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Published on Web 11/06/2002

Mimicking Biological Electron Transport in Sol−Gel Glass: Photoinduced Electron Transfer from Zinc Cytochrome *c* to Plastocyanin or Cytochrome *c* Mediated by Mobile Inorganic Complexes

Ekaterina V. Pletneva, Milan M. Crnogorac, and Nenad M. Kostić*

Contribution from the Department of Chemistry, Iowa State University, Ames, Iowa 50011

Received April 4, 2002. Revised Manuscript Received July 31, 2002

Abstract: Biomimetic studies of electron-transport chains are important for establishing the molecular mechanisms of long-range communications between proteins. We mimic these biological assemblies by encapsulating metalloproteins in sol−gel silica glass and letting mobile inorganic complexes shuttle electrons between the immobilized proteins. We present two examples of such rudimentary electron-transport chains. In both of them the immobilized electron donor is the zinc-substituted cytochrome *c*, Zncyt; the immobilized electron acceptor is either cupriplastocyanin, pc(II), or ferricytochrome *c*, cyt(III); and the mobile charge carrier Q/Q^- is the redox couple $FeEDTA^{-/2-}$ or $Ru(NH_3)_6^{3+/2+}$. The redox processes are photoinduced: Zncyt is excited by the laser pulse and converted to the triplet state, ^{3}Zncyt, which is a strong reducing agent. Visible absorption, circular dichroism, and electron paramagnetic resonance spectra of the metalloproteins show that encapsulation in sol−gel glass does not affect their intrinsic redox properties. The rigid silica glass spatially separates the proteins from each other. In this matrix, the electron-transfer reactions between ^{3}Zncyt and pc(II) and between ^{3}Zncyt and cyt(III), which occur fast in solution, are completely suppressed in the absence of a charge carrier Q/Q^-. The reactivity of $FeEDTA^-$ and $Ru(NH_3)_6^{3+}$ (as quenchers Q of ^{3}Zncyt) is minimally affected by the interior of the sol−gel glass. In the glass, the second-order rate constants for the excited-state electron transfer, from ^{3}Zncyt to Q, are $(8.9 \pm 0.6) \times 10^6$ and $(8.0 \pm 2.4) \times 10^6$ M^{-1} s^{-1} for $FeEDTA^-$ and $Ru(NH_3)_6^{3+}$, respectively. This reaction is followed by the ground-state back electron transfer, from Q^- to $Zncyt^+$. In the "monoprotein" glasses Zncyt/Q, the respective second-order rate constants for this back electron-transfer reaction are $(4.9 \pm 0.2) \times 10^7$ and $(7.8 \pm 2.7) \times 10^7$ M^{-1} s^{-1}. In the "diprotein" glasses Zncyt/Q/pc(II) and Zncyt/Q/cyt(III), containing also the acceptor protein pc(II) or cyt(III), $Zncyt^+$ decays on two time scales. The faster and major component of this decay is analogous to the only mode of the decay in the Zncyt/Q glasses and is a second-order process. Between 25 and 40% of the initially formed $Zncyt^+$, however, lives longer ($k_{slow} = 1.1 \pm 0.2$ s^{-1}) and decays by a first-order process. We attribute the lengthening of the $Zncyt^+$ lifetime to a partial escape of the photogenerated Q^- into the glass pores, where it reacts with the immobilized pc(II) or cyt(III). Indeed, the visible absorption spectra show the photoinduced reduction of pc(II) and cyt(III). Evidently, the small inorganic complexes, $FeEDTA^{-/2-}$ and $Ru(NH_3)_6^{3+/2+}$, move through the glass pores, react with the encapsulated metalloproteins, and establish the interprotein electron transfer. Each interprotein reaction now occurs in two steps: a mobile charge carrier Q receives an electron from ^{3}Zncyt, and Q^- then delivers an electron to pc(II) or cyt(III). Ultimately, the energy of visible light is converted to reducing equivalents for plastocyanin and cytochrome *c*. The sequential electron transfer described here resembles the events in a rudimentary electron-transport chain. Our findings demonstrate the promise of integrating proteins, with their optimally adjusted redox sites, in photocatalytic materials.

Introduction

Electron-transfer reactions play essential roles in photosynthesis, respiration, nitrogen fixation, DNA biosynthesis and repair, and many other biochemical processes. In biological systems, electron-transfer proteins often combine to form electron-transport chains, which ferry electrons over long distances. The intricate organization of the constituents of the chain and stepwise electron transfer between them prevent charge recombination and ensure the production of useful chemical products.

Mimicking biological electron-transport chains is of great interest for technological and scientific reasons. Studies of such systems are important for establishing the molecular mechanisms of long-range communications between proteins and under-

* To whom correspondence should be addressed. E-mail: nenad@iastate.edu.

10.1021/ja020489r CCC: $22.00

standing the mechanism of photoinduced charge separation realized by Nature.

We mimic a biological electron-transport chain by immobilizing protein components of the chain and letting small inorganic complexes shuttle electrons between these proteins. Our design of such a biomimetic system is guided by two principles. Sol−gel silica glass provides separated compartments to the relatively large protein components of the electron-transport chain. Mobility of the relatively small electron carriers between the compartments ensures communications between the proteins.

The sol−gel trapping technique offers several advantages over other immobilization techniques.[1,2] The sol−gel method proved compatible with proteins, and several of them, mostly enzymes, have been encapsulated in sol−gel glass. The method, unlike covalent binding or cross-linking of protein molecules to a suitable support, is independent of the functionalities on the protein. Because the pores of the sol−gel glass contain water, the protein molecules are solvated and retain their structure and reactivity. Because sol−gel glasses are porous, relatively small molecules can diffuse in and out of gels and reach the larger protein molecules entrapped inside. Because the glasses are transparent to the UV and visible light, the protein reactions can be studied by optical spectroscopic methods.

The feasibility of electron-transfer reactions in sol−gel glass has been demonstrated by several research groups,[3−5] including ours.[6] In these materials, electron transfer is possible between the species immobilized in the glass matrix and mobile species in the intrapore solution. Because sol−gel glasses are transparent, reactions can be induced photochemically. The studies of the latter kind are especially important because of their potential applicability in solar energy conversion.

