Spectroscopy and Photochemistry of Spinach Photosystem I Entrapped and Stabilized in a Hybrid Organosilicate Glass

Hugh O'Neill[†] and Elias Greenbaum*[,‡]

Department of Biochemistry & Cellular and Molecular Biology, University of Tennessee, Knoxville, Tennessee 37996, and Chemical Sciences Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37831-6194

Received December 20, 2004. Revised Manuscript Received February 20, 2005

Photosystem I (PSI) is part of the photosynthetic apparatus of higher plants and algae. It is one of the naturally occurring molecular photovoltaic structures whose primary function is to convert solar energy into chemical energy. The immobilization and stabilization of this large transmembrane protein complex in a solvent-limited and optically clear organosilicate glass are reported. This was achieved by a modification of the sol-gel process whereby in the first instance the methanol formed during the hydrolysis of the alkoxide precursor was removed before adding the enzyme preparation. In the second instance, glycerol was used as a nonsurfactant templating agent. It was present at a final concentration of 50% (v/v) after the solvent removal process was completed. In addition to stabilizing PSI, it may also play an important structural role, as its omission results in opaque and brittle glasses. The PSI complexes retained their activity during the immobilization procedure and after 96% (w/w) of the solvent in the sol-gel matrix was removed. The ability of the P700 reaction centers in entrapped PSI to undergo photochemical oxidation indicated that the intramolecular electron-transfer apparatus of the reaction centers was functional. In addition, it was demonstrated that their intermolecular electron-transfer function was also intact, as evidenced by PSI-mediated photodependent hydrogen production. The results demonstrate the validity of this novel approach for investigation of complex biological electron-transfer processes under nonnative conditions.

Introduction

Oxygenic photosynthesis as carried out by plants, algae, and cyanobacteria is a fundamental metabolic process that supports the existence of all higher life forms on earth. It generates chemical reducing potential that is used to convert carbon dioxide into organic matter and oxygen that is essential for oxidative metabolism. The initial steps in photosynthesis are driven by photon absorption in Photosystems II and I, two large multisubunit transmembrane light-activated protein complexes that act in series.[1,2] Electrons from the photooxidation of H_2O by Photosystem II (PSII) are shuttled via a plastoquinone pool and cytochrome b_6f to the electron relay protein plastocyanin. Photosystem I (PSI), activated by the absorption of a photon, accepts an electron from plastocyanin and translocates it across the thylakoid membrane to ferredoxin. The reducing potential of ferredoxin is then used in many metabolic processes including NADPH formation and nitrate assimilation. In the dark, NADPH and ATP are used to reduce carbon dioxide to carbohydrates.

PSI is composed of a light-harvesting complex and a reaction center with 12 core subunits.[3] Its molecular mass is approximately 525 kDa. The light-harvesting complex serves as an accessory antenna to harvest and funnel absorbed photons to a special chlorophyll dimer in PSI called P700.[4] After absorption of a photon, the photochemistry of P700 induces a charge separation ($P700^+A_0^-$) in the reaction center by rapid electron transfer to peripheral iron sulfur complexes (F_A and F_B) via the intermediates A_0, A_1, and F_x.[5,6] This results in the generation of a weak oxidant ($E_o' = +0.4$ V) at the primary electron donor (P700) and a strong reductant ($E_o = -0.7$ V) at the terminal electron acceptor (4Fe−4S center).[7] The photochemical reaction is completed within 100−150 ns[8] and generates approximately a 1-V potential difference[9] over a distance of 6 nm.[3] The quantum yield of this photochemical reaction is close to 100%.[10,11] To our knowledge, a comparable structure, with such sophisticated molecular organization, has not been demonstrated by a synthetic approach.

In this paper, a strategy for the entrapment of spinach PSI using the sol-gel technique is reported. The combination of advanced inorganic materials with biological molecules is a

* Corresponding author. Tel: 865-574-6835; fax: 865-574-1275; e-mail: greenbaum@ornl.gov.
[†] University of Tennessee.
[‡] Oak Ridge National Laboratory.

(1) Chitnis, P. R. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **2001**, *52*, 593−626.
(2) Barber, J. *Curr. Opin. Struct. Biol*. **2002**, *12*, 523−530.
(3) Ben-Shem, A.; Frolow, F.; Nelson, N. *Nature* **2003**, *426*, 630−635.
(4) Trissel, H.-W.; Wilhelm, C. *Trends Biochem. Sci.* **1993**, *18*, 415−419.
(5) Owens, T. G.; Webb, S. P.; Alberte, R. S.; Mets, L.; Fleming, G. R. *Proc. Natl. Acad. Sci U.S.A.* **1987**, *84*, 1532−1536.
(6) Golbeck, J. H. *Biochim. Biophys. Acta* **1987**, *895*, 167−204.
(7) Barber, J.; Andersson, B. *Nature* **1994**, *370*, 31.
(8) Brettel, K. *Biochim. Biophys. Acta*. **1997**, *1318*, 322−373.
(9) Lee, I.; Lee, J. W.; Stubna, A.; Greenbaum, E. *J. Phys. Chem. B* **2000**, *104*, 2439−2443.
(10) Zankel, K. L.; Reed, D. W.; Clayton, R. K. *Proc. Natl Acad. Sci. U.S.A.* **1968**, *61*, 1243.
(11) Hiyama, T. *Physiol. Veg*. **1985**, *23*, 605−610.

