the present study was to determine the solubility, stability, and catalytic activity in a range of organic solvents of the pure enzyme in its PEG-modified state.

MATERIALS AND METHODS

Preparation of PEG-Modified Hydrogenase

Pyrococcus furiosus was cultured at 90°C, and its hydrogenase was purified as previously described (Bryant and Adams, 1989). Three different PEG reagents with average molecular weights of 3500–5000 (Sigma Chemical Co., St. Louis, MO) were used in this study: polyoxyethylene glycol *p*-nitrophenyl carbonate (PEG-pNPC), methoxypolyethylene glycol *p*-nitrophenyl carbonate (MPEG-pNPC), and methoxypolyethylene glycol cyanuric chloride (MPEG-CC). In a typical modification reaction, hydrogenase (10 mg in 1 mL of 50 m*M* Tris-HCl, pH 8.0, containing 2 m*M* sodium dithionite) was reacted with the PEG reagent (100 mg in 1 mL of 0.2*M* borate buffer, pH 8.8) for 2 h at 23°C in a stirred, argon-flushed serum bottle. The unreacted reagent was removed from the reaction mixture by dialysis for 24 h in a cellulose membrane (12,000 MW cut-off, Spectra/Por, Los Angeles, CA) against 6 × 2.0 L of argon-sparged 10-m*M* potassium phosphate buffer, pH 8.0. Each dialysis was performed for 1 h with 10 h for the final dialysis step.

Enzyme Assays

Hydrogenase activity was routinely measured by H_2 evolution using methyl viologen reduced by sodium dithionite as the electron donor. The assay was performed at 80°C in a 9.25-mL vials fitted with butyl rubber stoppers and aluminum seals. The 1.4-mL assay mixture contained 50 m*M* EPPS (*N*-(2-hydroxyethyl)-piperazine-*N'*-(3-propanesulfonic acid)) buffer, pH 8.0, 3 m*M* methyl viologen, and 30 m*M* sodium dithionite. After pre-incubation at 80°C for 4 min, the reaction was started by addition of the enzyme and terminated after incubation at 80°C for 5 min by injecting 0.4*M* EDTA, pH 8.0. H_2 production was measured by gas chromatography (Bryant and Adams, 1989). Sulfur reductase activity was measured at 80°C using the same stoppered vials, but with H_2 in the gas headspace. The 1-mL assay mixture contained 10 m*M* potassium phosphate buffer, pH 8.0, and 100 mg of elemental sulfur (Baker, Phillipsburg, NJ). Benzyl viologen (25 m*M*) was also added as indicated. The vials were pre-incubated in a water bath at 80°C, and the reaction was started by addition of enzyme. The amount of H_2S produced was measured by gas chromatography using a Porapak QS column (3 ft × 1/8 in., Hewlett Packard, Wilmington, DE). The column, injector, and thermal conductivity detector were at 70, 125, and 125°C, respectively. Helium was used as a carrier gas at a flow rate of 20 mL/min.

The H_2 oxidation activity of the hydrogenase was measured at 80°C in stoppered cuvettes using either benzyl viologen (4 m*M*), methyl viologen (1 m*M*), or methylene blue (0.01 m*M*) as the electron acceptor. The 2.0-mL reaction mixture also contained 50 m*M* EPPS buffer, pH 8.0, under H_2. The cuvette was preincubated for 3 min, and the reaction was started by adding the enzyme. The activity was measured spectrophotometrically by reduction of benzyl viologen (7,800 $M^{-1}cm^{-1}$ at 580 nm), methyl viologen (9,700 $M^{-1}cm^{-1}$ at 580 nm), or methylene blue (30,500 $M^{-1}cm^{-1}$ at 670 nm). Specific activity is expressed as unit/mg of protein in which 1 unit equals 1 mole of product (H_2 or H_2S) produced or substrate (H_2) oxidized per min. Sulfur-reductase activity of the PEG-hydrogenase in toluene was determined as described above, except that toluene replaced the aqueous buffer. For assays in toluene/water mixtures, toluene and the buffer (10 m*M* potassium phosphate, pH 8.0) were separately sparged with H_2, and added separately to the assay vial. The vials were shaken at 150 rpm to ensure efficient mixing. Because the H_2S produced is distributed between the gas and two liquid phases (water and toluene), the total amount of H_2S produced was calculated from a standard curve. This was prepared by injectng up to 1 mL of 100% H_2S (40 μmole)) into 9.25 mL stoppered vials containing from 0–100% toluene (1 mL). Vials were incubated at the appropriate temperature for 1 h prior to analyzing for H_2S in the gas phase.