In their elegant studies of photoinduced electron transfer, Avnir and co-workers took advantage of both solid and liquid phases of the sol−gel glass and simultaneously incorporated in the glass mobile and immobile redox-active components.[4,7] In these artificial photosynthetic materials, a mobile electron acceptor shuttles electrons from an immobilized donor to another electron acceptor, immobilized elsewhere within the gel. For the pyrene−viologen system, this strategy has led to charge-separated pairs that live for hours.[7] Despite this success of the sol−gel method in charge separation with small molecules, charge separation involving proteins or stepwise interprotein electron transfer in sol−gel glass has not been reported.

The heme protein cytochrome *c*[8−10] and the blue copper protein plastocyanin[11−13] make an excellent model system for studies of interprotein electron transfer. Their oxidoreduction reactions with various chemical and biochemical agents have been much studied, and their three-dimensional structures are known in detail.

Replacement of the iron ion with the zinc(II) ion does not significantly perturb the structure of cytochrome *c*[14−16] and its interactions with other proteins.[17−19] The excited triplet state of the zinc porphyrin, ^{3}Zncyt, is produced by a laser flash. The triplet ^{3}Zncyt has been used as a strong reducing and oxidizing agent in several studies.[20−24] The advantage of zinc cytochrome *c*, Zncyt, over the native (iron-containing) species is that the reaction between ^{3}Zncyt and its redox partner can be photoinduced and is not dependent on external redox agents. This advantage makes Zncyt especially attractive for studies of encapsulated proteins since the electron-transfer reactions can be triggered almost instantaneously.

Previous studies in this laboratory dealt with photoinduced electron-transfer reactions between zinc cytochrome *c* encapsulated in sol−gel glass and charged metal complexes and neutral organic compounds introduced in the porous glass by diffusion.[6,25] These studies revealed interesting electrostatic and structural properties of the glass interior and solutions confined therein, which make sol−gel materials quite different from free solution as a reaction medium.

Here we first report a study of photoinduced electron transfer between the proteins zinc cytochrome *c* and plastocyanin encapsulated in sol−gel glass. The reaction between these two proteins in solution is well-studied.[22,26−31] The triplet ^{3}Zncyt is oxidatively quenched by cupriplastocyanin, pc(II), according to eq 1. The resulting cation radical, Zncyt$^+$, returns to the ground state, Zncyt, in the thermal (so-called back) electron-transfer reaction with cuproplastocyanin, pc(I), according to eq 2.

$$^3\text{Zncyt} + \text{pc(II)} \xrightarrow{k_1} \text{Zncyt}^+ + \text{pc(I)} \quad (1)$$

$$\text{Zncyt}^+ + \text{pc(I)} \xrightarrow{k_2} \text{Zncyt} + \text{pc(II)} \quad (2)$$

The encapsulated proteins zinc cytochrome *c* and plastocyanin are spatially separated from each other by the rigid sol−gel matrix. Their "communications" are mediated by smaller redox-active molecules Q and Q$^-$ that can diffuse in the pores of the matrix and react with the immobilized protein molecules

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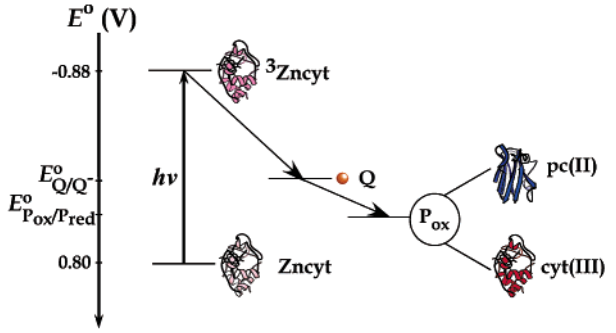
Scheme 1. Rudimentary Electron-Transport Chain in Sol−Gel Glass[a]



[a] The electron transfer between the immobilized protein zinc cytochrome *c*, Zncyt, and the immobilized redox protein P_{ox} occurs stepwise, via mobile electron carriers Q/Q^-. The redox processes are photoinduced. The initial electron donor is the triplet excited state ^{3}Zncyt; the ultimate electron acceptor P_{ox} is either cupriplastocyanin, pc(II), or ferricytochrome *c*, cyt(III). The species Q oxidatively quenches the triplet ^{3}Zncyt, and species Q^- then reduces P_{ox}. The reduction potentials $E°$ (versus NHE) of plastocyanin and cytochrome *c* are 0.39 and 0.26 V, respectively. The reduction potentials $E°$ (versus NHE) of the carriers $FeEDTA^{-/2-}$ and $Ru(NH_3)_6^{3+/2+}$ are 0.12 and 0.05 V, respectively.[47]

according to eqs 3−5. We chose the well-studied inorganic complexes $FeEDTA^{-/2-}$ and $Ru(NH_3)_6^{3+/2+}$ to act as electron shuttles. The unidirectionality of the shuttling reactions is assured by the difference in the redox potentials, as indicated in Scheme 1.

$$^3\text{Zncyt} + \text{Q} \xrightarrow{k_3} \text{Zncyt}^+ + \text{Q}^- \quad (3)$$

$$\text{Zncyt}^+ + \text{Q}^- \xrightarrow{k_4} \text{Zncyt} + \text{Q} \quad (4)$$

$$\text{pc(II)} + \text{Q}^- \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} \text{pc(I)} + \text{Q} \quad (5)$$

To test the generality of this design principle, we also studied interprotein electron-transfer reactions in sol−gel glass between zinc cytochrome *c*, Zncyt, and ferricytochrome *c*, cyt(III), according to eqs 3, 4, and 5′;

$$\text{cyt(III)} + \text{Q}^- \underset{k_{-5'}}{\overset{k_{5'}}{\rightleftharpoons}} \text{cyt(II)} + \text{Q} \quad (5')$$

see Scheme 1. For both systems, Zncyt/Q/pc(II) and Zncyt/Q/cyt(III), energy of light is ultimately converted into reducing equivalents: we observe photoinduced reduction of plastocyanin and cytochrome *c*. Our experimental design also extends the lifetime of the cation radical $Zncyt^+$. To our knowledge, this study is the first protein mimic of a rudimentary electron-transport chain in sol−gel glass.