10.1021/cm047763w CCC: $30.25
Published on Web 04/21/2005

subject that is currently of great interest from both a fundamental and applied research perspective. Biological electron-transfer reactions play a central role in many cellular metabolic processes such as respiration and photosynthesis. The entrapment of biomolecules in inorganic materials allows the electron-transfer pathways to be emulated under nonnative conditions.[12] This biomimetic approach is important for elucidation of the fundamental mechanisms of intramolecular and intermolecular biological electron transfer. Moreover, the light-transducing properties of PSI make it an attractive component in molecular-scale optoelectronic and photovoltaic devices. To realize such a device based on PSI, it is necessary to devise a strategy to entrap the reaction centers in a solid-state environment such that their optical and photocatalytic properties are preserved and accessible.[13]

Sol-gel chemistry is an ambient-temperature process for the synthesis of inorganic glasses.[14] The utility of this technique for the immobilization of a range of biomolecules and cells in silica glasses has been demonstrated.[15] These biofunctionalized glasses make it possible to retain the unique reactivities of biomolecules in a solid-state environment and also provide morphological and structural control that is not available when they are dissolved in aqueous media.[16] Sol−gels provide a host matrix with good chemical, thermal, and mechanical stability. In addition, they are also nontoxic, hydrophilic, and biologically inert. Although these properties are advantageous for the entrapment of all enzymes, it is the transparent nature of the sol-gel matrix that is equally important for the immobilization of photoactive biomolecules with functional optical properties. This technique has been applied to the development of optical biosensors based on enzymes that contain chromophoric or fluorescent groups.[17]

The sol-gel technique has been employed in the immobilization of other photoactive proteins such as bacteriorhodopsin (BR)[18,19] and phycoerythrin.[20] The most widely studied photoactive system is BR, a light-activated transmembrane protein found in halobacteria.[21] Under light irradiation, BR undergoes a conformational change in its active site that pumps protons across the cell membrane. The proton-pumping process is accompanied by a sequential shift in the absorption maximum of retinal, a chromophore bound to BR. This photocycle has been used as the basis for using BR as the fundamental component in bioelectronic devices[22] such as optical memories based on photochromic storage[23] and holographic storage.[24] Phycoerythrin is another example of a light-transducing protein that has potential for signal transduction and biosensor applications.[20] This is a water-soluble protein that is found in the outer membrane of marine cyanobacteria. It harvests and funnels ambient light to the photoreactive center to drive photosynthesis with greater than 90% efficiency. As a dried thin film, it displays interesting photovoltaic and photoconducting behavior and has been proposed as a promising candidate for signal transduction and biosensor applications.[20]

The sol-gel process is initiated by the hydrolysis of a metal alkoxide such as tetramethyl orthosilicate in the presence of an acid catalyst to form a sol solution. This is followed by base-catalyzed condensation of the colloidal sol solution in the presence of the desired biomolecule preparation to produce a sol-gel.[25] When fully dehydrated, these glasses are called xero-gels. They are optically transparent and microporous with pores sizes less than 15 Å.[16] Recently, a nonsurfactant templating approach for the biocompatible synthesis of mesoporous sol-gels has been reported. The pore size of the gels could be increased to 30−40 Å.[26,27] This is important for enzyme-catalyzed reactions for which mass-transfer rates are a consideration. Other modifications to the process, such as the addition of polymers to enhance and stabilize enzyme activity, have also been demonstrated.[28,29]

Several approaches have been used to immobilize PSI to study its properties while maintaining its optoelectronic properties. These include the incorporation of PSI into lipid films[30] and polymer matrixes such as albumin-glutaraldehyde[31] and acrylamide.[32] PSI has also been contacted with metallic platinum and anchored to a metal surface.[33] Previous work by Lee et al. has demonstrated that PSI reaction centers can be self-assembled and oriented on organosulfur-modified gold electrodes and are stable nanometer-scale diodes.[34,35] The photovoltaic properties of the protein were stable during long-term storage.[36] Scanning surface probe microscopy was used to measure the light-induced electrostatic potentials

(12) Pletneva, E. V.; Crnogorac, M. M.; Kostič, N. M. *J. Am. Chem. Soc.* **2002**, *124*, 14342−14354.
(13) Gust, D.; Moore, T. A.; Moore, A. L. *Acc. Chem. Res.* **2001**, *34*, 40−48.
(14) Brinker, C. J.; Scherer, G. W. *Sol-Gel Science*; Academic Press: New York, 1990.
(15) Livage, J.; Coradin, T.; Roux, C. *J. Phys.: Condens. Matter* **2001**, *13*, R673−R691.
(16) Dave, B. C.; Dunn, B.; Valentine, J. S.; Zink, J. I. *Anal. Chem.* **1994**, *66*, 1120−1127.
(17) Jin, W.; Brennan, J. D. *Anal. Chim. Acta* **2002**, *461*, 1−36.
(18) Shuguang, W.; Ellerby, L. M.; Cohan, J. S.; Dunn, B.; El-Sayed, M. A.; Selverstone Valentine, J.; Zink, J. I. *Chem. Mater.* **1993**, *5*, 115−120.
(19) Shamansky, L. M.; Luong, K. M.; Han, D.; Chronister, E. L. *Biosens. Bioelectron.* **2002**, *17*, 227−231.
(20) Chen, Z.; Samuelson, L. A.; Akkara, J.; Kaplan, D.; Gao, H.; Kumar, J.; Marx, K. A.; Tripathy, S. K. *Chem. Mater.* **1995**, *7*, 1779−1783.
(21) Birge, R. R. *Biochim. Biophys. Acta* **1990**, *1016*, 293.
(22) Wise, K. J.; Gillespie, N. B.; Stuart, J. A.; Krebs, M. P.; Birge, R. R. *Trends Biotechnol.* **2002**, *20*, 387−394.
(23) Tallent, J. R.; Stuart, J. A.; Song, Q. W.; Schmidt, E. J.; Martin, C. H.; Birge, R. R. *Biophys. J.* **1998**, *75*, 1619−1634.
(24) Juchem, T.; Hampp, N. *Opt. Lasers Eng.* **2000**, *34*, 84−100.
(25) Ellerby, L. M.; Nishida, C. R.; Nishida, F.; Yamanaka, S. A.; Dunn, B.; Selverstone Valentine, J.; Zink, J. I. *Science* **1992**, *255*, 1113−1115.
(26) Wei, Y.; Jin, D.; Ding, T.; Shih, W.-H.; Liu, X.; Cheng, S. Z. D.; Fu, Q. *Adv. Mater.* **1998**, *3*, 313−316.
(27) Wei, Y.; Xu, J.; Feng, Q. M.; Lin, M.; Dong, H.; Zhang, W.-J.; Wang, C. *J. Nanosci. Nanotechnol.* **2001**, *1*, 83−91.
(28) O'Neill, H.; Angley, C. V.; Hemery, I.; Evans, B. R.; Dai, S.; Woodward, J. *Biotechnol. Lett.* **2002**, *24*, 783−790.
(29) Chen, Q.; Kenausis, G. L.; Hellar, A. *J. Am. Chem. Soc.* **1998**, *120*, 4582−4585.
(30) Munge, B.; Das, S. K.; Ilagan, R.; Pendon, Z.; Yang, J.; Frank, H. A.; Rusling, J. F. *J. Am. Chem. Soc.* **2003**, *125*, 12457−12463.
(31) Bonenfant, D.; Carpentier, R. *Appl. Biochem Biotechnol.* **1990**, *26*, 59−71.
(32) Govorunova, E.; Dér, A.; Toth-Boconadi, R.; Keszthelyi, L. *Bioelectrochem. Bioenerg.* **1995**, *38*, 3−56.
(33) Lee, J. W.; Lee, I.; Greenbaum, G. *Biosens. Bioelectron.* **1996**, *11*, 375−387.
(34) Lee, I.; Lee, J. W.; Warmack, R. J.; Allison, D. P.; Greenbaum, E. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 1965−1969.
(35) Lee, I.; Lee, J. W.; Greenbaum, E. *Phys. Rev. Lett.* **1997**, *79*, 3294−3297.
(36) Lee, I.; Justus, B. L.; Lee, J. W.; Greenbaum, E. *J. Phys. Chem. B* **2003**, *107*, 14225−14230.