Determination of Solubility and Stability PEG-Proteins in Solvents

Lyophilization was carried out by freezing the dialyzed PEG-hydrogenase (containing 2.5 mg of hydrogenase, as determined by the protein assay, in 0.5 mL of 10 m*M* potassium phosphate buffer, pH 8.0, 2 m*M* dithionite) in liquid N_2 in a 2-mL centrifuge tube, and then drying under vacuum. The lyophilized, modified hydrogenase (2.5 mg) was resuspended in 0.5 mL of the indicated organic solvent, and its solubility was determined after incubation at an appropriate temperature by visual inspection. Stability of the modified hydrogenase in organic solvents was determined by measuring residual H_2-evolution activity after incubation in the solvent. The solvent-solubilized PEG-hydrogenase was placed in an argon-flushed micro-vial (Kimble, Loveland, CO) fitted with open-top closure and silicone septum, and incubated at the desired temperature. Aliquots of the sample were removed and assayed directly for H_2-evolution activity in aqueous buffer under standard conditions.

Other Methods

Sodium dodecyl sulfate-polyacrylamide gel-electrophoresis (SDS-PAGE) was performed as described by Laemmli

(1970). Protein concentration was determined by the Lowry method with bovine serum albumin as the standard (Lowry et al., 1951). The molecular weight of the PEG-modified hydrogenase was estimated using a Sephacryl HR 300 column (1 × 30 cm) using thyroglobulin (669,000 Da), apoferritin (443,000 Da), β-amylase (200,000 Da) and ovalbumin (45,000 Da) as standards.

RESULTS AND DISCUSSION

Modification of Pf Hydrogenase

Three different PEG reagents were used to modify the hydrogenase, PEG-pNPC, MPEG-pNPC, and MPEG-CC. The specific activities of the three different kinds of modified enzymes were very similar and were also comparable to that of the unmodified native enzyme (~ 200 units/mg of protein), showing that the modification procedure did not cause significant inactivation of the enzyme. However, the solubility of the hydrogenase modified by either PEG-pNPC or MPEG-pNPC in toluene, which was used as the model organic solvent, varied considerably from preparation to preparation for, as yet, unknown reasons. In contrast, the MPEG-CC-modified enzyme was consistently soluble in toluene, up to at least 5 mg/mL. The MPEG-CC enzyme, hereafter termed PEG-hydrogenase, was therefore used for all further studies.

Analysis of PEG-hydrogenase by denaturing electrophoresis (SDS-PAGE) indicated that the PEG molecules do not dissociate from the enzyme, even after heating at 100°C for 30 min, and are assumed to be covalently attached. The molecular weight of the native and modified forms of the hydrogenase were estimated by gel filtration to be 151 ± 10 kDa and 830 ± 50 kDa, respectively (data not shown). The former value is in agreement with the molecular weight (153 kDa) calculated from the sequence of the enzyme (Pedroni et al., 1995). The higher molecular weight of PEG-hydrogenase can be accounted for by about 200 molecules of PEG (average MW 3350), but PEG reacts predominantly with lysine residues of which the hydrogenase only contains 105 (Pedroni et al., 1995). Hence, the modified enzyme is likely an aggregate of at least two enzyme molecules in addition to PEG. Nevertheless, it is clear that a high-molecular-weight form of the enzyme has been produced and that the PEG is covalently attached.