Experimental Procedures

Chemicals. Distilled water was demineralized to a resistivity greater than 16 MΩ·cm. Chromatography resins and tetramethyl orthosilicate were purchased from Sigma Chemical Co. The complex $Ru(NH_3)_6Cl_3$ was obtained from Alfa Aesar Chemical Co. and purified by a published method.[32] The salt $NaFeEDTA·3H_2O$ was synthesized according to a published procedure.[33] Hydrogen fluoride, nitrogen, and ultrapure argon were purchased from Air Products Co. All other chemicals were obtained from Fisher Chemical Co.

Buffer. All experiments were done in a sodium cacodylate buffer at pH 6.0 and ionic strength of 1.00 M, unless specified otherwise. The ionic strength was adjusted with NaCl. At pH 6.0, formation of the oxo-bridged complex $(FeEDTA)_2O^{4-}$ in dilute solutions of $FeEDTA^-$ is minimized,[33] and all the proteins (zinc cytochrome *c*, cytochrome *c*, and plastocyanin) are stable.

Proteins. Horse-heart cytochrome *c* was obtained from Sigma Chemical Co. Iron was removed, and the free-base protein was purified and reconstituted with zinc(II) by a modification[16] of the original procedure.[17] The criteria of purity were the absorbance ratios $A_{423}/A_{549} > 15.4$ and $A_{549}/A_{585} < 2.0$ and also the rate constant for natural decay of the triplet state, $k_d < 100\ s^{-1}$.

Recombinant spinach plastocyanin was prepared according to the published procedure for overexpression in *Escherichia coli*.[34] The criterion of purity was the absorbance ratio $A_{278}/A_{597} < 1.2$.

Before the experiments, plastocyanin and cytochrome *c* were oxidized with an excess of $K_3Fe(CN)_6$, which was then removed with Centricon-3 ultrafiltration cells, obtained from Millipore Co. Concentrations of proteins were determined on the basis of their known absorptivities: $\epsilon_{423} = 243\ mM^{-1}\ cm^{-1}$ for zinc cytochrome *c*,[17] $\epsilon_{530} = 11.1\ mM^{-1}\ cm^{-1}$ for ferricytochrome *c*,[35] and $\epsilon_{597} = 4700\ M^{-1}\ cm^{-1}$ for cupriplastocyanin.[34] All the proteins were stored in liquid nitrogen.

The Sol−Gel Method. The silica hydrogel, which we refer to simply as glass, was prepared by a modification of the published procedure.[6] A mixture of 3.94 g of tetramethyl orthosilicate and 1.8 mL of 0.15 mM HCl was kept in an ice-cooled ultrasonic bath for 1 h. Clear, homogeneous sol of orthosilicic acid was formed. Upon addition of 10 mL of the aforementioned buffer, gelation began. Solution in the same buffer of zinc cytochrome *c*, of cupriplastocyanin, of a mixture of zinc cytochrome *c* and cupriplastocyanin, or of a mixture of zinc cytochrome *c* and ferricytochrome *c* was added to the polymerizing gel. The sol was mixed and quickly transferred into the "molds": 10 mm × 10 mm × 45 mm or 10 mm × 4 mm × 45 mm polystyrene cuvettes having all sides transparent. The volume of the added sol was 2.5 mL for the larger cuvettes and 1.0 mL for the smaller ones. The gelation occurred very rapidly, and the samples became solid in less than 10 min. The aging lasted for 5 days at 4 °C, under the Parafilm seal. The monoliths were washed several times a day with the same buffer with which they had been made, to remove methanol and thereby prevent protein denaturation. During these washings, the buffer was added directly to the "molds", where there was ample space for perfusion of the monolith. This washing is a standard procedure in preparation of protein-containing sol−gel materials, as described elsewhere.[6] Because the gels were not dried, the monoliths essentially retained their original dimensions. The larger monoliths were used for spectroscopic characterization and uptake experiments. The smaller monoliths were used for kinetic experiments, to economize with the proteins.

Introduction of Mobile Quenchers into the Protein-Doped Glass. The glass monoliths were soaked in buffered solutions of $FeEDTA^-$ and $Ru(NH_3)_6^{3+}$ for 1.5 and 3.5 days, respectively. Control experiments showed that these times were more than sufficient for complete uptake of the quenchers. The soaking solutions were changed three to four times for thorough equilibration with the porous glass. Concentrations of the quenchers inside the glasses will be discussed in detail below.

Deoxygenation of the Samples. To prevent quenching of the triplet state of zinc cytochrome *c* by dioxygen, each sample for kinetic experiments was thoroughly deoxygenated. Samples were gently flushed with ultrapure argon that had been passed through water to avoid evaporation of the solvent. When the sample was an aqueous solution, argon flushing lasted for 15−20 min. After that, the rate constant for the ^{3}Zncyt decay ceased changing, and the samples were ready for the measurements. When the sample contained a monolith of sol−gel glass,

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argon flushing lasted for 12−24 h for complete removal of dioxygen from the pores.[6] Then a solution containing glucose, glucose oxidase, and catalase[36] was added to the soaking solution, and the cuvette was sealed with a septum and several layers of Parafilm. Glass samples were then incubated for an additional 10−12 h at 4 °C. In control experiments, the quenching of ^{3}Zncyt by dioxygen was monitored periodically; these experiments proved our deoxygenation method to be thorough. Addition of the enzyme mixture did not affect the observed kinetics; the same results were obtained with samples deoxygenated by combination of argon flushing and enzymatic reaction and with samples deoxygenated by argon flushing for more than 24 h. As a special precaution, during the prolonged kinetic experiments the enzymes were not used. These samples were deoxygenated by flushing with argon for 24−36 h, sealed with a septum, and kept under a constant pressure of argon.