above single photosynthetic reaction centers, and the measured potentials were approximately 1 V, in good agreement with previously reported data.[9]

We report here the employment of the sol-gel technique to entrap and stabilize PSI. As a prerequisite for this study, the development of a strategy to stabilize the protein and preserve the optical properties of the silica glasses during the solvent removal process was essential. Two modifications to a previously described method[25] were necessary to achieve this goal. The methanol released during the hydrolysis of the alkoxide precursor was removed by evaporation, and glycerol was used as a templating agent. Previous reports[37,38] have employed these modifications separately. In this study, the combination of both was required to attain a stable protein in an optically clear glass. The spectrophotometric, photochemical, and photocatalytic characteristics of the immobilized enzyme were examined to evaluate the properties of the reaction centers as the sol-gels were dehydrated. The effect of the solvent removal process on the properties of the entrapped reaction centers, particularly on their ability to carry out electron-transfer reactions, was investigated.

Experimental Section

Isolation of PSI. Commercially obtained "baby" spinach was washed with distilled water, finely chopped in a food processor (Cuisinart), suspended in two volumes of STNM (0.05 M Tris-HCl pH 7.8, 0.4 M sucrose, 10 mM NaCl, and 5 mM $MgCl_2$), and homogenized by three 10-s bursts with a PolyTron (Kinematica Inc., Switzerland). All other steps were carried out as described by Bruce and Malkin[39] with minor modifications. Protease inhibitors were not included in the protocol. After the second sodium bromide wash, the membrane pellet was resuspended in H_2O to a final chlorophyll concentration of 1 mg/mL and was solubilized by addition of Triton X 100 (0.7% (w/v)) at 20 °C for 1 h. Ultracentrifugation of the solubilized membranes was carried out in an SW28 rotor (Beckman) at 27 000 rpm for 18 h. The chlorophyll/P700 ratio of the isolated complex was 350, with a protein concentration of 0.95 mg/mL and a chlorophyll concentration of 0.18 mg/mL. The final preparation was stored at −80 °C prior to use.

Isolation of Plastocyanin. The method employed for the isolation and purification of plastocyanin from market spinach leaves was adapted from previously reported protocols. The initial part of the purification, to the first dialysis step, was carried out as described by Ellefson et al.[40] The latter part of the purification followed the procedure described by Christensen et al.[41] with minor modifications. Hydrophobic interaction chromatography was carried out using a Phenyl FF column (16/10) attached to a Pharmacia FPLC system. The equilibration buffer A was 20 mM potassium phosphate pH 7.5, 2.0 M $(NH_4)_2SO_4$, and the limit buffer B was 20 mM potassium phosphate pH 7.5, 1.0 M $(NH_4)_2SO_4$. Prior to loading plastocyanin, the column was washed with six column volumes of buffer A and then with an additional three volumes of the same buffer. A gradient from 10 to 100% B in six column volumes was used to elute plastocyanin. Ion-exchange chromatography was carried out on Hi-Trap Q Sepharose (Amersham Biosciences) as described, except that the oxidized protein was loaded on the column. Two peaks of plastocyanin eluted: a blue peak of the oxidized form at 31% and a colorless reduced form at 36% limit buffer. Fractions with an absorbance ratio A_{278}/A_{597} of 1.0−1.1 in the oxidized state were pooled. The concentration of the oxidized form was calculated using $\epsilon_{597} = 4500\ M^{-1}$.[42] Protein yields of 7.5 mg/kg spinach were typically obtained and are in good agreement with previous studies.[40,41]

Analytical Assays. Protein assays were carried out using a modified Lowry assay protocol (Pierce) with bovine serum albumin as the standard protein. Chlorophyll determinations were made in 80% acetone with the extinction coefficients calculated by Arnon.[43] P_{700} measurements were carried out using the chemical assay method described by Markwell et al.[44]

Preparation of Silica Gels. A mixture of tetramethyl orthosilicate (TMOS; 6 g), H_2O (2.05 g), and 40 mM HCl (0.08 g) was sonicated (Laboratory Supply Company Sonicator, Model G112SPIT) at 4 °C until a homogeneous solution was obtained. The sample was then placed under vacuum (100 kPa) at 20 °C for 30 min. The resulting viscous liquid was diluted with 1.4 g H_2O. This sol solution was stable for several hours at 4 °C. In a typical immobilization reaction, 0.1 mL PSI, 0.3 mL 10 mM sodium phosphate buffer pH 7.2, and 0.5 g glycerol were mixed with 1 mL of sol solution in a 4-mL plastic cuvette. Gelation occurred within a 5-min period. The samples were stored at room temperature in the dark and desiccated under silica gel.