Stability and Catalytic Activity of PEG-Hydrogenase in Aqueous Solution

PEG-hydrogenase in aqueous solution was not as stable as the unmodified native enzyme, as indicated in Figure 1. For example, the modified enzyme lost ~ 50% of its H_2 evolution activity after a 10 min incubation at 100°C, whereas the half-life of the native enzyme was about 1 h. Similarly, the PEG-hydrogenase retained only about 50% of its activity

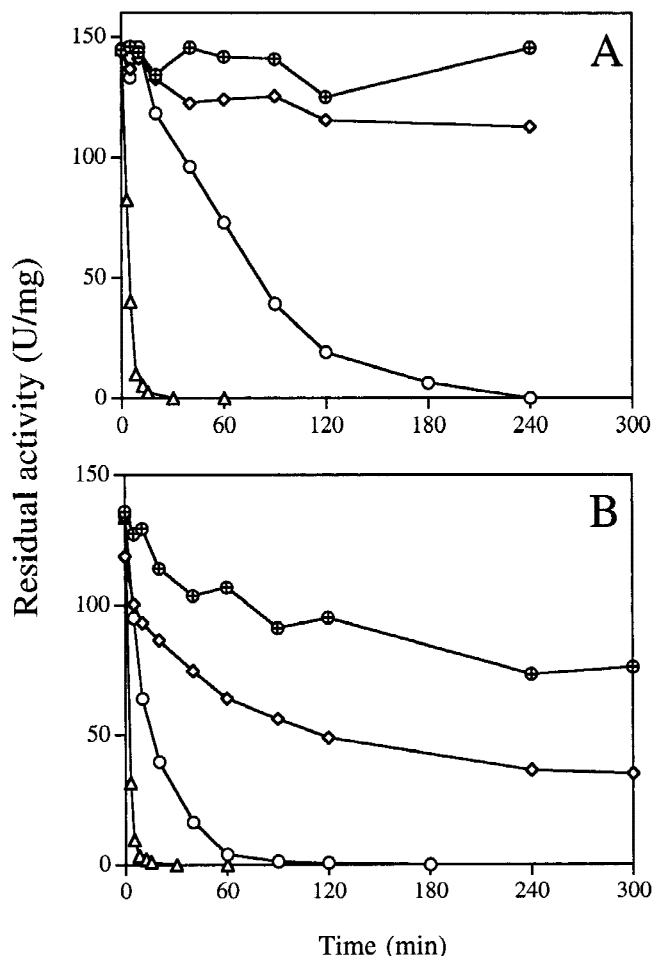

Figure 1. Thermostability of native and PEG-hydrogenase in aqueous solution. Residual H_2 evolution activities were determined after incubating the native (A) or modified (B) forms of the enzyme for the indicated periods. The incubation temperatures were 80 (⊕), 90 (◇), 100 (○), or 110 °C (△).

after a 5 h incubation at 80°C, but the native enzyme was completely unaffected by this treatment. Nevertheless, the latter result showed that the modified enzyme was stable under assay conditions (≤ 10 min at 80°C). The H_2-evolution activity of the native enzyme and PEG-hydrogenase were determined at various temperatures, and the results are given in Figure 2. The temperature for maximum activity of the modified hydrogenase was 80°C, while the optimum temperature for the native enzyme was 90°C. The activity of PEG-hydrogenase was higher at lower temperatures than that of native enzyme. For example, the native enzyme exhibits very low, but readily measured activity at 30°C, but the modified enzyme exhibits twice the activity at this temperature. These data are consistent with the lower thermal stability of the modified enzyme. It is possible that the attachment of PEG may lead to a somewhat more flexible enzyme that has a lower stability at high temperatures, but more flexibility at low temperatures, thus enabling a higher rate of catalysis.