Spectroscopic Measurements. Absorption spectra were recorded with a Perkin-Elmer Lambda 18 spectrophotometer. A protein-doped glass monolith in the sample beam was examined against an undoped glass monolith in the reference beam. Circular dichroism spectra were recorded at 25.0 ± 0.1 °C with the instrument JASCO J-710 equipped with a Peltier thermoelectric cell. These spectra gave the ellipticity of the protein samples. Absorption spectra of the same samples, measured immediately afterward, gave the protein concentrations; molar ellipticity was then calculated and normalized to the mean residue value. Electron paramagnetic resonance (EPR) spectra were recorded at 130 K with a Bruker ER 200D-SRC spectrometer operating at 9.57 GHz.

Kinetic Measurements. Laser flash photolysis experiments were performed with a Continuum Minilite II Q-switched Nd:YAG laser, which delivered 3.5-ns pulses at 532 nm. The laser has a built-in attenuator. The laser output was calibrated with a Scientech power meter, model H410D. In the reported experiments, the output power was adjusted to 1 mJ/pulse. The probe light beam from a 250-W QTH lamp (Oriel 66198) passed through an interference filter (Optometrics USA) having a bandwidth of 10 nm. Another interference filter, for the detection light, was placed in front of the home-built photodiode (Hamamatsu Si PIN photodiode model 53071, 40-MHz cutoff frequency). The transient signals from the photodiode were digitized using a LeCroy LT 322 oscilloscope. To improve the signal-to-noise ratio, kinetic traces were averaged 50−300 times. Decay of the triplet state ^{3}Zncyt was monitored at 458 nm. Appearance and disappearance of the cation radical Zncyt$^+$ were monitored at 676 nm. Reduction and reoxidation of plastocyanin were monitored at 600 nm.

Slow reactions in sol−gel glasses were monitored spectrophotometrically with a Perkin-Elmer Lambda 18 spectrophotometer. Before the measurements, a glass sample was exposed to 200 pulses of the Nd:YAG laser. The processes in the irradiated samples were always compared to those in the analogously prepared samples that were kept in the dark.

All kinetic measurements were done at a temperature of 25 ± 2 °C. The concentration of zinc cytochrome *c* was always 10 μM. The concentration of the triplet ^{3}Zncyt depended on the excitation power and was kept constant at ca. 1 μM. In all the measurements, the mole ratio of Q to ^{3}Zncyt was greater than 10:1 so that the conditions for the pseudo-first-order reaction always prevailed. Concentrations of other components will be specified below.

Fittings of Kinetic Data. Kinetic data were analyzed with the software SigmaPlot v. 5.0, from SPSS Inc. All error margins include two standard deviations and correspond to the confidence limit greater than 95%.

The rate constants were obtained from changes with time of the absorbance at 458, 600, and 676 nm. The change at 458 nm corresponds to the disappearance of the triplet ^{3}Zncyt and is usually monoexponential; it is described by eq 6, in which a_1^{458} is the amplitude and k_1 is the rate constant. The change at 676 nm is caused by both the triplet ^{3}Zncyt and the cation radical Zncyt$^+$ and is described by eqs 7−9.[37] The contribution of the triplet ^{3}Zncyt to the absorbance change at 676 nm is given in eq 8, in which a_t is the instantaneous absorbance after the laser flash. The contribution of the cation radical Zncyt$^+$ to the absorbance change at 676 nm is given in eq 9, in which a_c is the amplitude and k_2 is the rate constant for the decay of Zncyt$^+$. To determine the second-order rate constant for the thermal back-reaction, k_b, traces at 676 nm were fitted to eq 10,[29] in which a_1^{676} and a_2^{676} are the amplitudes for appearance and disappearance of Zncyt$^+$, and c_0 is the concentration of Zncyt$^+$. The change at 600 nm is caused by the triplet ^{3}Zncyt, the cation radical Zncyt$^+$, and pc(II), and is described by eqs 8, 9, 11, and 12. In eq 12, a_{Cu} is the amplitude, and k_{red} and k_{ox} are the rate constants for reduction and oxidation of the copper site in plastocyanin, respectively. In the studies of oxidative quenching of ^{3}Zncyt by cupriplastocyanin in bulk solution, the equalities $k_{red} = k_1$ and $k_{ox} = k_2$ hold, and the simpler eq 13 is obtained.

$$\Delta A_{458} = a_1^{458} \exp(-k_1 t) + b \tag{6}$$

$$\Delta A_{676} = \Delta A_{triplet} + \Delta A_{cation} \tag{7}$$

$$\Delta A_{triplet} = a_t \exp(-k_1 t) + b \tag{8}$$

$$\Delta A_{cation} = a_c\{\exp(-k_2 t) - \exp(-k_1 t)\} + b \tag{9}$$

$$\Delta A_{676} = -a_1^{676} \exp(-k_1 t) + a_2^{676} \frac{1}{1 + c_0 k_b t} + b \tag{10}$$

$$\Delta A_{600} = \Delta A_{triplet} + \Delta A_{cation} + \Delta A_{Cu} \tag{11}$$

$$\Delta A_{Cu} = a_{Cu}\{\exp(-k_{red} t) - \exp(-k_{ox} t)\} + b \tag{12}$$

$$\Delta A_{600} = a_1^{600} \exp(-k_1 t) + a_2^{600} \exp(-k_2 t) + b \tag{13}$$

The second-order rate constants for the quenching of ^{3}Zncyt were obtained from the corresponding pseudo-first-order rate constants (measured at 5−10 different concentrations of the quencher Q) by least-squares fittings.