Photochemical Activity of PSI. The photochemical activity of PSI was determined from the light-induced P700 absorption changes at 810 nm using the dual-wavelength emitter-detector unit ED-P700DW-E connected to a PAM 101 Fluorimeter (Walz, Effeltrich, Germany). Data were recorded and stored by interfacing the PAM 101 fluorimeter with a personal computer (Gateway) via a DATAQ serial interface. The sample was placed in the holder and dark adapted before illumination with white light (1400 μmol/m^2/s, Schott KL 1500 light source) to excite P700 over a 1-min period. The actinic light was then extinguished, and the rereduction of $P700^+$ was followed for an additional minute.

Coimmobilization of PSI and Plastocyanin. Plastocyanin (20.2 nmol) and PSI (0.294 nmol; 220 μg chlorophyll) were mixed with 1.5 g glycerol, 0.75 g 10 mM sodium phosphate pH 7.11, and 2.73 g sol solution (as prepared previously). The reaction components were mixed well and transferred to a covered cylindrical glass vessel [1.1 × 9.1 cm (I. D.)] equipped with gas inlet and outlet ports. The contents covered the bottom of the vessel to a depth of approximately 1 mm. Gelation occurred within several minutes; the reaction vessel was stored in a silica gel desiccator during aging in the absence of light.

Photocatalyzed Hydrogen Evolution. The experimental apparatus used for the continuous measurement of hydrogen was described previously[45] with the following modifications: Humidified helium was used as the carrier gas, and a Dolan-Jenner light source equipped with a red filter (90% T at λ > 590 nm) and a 150-W halogen projector bulb were used to illuminate the reactions. The light intensity was measured with a Licor model LI-189 quantum flux meter. The immobilized proteins were washed with several changes of 10 mM sodium phosphate for 4 h at room

(37) Gill, I.; Ballesteros, A. *J. Am. Chem. Soc*. **1998**, *120*, 8587−8598.
(38) Ferrer, M. L.; del Monte, F.; Levy, D. *Chem. Mater*. **2002**, 3619−3621.
(39) Bruce, B. D.; Malkin, R. *J. Biol. Chem.* **1988**, *263*, 7302−7308.
(40) Ellefson, W. L.; Ulrich, E. A.; Krogman, D. W. *Methods in Enzymology;* San Pietro, A., Ed.; Academic Press: New York, 1980; Vol. 69, pp 223−230.
(41) Christensen, H.E. M.; Conrad, L. S.; Ulstrup, J. *Photosynth. Res*. **1991**, *28*, 89−93.
(42) Sykes, A. G. *Chem. Soc. Rev.* **1985**, *14*, 283−315.
(43) Arnon, D. I. *Plant Physiol*. **1949**, *24*, 1−15.
(44) Markwell, J. P.; Thornber, J. P.; Skrdla, M. P. *Biochim. Biophys. Acta* **1988**, *591*, 391−399.
(45) Millsaps, J. F.; Bruce, B. D.; Lee, J. W.; Greenbaum, E. *Photochem. Photobiol*. **2001**, *73*, 630−635.

temperature in the dark to allow the diffusion of glycerol from the gels. This buffer was removed and replaced with a solution comprised of 5.0 mM sodium ascorbate and 0.5 mM sodium hexachloroplatinate in 10 mM sodium phosphate pH 7.11. The reaction vessel was assembled into the experimental apparatus, and the contents were allowed to equilibrate with the humidified carrier gas. After a baseline had been achieved, the 2-h light-on/1-h light-off cycle was commenced, and the photocatalyzed production of H_2 was recorded as described previously. The same conditions were employed with the native enzymes except that the reaction was stirred.

Results and Discussion

Entrapment of Photosystem I. Sol-gel chemistry is a room-temperature process regimen for the preparation of optically clear metal oxide glasses and ceramics. It is initiated by the hydrolysis of a chemical precursor that passes through a solution state and a gel state before being dehydrated to a glass or ceramic. The preparation of a metal oxide by the sol-gel method proceeds via three basic steps. First, a sol solution is formed by the partial hydrolysis of a metal alkoxide precursor to form reactive monomers followed by polycondensation of the monomers to form colloidlike oligomers. This is followed by gel formation, known as gelation, which involves additional hydrolysis to promote polymerization and cross-linking leading to the formation of a three-dimensional matrix. Further increase in cross-linking is promoted by solvent removal and results in gel shrinkage. This process is called aging, and in the last step a glass matrix called a xero-gel is formed.

Initial immobilization studies were carried out using a previously described method.[25] The PSI preparation, in 10 mM sodium phosphate buffer pH 7.2, was mixed with the prehydrolyzed sol solution (H_2O/Si ratio of 2) and was aged in air at room temperature in the absence of light. The immobilized PSI displayed no photochemical activity after 48 h. However, if the PSI sol-gel was placed in a buffered solution or H_2O immediately after gelation and aged in this medium, it did retain photochemical activity for at least 1 month. It was postulated that the loss in PSI activity was due to a combination of the relatively high concentration of methanol in the gels immediately after gelation [26.9% (w/v)] and the conformational restrictions imposed on PSI after immobilization. Methanol is a known protein denaturant, and by placing the gels in aqueous-buffered solution, the methanol freely diffused away from the immobilized proteins. However, it is expected that the extent of siloxane chain cross-linking would be also reduced, and hence the pore size of these gels would be larger, as H_2O promotes the hydrolysis of SiO_2 bonds.[14] The gels aged under these conditions lost their optical clarity and in some cases cracked extensively when removed from the solvent in the final drying step. This has also been observed in other studies. For example, BR was aged under a buffered solution and it was necessary to maintain at least 20% solvent to maintain the optical properties of the gel.[46] BR sol-gels that were dried to the xero-gel state were not optically clear.[47]