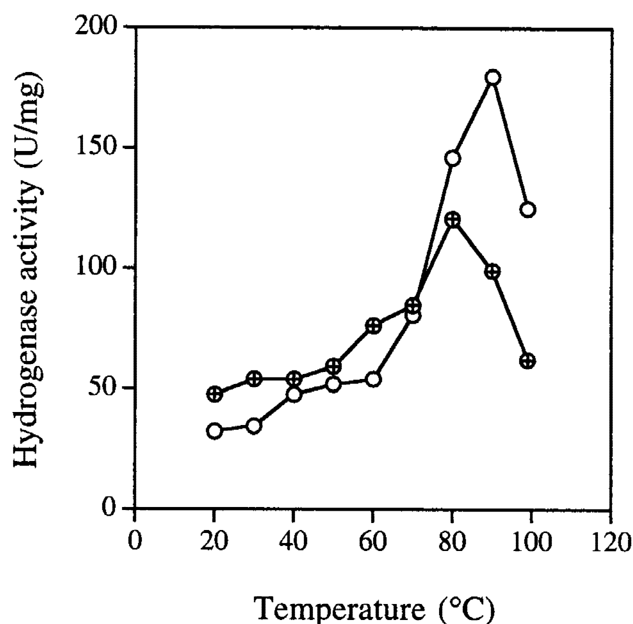

Figure 2. Effect of temperature on the H_2 evolution activity of native (○) and PEG-modified (⊕) hydrogenase. The enzymes were assayed under standard assay conditions except the temperature was varied as indicated.

Solubility and Stability of the PEG-Hydrogenase in Organic Solvents

The solubility and stability of PEG-hydrogenase was investigated in six different organic solvents (Log P range of −1.35 ~ 4.05). The results are shown in Table I, where stability was determined by removing samples of the solvent-dissolved enzyme, and determining residual H_2-evolution activity in aqueous solution at 80°C under standard conditions. There was a correlation between the hydrophobicity of the organic solvent and solubility of PEG-hydrogenase in the same solvent, with a higher solubility observed in the more hydrophilic solvents. For example, PEG-hydrogenase is readily soluble at 25°C in four solvents with Log P values of 2.0 or lower, is not soluble in toluene (Log P = 2.6) at 25°C, but becomes soluble after a 10 min incubation at 40°C, yet is insoluble in heptane (Log P = 4.05). A correlation was also found between the hydrophobicity of solvent and the stability of the PEG-hydrogenase in the solvent, with the enzyme being more stable in the more hydrophobic solvent. For example, although readily soluble, PEG-hydrogenase is not stable in DMSO, ethanol, or 1-pentanol as it quickly (within 1 min) loses its activity at 25°C (Table I). However, the modified enzyme is relatively more stable in benzene and toluene with $T_{1/2}$ values of more than 6 h at 25°C. It was suggested above that the lower thermal stability of PEG-hydrogenase in aqueous buffer could be due to the increased flexibility of the modified enzyme in buffer. Placement of the PEG-enzyme in a more hydrophobic solvent (i.e., toluene vs. DMSO) may therefore, increase the constraints placed upon the enzyme due to strong non-polar interactions between the PEG matrix and the solvent. However, this remains speculation in the absence of data on the physical properties of the enzyme in the various media.

S° Reduction Activity of PEG-Hydrogenase

Like the unmodified enzyme, PEG-hydrogenase readily catalyzed the H_2-dependent reduction of S° to H_2S in an aqueous medium at 80°C. The rate of H_2S production was approximately an order of magnitude lower than the rate of H_2 production from reduced methyl viologen. However, the PEG-modified form of pure hydrogenase was not active in pure toluene nor in the other solvents listed in Table I. H_2S could not be detected in the gas phase, and the amount of H_2 did not diminish over time (up to 3 h at 80°C). One explanation for this is that the PEG-hydrogenase is not stable in toluene and is rapidly denatured in toluene at the assay temperature 80°C. To investigate this possibility, S° reduction assays were performed in pure toluene, but at lower temperatures (40 and 60°C) to minimize thermal inactivation of the enzyme. However, no H_2S was detected at either temperature after 3 h and even after 18 h. Enzyme stability therefore, appears not to be the primary reason for the lack of activity of the PEG-modified enzyme.

However, H_2S was detected if water was also present in the assay medium. As shown in Figure 3, the initial rate of H_2S production (measured up to 30 min) increased with increasing water concentrations up to 50% (v/v). Note that the amount of H_2S produced in the gas phase in these reactions was dependent upon the amount of water present, see Figure 3. In these assays the total amount of H_2S was measured, so the amount of H_2S in gas phase is not an

Table I. Solubility and stability of the PEG-Hydrogenase in solvents.