Yield of Electron-Transfer Products from ^{3}Zncyt Quenching. The yield of the cation radical Zncyt$^+$ was estimated according to eq 14,[22] in which ΔA_{676} is the maximum change and ΔA_{458} is the change coincident with the pulse (at time zero), in absorbance at the specified wavelength; $\Delta\epsilon_{458}$ and $\Delta\epsilon_{676}$ are the differences in the molar absorptivities (extinction coefficients) at these two wavelengths. The absorptivity at 458 nm increases by 3.5×10^4 M^{-1} cm^{-1} as Zncyt is excited to ^{3}Zncyt. The difference in absorptivities at 676 nm between Zncyt$^+$ and Zncyt is 1.4×10^4 M^{-1} cm^{-1}.[21] The yield of the electron-transfer products was determined in the presence of a very high excess of the quencher, whose concentrations were 100 μM and greater.

$$Y_{ET} = \frac{[\text{Zncyt}^+]}{[^3\text{Zncyt}]} = \frac{\Delta A_{676}}{\Delta A_{458}} \frac{\Delta\epsilon_{458}}{\Delta\epsilon_{676}} \tag{14}$$

Results and Discussion

Introduction of Proteins into the Sol−Gel Glass. Doping of the silica glass with zinc cytochrome *c* and cupriplastocyanin results in glass monoliths having uniform color and constant absorbance in different regions, evidence for homogeneous distribution of the two proteins. After the gelation was complete, the proteins were completely immobilized: repetitive washing

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of crushed gels with water did not result in detectable extraction of either zinc cytochrome *c* or plastocyanin from the gels.

Previous studies in this laboratory have shown that encapsulation of zinc cytochrome *c* and cytochrome *c* in sol−gel glass only slightly perturbs the polypeptide backbone and does not detectably perturb the heme group, which is the redox agent.[6] We now report that the encapsulation procedure is generally noninvasive toward plastocyanin, too.

The visible absorption spectrum of cupriplastocyanin shows a major band at 597 nm, attributed to the charge-transfer transition $S_{Cys}\pi \rightarrow$ Cu $d_{x^2-y^2}$ at the copper site.[38] As Figure S1 in the Supporting Information shows, the absorption spectrum of cupriplastocyanin encapsulated in sol−gel glass is nearly identical to that of the protein in solution. When the glass doped with 30 μM cupriplastocyanin was soaked first in a 1.0 mM solution of ascorbic acid and next in a 1.0 mM solution of K_3-$Fe(CN)_6$, complete reduction and then reoxidation of the copper site were evident from disappearance and appearance of the characteristic band at 597 nm (data not shown). These results indicate that encapsulation does not affect the intrinsic redox properties of plastocyanin.

In previous studies in this laboratory, circular dichroism (CD) spectroscopy has proved very useful in characterizing encapsulated proteins.[6,39] Different regions of the CD spectrum provide information about protein secondary and tertiary structures and about the local environment of the protein chromophore. Figure 1 shows the CD spectra of cupriplastocyanin in solution and in sol−gel glass.

As illustrated in Figure 1a, the far-UV CD spectra of cupriplastocyanin in solution and in sol−gel glass are nearly identical. Evidently, encapsulation does not perturb the secondary structure of the protein.

The near-UV CD spectra of cupriplastocyanin in solution and in sol−gel glass are shown in Figure 1b. This region of the CD spectrum is sensitive to changes in tertiary structure of the protein and to the changes in the local environments of aromatic residues. The CD bands at ca. 252 nm and in the region from 278 to 284 nm are associated with phenylalanine and tyrosine residues, respectively. The CD bands in the region from 295 to 330 nm are due to intrahistidine and histidine charge-transfer transitions. As Figure 1b shows, the positive band in the phenylalanine region of the spectrum is unaffected by encapsulation, while other bands of the near-UV CD spectrum are slightly perturbed. The observed perturbations are comparable to those observed upon reduction of the copper site (in cuproplastocyanin) and removal of the copper ion (in apoplastocyanin).[40,41] The conformational changes upon encapsulation must be slight since not all the aromatic residues are affected by them. These changes are consistent with a flexible tertiary structure of plastocyanin.[41] The residues Tyr83 and His87 in plastocyanin belong to a flexible protein loop,[42] even a small constraint of which by the sol−gel matrix may be evident in the CD spectrum of the protein. On the other hand, all the phenylalanine residues belong to relatively rigid parts of the protein,[43] and the CD spectra in the region around ca. 252 nm are identical for the protein in solution and in sol−gel glass.

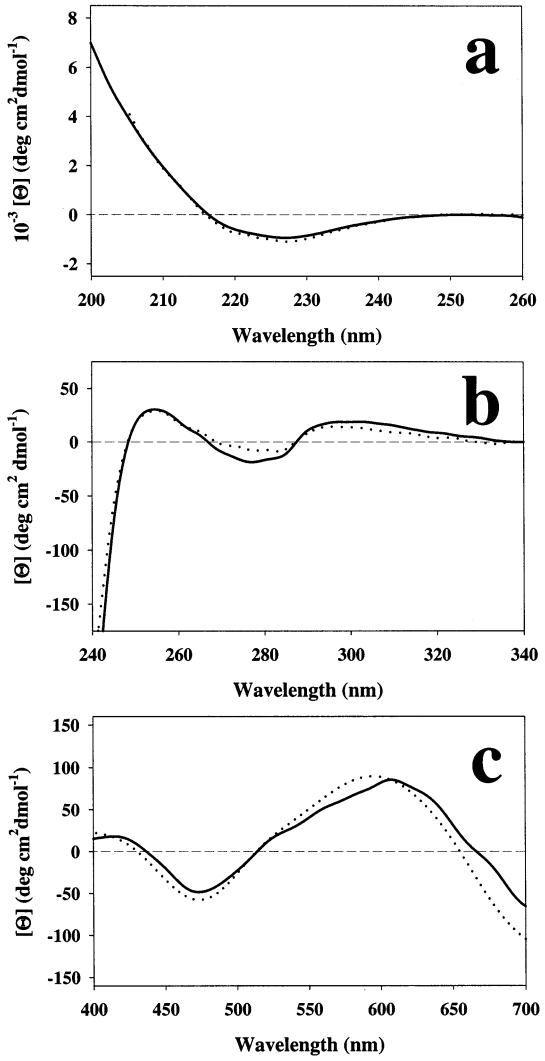

Figure 1. CD spectra at 25 °C of cupriplastocyanin in a sodium cacodylate buffer at pH 6.0 and ionic strength of 1.00 M (solid line) and in sol−gel glass infused with the same buffer (dotted line): (a) far-UV region, (b) near-UV region, and (c) visible region. Molar ellipticity is normalized to the mean residue value.