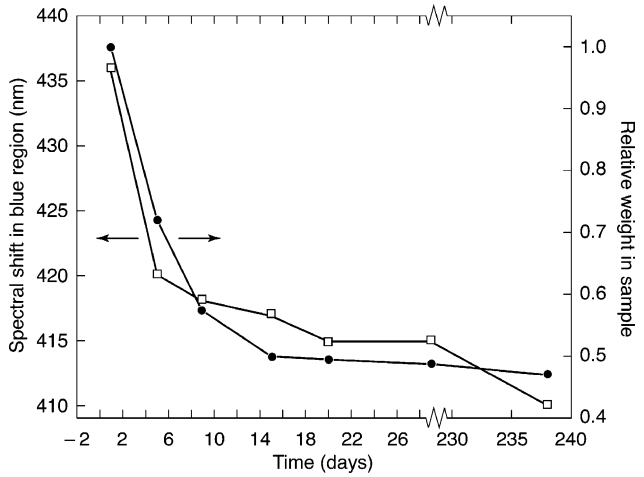

Figure 1. Comparison of the weight loss due to solvent removal (●) with the peak shift observed in the blue region of the visible spectrum (□). The weight is expressed as a fraction of the weight of the sample directly after gelation.

Table 1. Drying Data for PSI Immobilized in Sol−Gels

	weight of gel (g)	solvent removal (%)	H_2O remaining (mmol)
day 0	2.09 ± 0.03	0.0	64.4
day 29	1.03 ± 0.03	91.4	5.5
day 238	0.98 ± 0.02	95.7	2.8
after 120 °C for 18 h	0.93 ± 0.02	100	0

To circumvent these problems, a modified sol-gel immobilization method was developed to enable the immobilization of PSI in an active and stable form. The H_2O/Si ratio was increased from 2 to 3, and the methanol subsequently released during the hydrolysis reaction was removed under vacuum. Typically, the weight loss was 3.5 ± 0.2 g, which represented 91.4 ± 4.6% of the theoretical amount of methanol in the prehydrolyzed sol solution. The viscous sol solution that resulted was dispersed by the addition of 2 mol equiv of H_2O. As water is a product of the condensation reaction, it is likely that its addition promoted a partial hydrolysis of the siloxane bonds. This sol solution (pH ∼ 1) was stable for several hours at room temperature. The other major modification to the procedure was the addition of a relatively high concentration of anhydrous glycerol to the prehydrolyzed sol solution to stabilize PSI during the immobilization reaction. Its addition, at a final concentration of 27.9% (w/v), was sufficient to stabilize PSI and to maintain the optical properties of the sol-gels even in the xero-gel state. Gelation occurred within 5 min, and the gels were aged over an anhydrous silica gel desiccant at room temperature in the dark.

The removal of solvent was a gradual process that proceeded most rapidly during the first 12 days (Figure 1). The final water content in the gels was determined by gravimetric analysis after heating the glasses to 120 °C for 18 h under vacuum (30 in. Hg). Approximately 91% of the water in the gel was removed by the 29th day (Table 1). After nearly 8 months, this increased to 96%, suggesting that the additional water left in the glass (approximately 3 mmol) was bound to the silica matrix and the protein and may represent the minimum amount of water necessary to

(46) Wu, S. G.; Ellerby, L. M.; Cohan, J. S.; Dunn, B.; Elsayed, M. A.; Valentine, J. S.; Zink, J. I. *Chem. Mater.* **1993**, 115−120.
(47) Shamansky, L. M.; Luong, K. M.; Han, D.; Chronister, E. L. *Biosens. Bioelectron.* **2002**, *17*, 227−231.

maintain the catalytic activity of PSI. The volume of the gels decreased approximately 3-fold during the drying process.

These gels were optically clear and structurally stable throughout the aging process. The visible absorption spectrum of xero-gels without PSI had high optical clarity and uniform light transmittance across the visible region (see Figure 2). In contrast, gels to which no glycerol was added lost their optical clarity during the latter days of the drying process. This suggests that glycerol plays a role in maintaining the optical properties of the gel, perhaps by filling in the structural defects responsible for increased light scattering by the formation of hydrogen bonds with the siloxane backbone.

As described previously, the pore size of sol-gels is an important consideration for the immobilization of PSI. Xerogels are microporous materials with a typical pore diameter of ≤ 15 Å,[48,49] whereas the diameter of a single PSI molecule is approximately 150 Å.[3] Therefore, the restricted conformational freedom that results after entrapment in conventional sol-gels may pose another source of protein instability for entrapped PSI. It is proposed that glycerol plays a role as a templating agent in the sol-gel process to form a fluid barrier between PSI and the sol-gel matrix. This is supported by the fact that small polyalcohols such as glucose have been used in a similar role as nonsurfactant templating agents for the synthesis of mesoporous sol-gels with pore sizes greater than 30 Å.[26,27] In addition to glucose, sucrose was also tested in this study and both were found to produce optically clear gels and to be effective for the stabilization of the entrapped PSI. However, in the latter stages of the drying procedure, the gels shattered, presumably because of the crystallization of these reagents in the sol-gel.

Spectral Properties of PSI. The visible absorption spectra of PSI were recorded at intervals after immobilization to monitor spectral changes that occurred during solvent removal (Figure 2). Increased optical density was apparent in the gels that contained immobilized proteins. This has been observed in other sol-gel enzyme immobilization studies.[47,25]

The overall shape of the visible spectrum of PSI remained largely unchanged after immobilization and during the drying process compared with the native preparation (Figure 2). In the blue region of the spectrum, the 436-nm peak, which is associated with chlorophyll a absorption,[49] gradually shifted to 410 nm as water was removed from the gel (Figure 3). The spectral shift mirrored closely the weight change recorded during the drying process (Figure 1). Therefore, it can be attributed to the environmental changes associated with gelation and dehydration of the sol-gel matrix. The other peak associated with chlorophyll a absorption is in the red region of the spectrum. It also underwent a spectral shift from 678 to 672 nm, but it did not change significantly during the drying process and may be related to the chemistry of the immobilization procedure. In addition, there was also a 49% decrease in the magnitude of this chlorophyll peak in the dry xero-gel compared with the gel directly after immobilization (corrected for their relative background levels).