Solvent	Log P	Solubility	$t_{1/2}$ (25°C)[a]	$t_{1/2}$ (60°C)[a]
DMSO	−1.35	Readily soluble	<1	—
Ethanol	−0.26	Readily soluble	<1	—
1-Pentanol	1.33	Readily soluble	<1	—
Benzene	2.00	Readily soluble	>360	5
Toluene	2.60	After 10 min at 40°C	>360	10
Heptane	4.05	Insoluble	—	—

[a]The $t_{1/2}$ value, expressed in minutes, was determined by removing samples of the solvent-solubilized enzyme incubated at the indicated temperature, and then determining the residual H_2 evolution activity in aqueous buffer at 80°C.

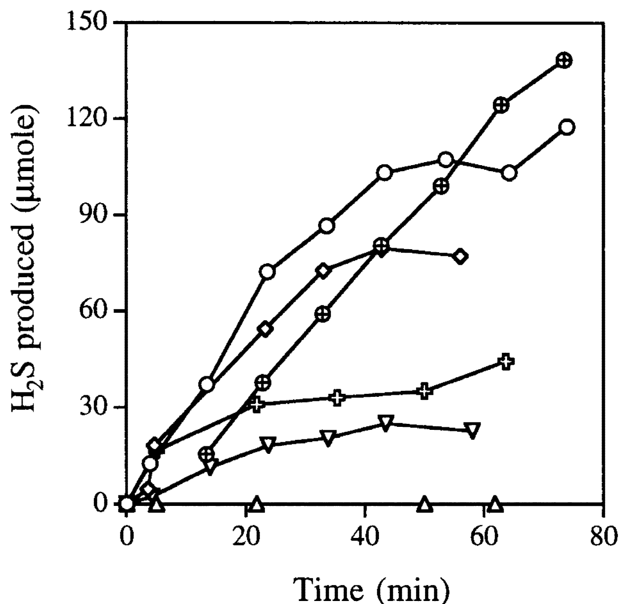

Figure 3. The effect of water on S° reduction activity of PEG-hydrogenase in toluene. S° reduction assays were carried out under standard assay conditions except the medium was varied as indicated: 0% water (△), 10% water (▽), 20% water (+), 30% water (◇), 50% water (○), 100% water (⊕).

artifact of the solvent mixture that was used (see Fig. 4). It should also be noted that the kinetics of H_2S production were also dependent upon the water content. Thus, the rates of the S° reduction decreased with time (after 30 min) with decreasing water concentrations (Fig. 3), whereas the rate in 100% water was linear for several hours (data not shown). Presumably, the decreasing rate of S° reduction at the higher toluene concentrations is due to the instability of the enzyme in toluene, see Table I.

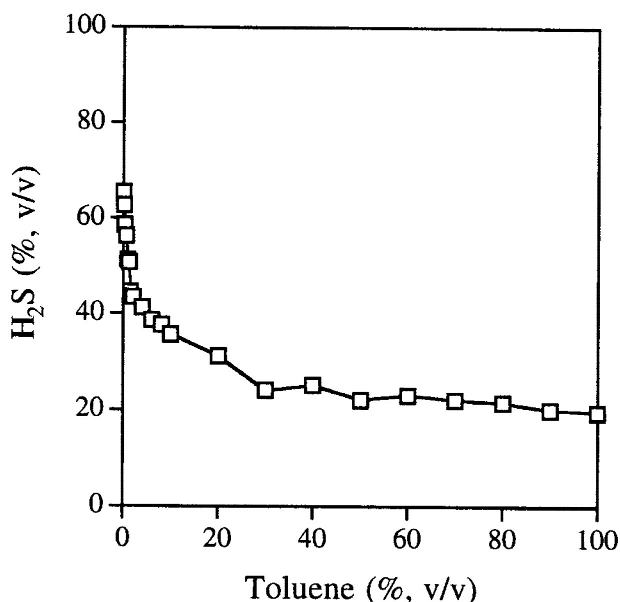

Figure 4. Solubility of H_2S in toluene-buffer mixture. The experiment was carried out as described in the Materials and Methods section.