Figure 1c shows the visible CD spectrum of plastocyanin. The relative position of the characteristic band does not change upon encapsulation, evidence that the copper site is unperturbed. The band shape changes only slightly upon encapsulation of plastocyanin, as was the case with the CD spectra of bacteriorhodopsin and zinc cytochrome *c*.[6,44] Detailed studies of the latter two proteins showed that they are not significantly perturbed in the glass, and we conclude that neither is plastocyanin.

EPR spectroscopy provides further evidence that the copper site in cupriplastocyanin is unaffected by encapsulation. As shown in Figure 2, the EPR spectra of cupriplastocyanin in solution and in sol−gel glass are nearly identical. The hyperfine

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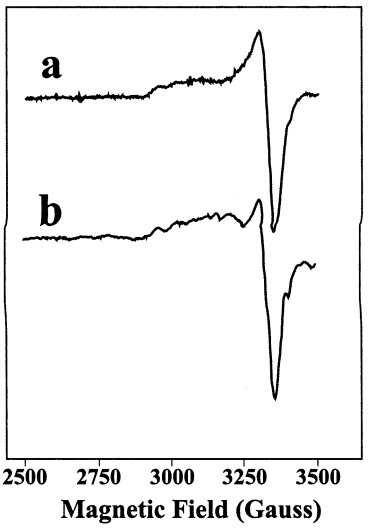

Figure 2. EPR spectra at 130 K of cupriplastocyanin (a) in a sodium cacodylate buffer at pH 6.0 and ionic strength of 1.00 M and (b) in sol−gel glass infused with the same buffer.

splitting in the parallel region, $A_{\parallel} = 6.4$ mT, is typical for a copper site of type 1.

We conclude that encapsulation in sol−gel glass changes only slightly the conformation of zinc cytochrome *c* and plastocyanin; both the heme group in the former and the copper site in the latter protein remain unperturbed and, thus, capable of the redox reactions.

Oxidative Quenching of ^{3}Zncyt by Cupriplastocyanin in Solution. In the absence of a quencher, the decay of the triplet excited state of zinc cytochrome *c* to its ground state in a sodium cacodylate buffer at pH 6.0 and ionic strength of 1.00 M is monoexponential with the rate constant k_d of 90 ± 10 s^{-1}. Upon addition of cupriplastocyanin, the decay of the triplet state ^{3}Zncyt accelerated but remained monoexponential. Studies in this laboratory have shown that cupriplastocyanin quenches the triplet state of zinc cytochrome *c* by electron transfer.[22,26,29] The rate constants for disappearance of ^{3}Zncyt and appearance of Zncyt$^+$ are equal within the error bounds; typical traces are shown in Figure 3. The second-order rate constants k_1, for the electron transfer from ^{3}Zncyt to cupriplastocyanin, and k_2, for the back electron transfer from cuproplastocyanin to Zncyt$^+$, are shown in Table 1. In solution, where the two proteins are free to diffuse, both of these interprotein electron-transfer reactions are fast.

Suppression of Interprotein Electron Transfer in Sol−Gel Glass Owing to Spatial Separation of the Proteins. The rate constant for the natural decay of the triplet ^{3}Zncyt in sol−gel glass, k_d, is 60 ± 10 s^{-1}. When both zinc cytochrome *c* and cupriplastocyanin were introduced in sol−gel glass, no quenching of the triplet state ^{3}Zncyt, that is, no electron transfer from ^{3}Zncyt to cupriplastocyanin, was observed. As Figure 4 shows, at all three wavelengths (458, 600, and 676 nm), the only observed process is the natural decay of the triplet state ^{3}Zncyt. Although both proteins are present in the glass, they do not interact with each other, presumably because the relatively rigid sol−gel matrix keeps them apart, in separate pores.

To verify this explanation, we monitored quenching of the triplet ^{3}Zncyt by cupriplastocyanin over the course of gelation. With our usual buffer at ionic strength of 1.00 M, the gelation was very rapid. To allow more time for sample deaeration and

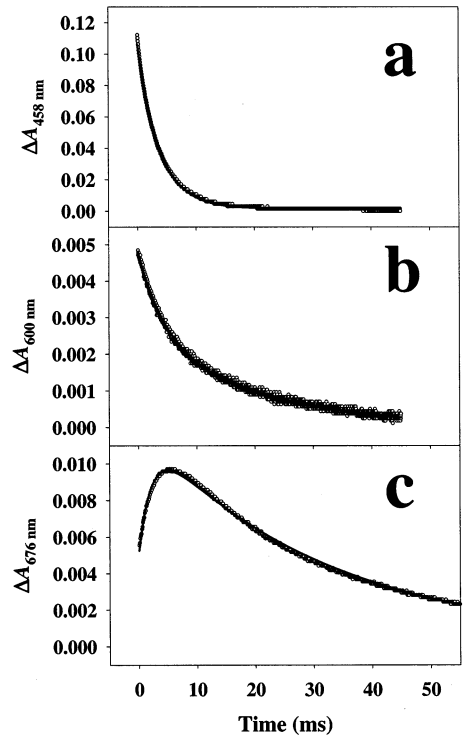

Figure 3. Changes in transient absorbance at 25 °C of the solution containing 10 μM zinc cytochrome *c* and 20 μM cupriplastocyanin in a sodium cacodylate buffer at pH 6.0 and ionic strength of 1.00 M. (a) Disappearance of the triplet ^{3}Zncyt, monitored at 458 nm. The line is a single-exponential fit. (b) Absorbance changes at 600 nm, caused by disappearance of the triplet ^{3}Zncyt, appearance and disappearance of the cation radical Zncyt$^+$, and reduction and reoxidation of the copper site in plastocyanin. The line is a fit to eqs 11−13. (c) Appearance and disappearance of the cation radical Zncyt$^+$, monitored at 676 nm. The line is a fit to eqs 7−9.