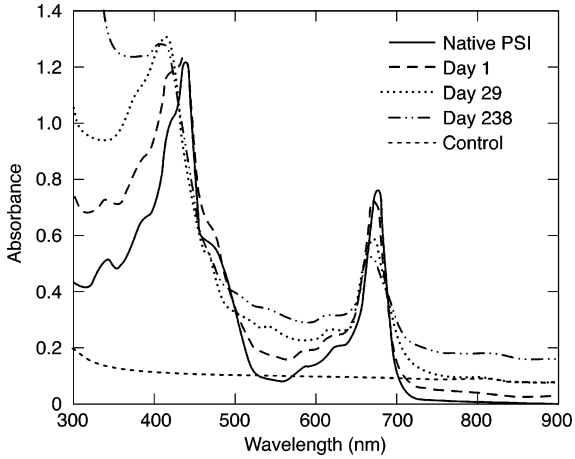

Figure 2. Visible absorption spectra of Photosystem I entrapped in sol−gel at intervals during the aging process compared with the solution spectrum of the native preparation. The spectrum of a control gel without PSI that was aged for 29 days is also shown.

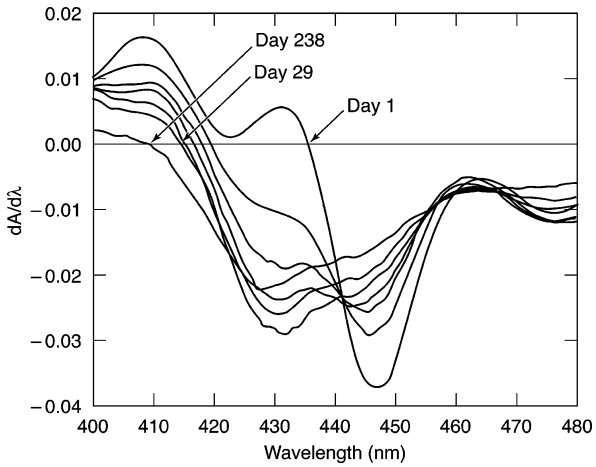

Figure 3. The first derivative of the blue region of the absorption spectrum of Photosystem I.

The shoulders at 470 nm and 650 nm originate from chlorophyll b[49] present in the PSI preparation. The shoulder at 470 nm disappeared as the solvent removal process progressed. In addition, the shoulder at 650 nm was also less evident as the absorptivity in this part of the spectrum increased during the dehydration process. These alterations to the absorption spectrum may also be associated with the environmental changes associated with gelation and dehydration of the sol-gel or may indicate that the chlorophyll b underwent some modification, perhaps oxidation of the aldehyde group at position 3 on the porphyrin ring.

There are two optical effects that must be considered when dealing with the absorption spectra of nonhomogeneous samples. These are absorption flattening and scattering. It is likely that both these optical effects contributed to the overall shape of the PSI spectra (Figure 2). Absorption flattening is an exception to the Beer−Lambert law. It occurs when large macromolecules with high optical absorption, such as chromophoric membrane proteins, have areas of high local concentration because of the formation of aggregates or

(48) Avnir, D.; Braun, S.; Lev, O.; Ottolenghi, M. *Chem. Mater.* **1994**, *6*, 1605−1614.
(49) Bassi, R.; Simpson D. *Eur. J. Biochem.* **1987**, *163*, 221−230.

microparticulates.[50] It is manifested as an underestimation of proper signal intensity. In the present work, the removal of water was accompanied by the shrinkage of the gel because of progressive cross-linking of the silica matrix. This also resulted in an increase in the effective concentration of the PSI molecules and therefore potentially led to high local concentrations of the proteins. These effects fulfill the criteria for the observation of absorption flattening and would explain why the optical density of the PSI peak at 678 nm decreased during the dehydration process (Figure 2).

Mie and Raleigh scattering are two common scattering processes that can be considered when studying light absorption and emission phenomena. Mie scattering is observed when the diameter of the particle is large compared with the wavelength of light and is weakly dependent on the wavelength of light.[51] In contrast, Raleigh scattering results when the diameter of the particle is small compared with the wavelength of light and is inversely proportional to the fourth power of the wavelength ($1/\lambda^4$).[51] It is more effective in the blue region of the spectrum. The absorption spectrum of the control sample exhibited little scattering across the visible region (Figure 2). In contrast, the apparent optical density of the protein-containing gels increases significantly in the blue region during the drying process. This may be attributed to a Raleigh scattering effect whereby the blue photons are scattered by the immobilized protein (15-nm diameter) in the gels, resulting in a higher apparent absorptivity in this region. Furthermore, as dehydration continued, the effective concentration of the proteins increased, resulting in greater absorbance in this region. It is likely that the increase in absorptivity at wavelengths greater than 750 nm was due to Mie scattering.

Photochemical Activity. The photochemical activity of PSI was monitored by near-infrared absorption spectroscopy using a dual-wavelength emitter-detector unit (ED-P700DW, Walz) attached to a standard PAM fluorimeter (Walz). The $P700^+$ cation radial produced by oxidation of a PSI reaction center (P700) during illumination with actinic light not only absorbs at 700 nm but also at 810−830 nm. The Walz emitter-detector unit records the difference in absorbance at 810 nm because of the $P700^+$ cation and at 860 nm, an isosbestic region of the spectrum.

The ED-P700DW emitter-detector unit was operated in remittance mode, and in this configuration, a semiquantitative comparison of the photooxidation of the P700 and $P700^+$ reduction profiles of the immobilized PSI and native PSI in solution was carried out. All samples were treated in an identical manner without the addition of exogenous reducing agents or electron mediators that are typically used in these reactions. Before the onset of illumination, the samples were dark adapted. After the onset of actinic light illumination (1400 μmol/m^2/s), the $P700^+$ population rose quickly and after 1 min the actinic light was extinguished and P700 was recovered. The P700 photooxidation and $P700^+$ reduction curves of PSI in solution were compared with PSI entrapped in sol-gel at various time intervals after immobilization (Figure 4).