The question therefore arises as to why the reduction of S° by H_2 catalyzed by PEG-hydrogenase is dependent upon water. There are two obvious mechanisms by which H_2S could be produced by hydrogenase from H_2 and S. In Equation (1), S° is directly reduced

$$H_2 + S^\circ \rightarrow H_2S \quad (1)$$

$$2H_2O + H_2 + S^\circ \rightarrow H_2S + 2OH^- + 2H^+ \quad (2)$$

by H_2, whereas in Equation (2), H_2 supplies the electrons, and water provides the protons (where the protons produced originate from H_2). However, the data shown in Figure 3 show that Equation (2) best describes the S°-reducing activity of PEG-modified hydrogenase. The stability of the enzyme is probably not a limiting factor at high concentrations of water, although it clearly has an effect at low concentrations ($\leq$ 20%, v/v; see Fig. 3).

It should be pointed out that Woodward and Kaufman (1996) previously reported that a PEG-modified form of impure Pf hydrogenase did catalyze the reduction of S° to H_2S in toluene. While the qualitative results of their study are verified in the current work, several factors necessitate quantitative correction of their findings. First, a miscalculation was made in the headspace sulfide concentrations in the original work (Woodward and Kaufman, 1996), which led to an overestimation of this value by nearly 3 orders of magnitude. Thus, while the reported reaction did take place, the amount of sulfide produced was in the μ*M* rather than m*M* range. Note that in the present study, with 10% (v/v) water in toluene, more than 20 m*M* sulfide is produced. However, this assumes that all of the H_2S remains in the liquid phase, which from Figure 4, is not the case. This leads to the second point regarding the previous report (Woodward and Kaufman, 1996), namely, that under reaction conditions (pH 8.4), appreciable sulfide is soluble in the liquid as well as the headspace and thus, an accurate representation of sulfide production must account for sulfide in both of the phases. Finally, while no water was exogenously added to the "100% toluene" media used by Woodward and Kaufman (1996), subsequent measurement of the water content of the toluene and lyophilized protein by Karl Fischer analysis has revealed that the mixture contained about 1000 ppm of water. On a molar basis, this amount of water exceeds the amount of sulfide produced. Hence, the PEG-hydrogenase catalyzed production of H_2S cannot be said to be truly anhydrous, and is more aptly described by Equation (2) rather than by Equation (1). Thus, the inability of the modified enzyme to catalyze S° reduction in a truly homogeneous non-aqueous phase may complicate potential applications.

In conclusion, while the study of hyperthermophilic proteins has the potential to greatly expand applications of enzymatic catalysis because of their greater thermal stability, the current study of Pf hydrogenase indicates that when such proteins are modified to permit solubility in organic media, there is a dramatic decrease in thermostability, in both aqueous and organic media. We speculate that the modified protein becomes more flexible and therefore, sus-

ceptible to thermal denaturation. In addition, the chemistry of its catalytic reaction has to be evaluated in the organic solvent. For example, the results presented herein suggest that PEG-hydrogenase uses water rather than H_2 as a source of protons for S° reduction, a conclusion not readily obvious from its properties in aqueous media. Interestingly, for enzymes suspended in organic solvents, addition of water is not only necessary for catalysis, but it also activates the enzyme due to lubricating effect of water (Affleck et al., 1992; Guinn et al., 1991; Halling, 1994; Zaks and Klibanov, 1988). However, more than the optimal amount of water can significantly decrease the thermostability of the enzyme (Zaks and Klibanov, 1984) and cause unwanted hydrolytic reactions (Almarsson and Klibanov, 1995). For PEG-hydrogenase, however, it is not known how its other properties are affected when it is in a solvent such as toluene. For example, how are the integrity and redox function of its metals centers altered, in particular, its nickel-iron catalytic site? Clearly, additional biochemical and spectroscopic analyses of PEG-hydrogenase are required before a more meaningful description can be made of its ability to dissolve in, and catalyze reactions in, organic solvents.

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