Table 1. Second-Order Rate Constants (in M^{-1} s^{-1}) for the Bimolecular Reactions in This Study[a]

rate constant	reaction	in solution	in sol–gel silica glass
k_1	^{3}Zncyt + pc(II)	$(1.2 \pm 0.1) \times 10^7$	0
	^{3}Zncyt + cyt(III)	$(6.7 \pm 0.3) \times 10^6$	0
k_2	Zncyt$^+$ + pc(I)	$(9.1 \pm 0.2) \times 10^7$	0
	Zncyt$^+$ + cyt(II)	$(8.4 \pm 0.3) \times 10^7$	0
k_3	^{3}Zncyt + FeEDTA$^-$	$(4.8 \pm 0.3) \times 10^7$	$(8.9 \pm 0.6) \times 10^6$
	^{3}Zncyt + Ru(NH$_3$)$_6^{3+}$	$(5.1 \pm 0.1) \times 10^7$	$(8.0 \pm 2.4) \times 10^6$
k_4	Zncyt$^+$ + FeEDTA^{2-}	$(1.1 \pm 0.1) \times 10^8$	$(4.9 \pm 0.2) \times 10^7$
	Zncyt$^+$ + Ru(NH$_3$)$_6^{2+}$	$(2.2 \pm 0.7) \times 10^8$	$(7.8 \pm 2.7) \times 10^7$

[a] The solvent was a sodium cacodylate buffer at pH 6.0 and ionic strength of 1.0 M.

to ensure continuous monitoring of quenching during polymerization, we used in this experiment a buffer at ionic strength of 2.5 mM. At this low ionic strength, the proteins zinc cytochrome *c* and plastocyanin associate because of the complementary charges on their surfaces, and the reaction in solution is biphasic.[22,26,29] The faster phase, k_f, is the unimolecular reaction within the persistent diprotein complex, Zncyt/pc(II), which exists prior to the laser flash. The slower phase, k_s, is the bimolecular reaction between unassociated proteins, within the transient (collisional) complex. The electron-transfer reaction between ^{3}Zncyt and pc(II) is gated by structural rearrangement of the diprotein complex Zncyt/pc(II), a process during which the two proteins remain docked in the same general orientation but wiggle with respect to each other.[30]

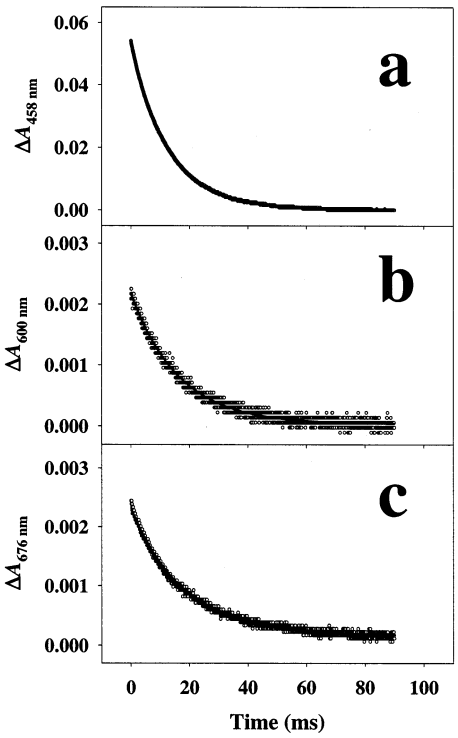

Figure 4. Changes in transient absorbance at 25 °C of the sol−gel glass doped with 10 μM zinc cytochrome *c* and 20 μM cupriplastocyanin in the absence of the redox mediator at (a) 458 nm, (b) 600 nm, and (c) 676 nm. At all three wavelengths, the changes in the spectra correspond to the natural decay of the triplet state ^{3}Zncyt. Each line is a single-exponential fit. The solvent is a sodium cacodylate buffer at pH 6.0 and ionic strength of 1.00 M.

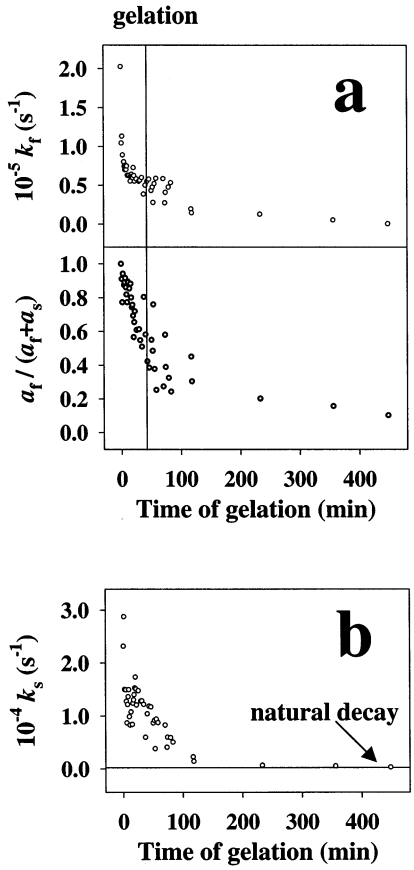

Figure 5. Dependence on the time of gelation of (a) the rate constant k_f for the unimolecular reaction within the persistent diprotein complex, Zncyt/pc(II), (upper panel) and the relative amplitude of this process in the overall quenching of the triplet ^{3}Zncyt (lower panel) and (b) the rate constant k_s for the bimolecular reaction between ^{3}Zncyt and pc(II). The solvent is a sodium phosphate buffer at pH 7.0 and ionic strength of 2.5 mM; temperature is 25 °C.

As Figure 5 shows, at the beginning of gelation both unimolecular and bimolecular reactions occur. The respective rate constants k_f and k_s decrease as the gelation progresses because of the increase in the solution viscosity.[31] Solution viscosity greatly impedes both the unimolecular rearrangement of the diprotein complex and the diffusion of the two unassociated proteins.[31] As shown in Figure 5a, the relative amplitude of the faster phase decreases during gelation, indicating the break-up of the diprotein complex. The two phases converge at higher viscosity. As shown in Figure 5b, after 3.5 h of gelation, quenching of ^{3}Zncyt is no longer observed; the only process is the natural decay of ^{3}Zncyt.

We conclude that *the silica matrix effectively inhibits intermolecular interactions of the encapsulated proteins*. The rigid silica cage restricts protein diffusion and thus eliminates the bimolecular electron-transfer reaction. Moreover, the suppression also of the unimolecular electron-transfer reaction implies that the individual protein molecules become isolated from each other in the sol−gel glass.