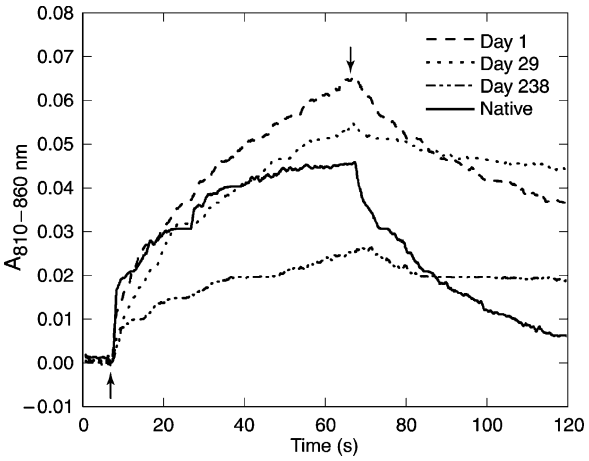

Figure 4. The P700 photooxidation and $P700^+$ reduction profiles of PSI at intervals after entrapment in sol-gels compared with the profile of the native preparation in solution. The up and down arrows indicate the start and finish of actinic light illumination.

Table 2. Photooxidation and Reduction Data for Photosystem I at Intervals after Entrapment in Sol−Gels Compared with the Native Preparation in Solution

	rate ($\Delta A_{810-860\ nm}$/s)	
	photooxidation of P700	reduction of $P700^+$
PSI in solution	$6.5 \times 10^{-2} \pm 1.4 \times 10^{-3}$	$9.9 \times 10^{-3} \pm 7.1 \times 10^{-5}$
day 1 after immobilization	$4.5 \times 10^{-2} \pm 4.4 \times 10^{-3}$	$1.1 \times 10^{-3} \pm 1.4 \times 10^{-4}$
day 29 after immobilization	$5.3 \times 10^{-3} \pm 9.0 \times 10^{-4}$	$4.0 \times 10^{-4} \pm 1.0 \times 10^{-4}$
day 238 after immobilization	$2.3 \times 10^{-3} \pm 5.9 \times 10^{-4}$	$3.0 \times 10^{-4} \pm 2.1 \times 10^{-4}$

The P700 reaction centers were able to be photooxidized and recovered, indicating that the samples were active directly after immobilization in the sol-gel and also after the solvent removal process was completed. The magnitude of the photochemical response of the immobilized samples was slightly greater than the native sample after immobilization and when the majority of the water had been removed (91.4%) after 29 days. However, the photochemical response decreased by approximately 50%, compared with the native sample, during storage for 8 months over desiccant in the dark. Although this indicates that the reaction centers lost some activity during long-term storage, a significant amount of activity did remain.

The initial rates of photooxidation and reduction of the P700 reaction centers, directly after illumination and when the lamp was extinguished, were also compared (Table 2). As the gel-aging process progressed, the rates became slower. Although the magnitude of the photochemical response was similar after the solvent removal was almost complete (>90%, 29 days), the rate of photooxidation had decreased 12.3-fold and recovery of the P700 after the lamp was extinguished was approximately 25-fold slower. After long-term storage, these rates were further decreased by 28-fold and 33-fold, respectively, when compared with the native enzyme.

The alterations to the kinetic properties of PSI after immobilization are related to changes in the physical and

(50) Gordon, D. J.; Holzwarth, G. *Arch. Biochim. Biophys.* **1971**, *142*, 481−488.
(51) Ingle, J. D.; Crouch, S. R. *Spectrochemical Analysis*; Prentice-Hall: Englewood Cliffs, NJ, 1988; pp 494−499.

chemical environment of the enzyme. During the aging process, the pore size of sol-gels decreased because of the extension of the silica framework, and this in turn reduced the conformational freedom of the entrapped enzyme molecules. The silica matrix bears a net negative charge, and so electrostatic interactions with the matrix are also to be expected. In addition, there is very little H_2O present in the gels. Therefore, it is not unexpected that these changes in the environment of the enzyme would be manifested as changes to the kinetic properties of the enzyme.

The enzyme reaction rates are also influenced by the dielectric properties of the surrounding medium.[52] Post-immobilization, the environment of PSI changed from one with a high dielectric constant (H_2O; $\epsilon = 81$ at 20 °C) to a medium composed mainly of silica glass ($\epsilon = 3.8$) with glycerol present ($\epsilon = 42.5$ at 20 °C). The increased ability of the medium surrounding PSI to support an electrostatic field may in part contribute to the stabilization of the $P700^+$ moiety as was observed in Figure 4. However, further investigation would be required to determine the relative contribution of the conformational constraints imposed by the matrix and the effect of removal of H_2O also.

Photocatalyzed Hydrogen Production. The light-dependent production of molecular hydrogen by platinum deposited at the reducing side of PSI was used as a measure of the catalytic activity of the reaction centers.[45] In this biomimetic reaction pathway, the source of electrons was sodium ascorbate with the electron relay protein plastocyanin serving as the mediator between ascorbate and the oxidizing side of PSI. The reaction is initiated by the PSI-catalyzed photoprecipitation of platinum from a sodium hexachloroplatinate solution at the oxidized end of the reaction center. Once sufficient platinum has been deposited (>50 atoms[53]), photoevolution of hydrogen proceeds.

Sol-gels that contained PSI coimmobilized with plastocyanin were prepared and aged as described in Materials and Methods. Although the primary role of glycerol in this study was to protect PSI during the immobilization process, it also acted as a templating agent. That is, the pores left after removal of glycerol facilitated greater access of reagents while maintaining the proteins in the gel. After assembly of the reaction apparatus and equilibration of the reagents, the immobilized sample was illuminated with red light. A characteristic light-dependent evolution of hydrogen was observed (Figure 5a). The rates and yield of H_2 observed were similar for an experiment carried out with the native enzymes in solution (Figure 5b). This confirms that the PSI preparation was active after immobilization and able to facilitate intermolecular electron transfer from sodium ascorbate via plastocyanin to platinum. This gives good confidence with respect to the structural and catalytic integrity of the PSI complexes.

Conclusions. In this study, we report the entrapment of PSI in an organo-hybrid sol-gel. PSI is one of the largest transmembrane protein complexes with at least 12 subunits, 4 different light-harvesting complexes, 45 transmembrane helices, and approximately 200 prosthetic groups.[3] The ability to stabilize this protein in a solvent-limited inorganic glass provides a new platform to investigate its complex structural and catalytic properties. It was found that the combination of methanol removal from the nascent sol solution and the addition of glycerol as a templating agent resulted in a clear organo-hybrid glass. The addition of glycerol played a dual role in the immobilization process. It not only stabilized the large PSI complexes, but its presence also resulted in sol-gels with high optical clarity. This is an important consideration for the investigation of the optoelectronic properties of PSI. The gels formed were structurally stable during the drying process and when it was completed.

The principal changes in the visible absorption spectrum of the protein occurred in the blue region and appeared to be associated with the change in the environment of the protein during the dehydration process. A similar effect was observed after dehydration of sol-gels that contained cytochrome *c*.[54] It is likely that a combination of the dielectric properties of the silica matrix and the glycerol and the net

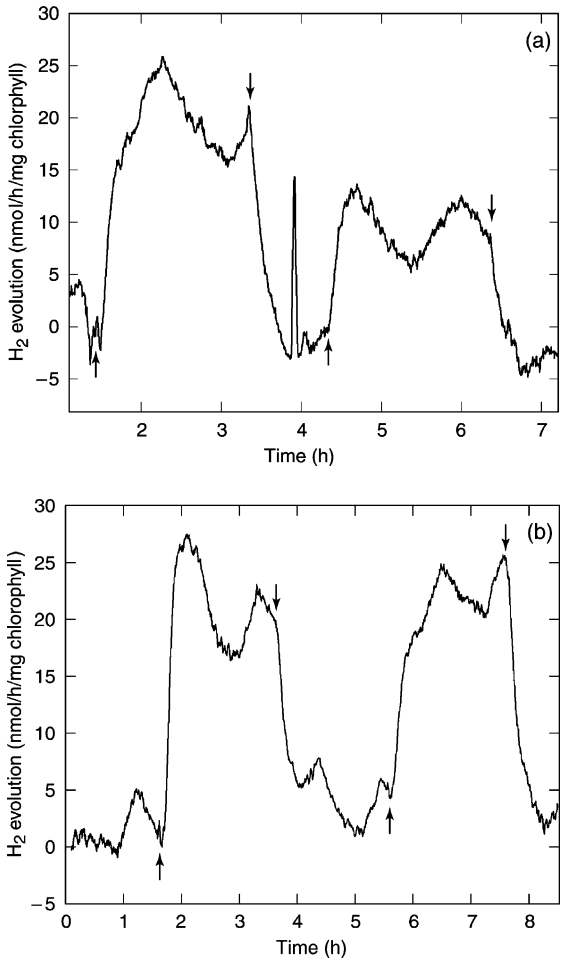

Figure 5. Light-dependent evolution of hydrogen by platinized Photosystem I reaction centers. a: Photosystem I and plastocyanin coimmobilized in sol-gel. B: Native enzymes in solution. The reactions were carried out with 0.294 nmol Photosystem I (220 μmol chlorophyll), 20.2 nmol plastocyanin, 0.5 mM Na_2PtCl_6, and 5.0 mM sodium ascorbate in 10 mM sodium phosphate pH 7.11 at 20 °C. Light cycles of 2 h on (↑) and 1 h off (↓) were used at a constant light intensity of 260 μmol/m^2/s.

(52) Thellier, M.; Vincent, J. C.; Alexandre, S.; Lassalles, J. P.; Deschrevel, B.; Norris, V.; Ripoll, C. *C. R. Biol.* **2003**, *326*, 149−159.
(53) Greenbaum, E. *J. Phys. Chem.* **1988**, *92*, 4571−4576.
(54) Dave, B. C.; Miller, J. M.; Dunn, B.; Valentine, J. S.; Zink, J. I. *J. Sol-Gel Sci. Technol.* **1997**, *8*, 629−634.

negative charge on the silica contribute to this spectral shift. In addition, optical effects such as absorption flattening and scattering were also evident in the spectra of the hybrid gels. The photocatalytic activity of the protein was retained during solvent removal, but the relative rates of P700 oxidation and $P700^+$ reduction were decreased. This is not unexpected because of conformational restrictions imposed on the protein because of the progressive removal of the solvent surrounding the protein. The small amount of H_2O (approximately 3 mmol) present in the glass after the dehydration process was complete may represent the minimum amount of solvent necessary to maintain the catalytic activity of PSI. PSI was coimmobilized in a sol-gel with plastocyanin, its natural electron relay protein. The deposition of platinum metal at the reducing side of PSI produced an active photodependent H_2 catalyst. The rate of H_2 evolution by platinized PSI was used as an ammeter for photocurrent measurement. The functionality of the immobilized proteins was demonstrated by the ability of the PSI complexes that were platinized in situ to catalyze photoinduced hydrogen production when sodium ascorbate was used as a sacrificial electron donor. This indicates that both the intra- and intermolecular electron-transfer apparatus of the entrapped PSI reaction centers are fully functional even after almost complete solvent removal. This study has direct relevance to the development of biobased optoelectronic devices. It provides detailed information on how the entrapment and subsequent dehydration procedures influence the behavior of the PSI reaction centers.

Acknowledgment. This work was supported by the Office of Basic Energy Sciences, U.S. Department of Energy, under contract number DE-AC05-00OR22725 with Oak Ridge National Laboratory, managed and operated by UT-Battelle, LLC. The authors thank B. Bruce, University of Tennessee, Knoxville, for assistance in preparing spinach Photosystem I.

CM047763W