The isolation of the individual dopant molecules in sol−gel matrices has been reported. Avnir and co-workers studied luminescence of pyrene encapsulated in sol−gel glass.[45] After the sol−gel glass was dried, no excimer emission was observed, suggesting that the pyrene molecules were completely separated. In another study from the same laboratory,[46] trypsin entrapped in sol−gel glass was stable toward autodigestion for several months, whereas trypsin immobilized in sol−gel glass by surface adsorption and kept under the same conditions became completely autodigested. Evidently, the sol−gel entrapment isolated the enzyme molecules and thus abolished their destructive intermolecular interactions.

Electron Transfer between Immobilized ^{3}Zncyt and Cupriplastocyanin Mediated by Mobile Inorganic Complexes. Now we will show that the interprotein electron transfer between immobilized ^{3}Zncyt and cupriplastocyanin can be revived with the help of the mobile redox mediators. Stepwise electron transfer (eqs 3 and 5) results in ultimate conversion of the energy of light into reducing equivalents for cupriplastocyanin. The design of this rudimentary electron-transport chain is illustrated in Scheme 1.

Both proteins, Zncyt and pc(II), are completely immobilized in the sol−gel glass but are redox-active. A mobile quencher having a suitable redox potential is free to diffuse in the intrapore liquid phase and thus serve as a charge carrier between the two proteins. We chose two well-studied inorganic complexes, namely, $FeEDTA^{-/2-}$ and $Ru(NH_3)_6^{3+/2+}$, to act as redox shuttles. After the initial photoinduced reaction in eq 3, the species Q^- migrates and reduces cupriplastocyanin according to eq 5. Chemical communication between the spatially separated proteins is thus established.

High ionic strength of the buffer was important for the construction of this rudimentary electron-transport chain. The

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oppositely charged zinc cytochrome *c* and cupriplastocyanin are less likely to associate under these conditions and to appear in the same pore. Because high ionic strength also weakens electrostatic interactions between the ionic quenchers and the siloxide anions on the silica surface, these mobile electron carriers can more easily diffuse through the glass pores. Moreover, numerous precedents show that high ionic strength greatly weakens the electrostatic interactions (both repulsive and attractive) between the ionic complexes $FeEDTA^{-/2-}$ or $Ru(NH_3)_6^{3+/2+}$, on one side, and the charged proteins cytochrome *c* or plastocyanin, on the other.[9−11,47] Finally, high ionic strength of the buffer prolongs the lifetime of the cation radical $Zncyt^+$, the electron-transfer product of quenching of the triplet ^{3}Zncyt by the oxidant Q.[29]

I. Inorganic Complexes as Mediators of Interprotein Communications in Sol−Gel Glass. The mobile quenchers $FeEDTA^-$ and $Ru(NH_3)_6^{3+}$ were introduced in sol−gel glass by soaking of a preformed glass monolith in a buffered solution of the quencher. Previous studies in this laboratory showed that diffusion of even small molecules inside the sol−gel glass is significantly hindered by the matrix.[6,25,48] Because the sol−gel glass may interact with the solute molecules, diffusion of these molecules between the glass and the surrounding solution may result in their uneven concentrations in solution and in sol−gel glass even when the system has reached equilibrium.

To investigate possible interactions of the quenchers with sol−gel glass and the effects of these interactions on the quenchers' mobility, the uptake experiments were carried out with glass monoliths that did not contain any proteins. The results of these experiments are shown in Figure S2 in the Supporting Information. Under our conditions, at high ionic strength, the uptake of $FeEDTA^-$ is finished in less than 5 h, while the much slower uptake of $Ru(NH_3)_6^{3+}$ takes about 72 h. At low ionic strength, the uptake of $Ru(NH_3)_6^{3+}$ is exceptionally slow: the complex ions continue to accumulate in the glass even after 2 weeks.[49]

The partitioning coefficient *P*, defined in eq 15,[6] is a quotient of the quencher's concentrations inside the glass monolith and in the solution surrounding the monolith. This coefficient is 0.95 for $FeEDTA^-$ and 1.38 for $Ru(NH_3)_6^{3+}$. Even at high ionic strength, there is an elevated uptake of $Ru(NH_3)_6^{3+}$ ions by sol−gel glass.[25]

$$P = [Q]_{in}/[Q]_{out} \tag{15}$$

At high ionic strength, electrostatic interactions between ionic molecules and sol−gel glass are negligible;[6,39] however, the results of the uptake experiments with the complexes $FeEDTA^-$ and $Ru(NH_3)_6^{3+}$ are different. Because both ions are much smaller than 20 Å, the estimated lower limit of the pore size,[6] uniform diffusion of the quencher probably is not obstructed by steric factors. We attribute the difference in the uptake of the two ions to extensive hydrogen-bonding with the surface silanol groups in the case of the $Ru(NH_3)_6^{3+}$; this interpretation is consistent with a theoretical prediction.[50−52] We will consider the difference in behavior of these two ions in the sol−gel glass when analyzing the kinetic data below.

II. Picking Up an Electron from ^{3}Zncyt: Oxidative Quenching of ^{3}Zncyt by Q. Both $FeEDTA^-$ and $Ru(NH_3)_6^{3+}$ quench the triplet state ^{3}Zncyt by electron transfer according to eq 3. Upon addition of either complex, the decay of the triplet state ^{3}Zncyt became faster. The electron-transfer intermediate, the cation radical $Zncyt^+$, was observed directly by monitoring the absorbance changes at 676 nm. The rate constants for disappearance of ^{3}Zncyt, monitored at 458 nm, and appearance of $Zncyt^+$, monitored at 676 nm, were equal within the error bounds. The observed pseudo-first-order rate constants depended linearly on the quencher concentration; at no case did we detect saturation behavior (leveling off), even at high concentrations of the quencher. These results indicate a simple collisional mechanism of quenching, in which the binding of Zncyt and Q is unmeasurably low and a persistent Zncyt/Q complex is not involved. The second-order rate constants for the forward electron transfer from ^{3}Zncyt to Q, k_3, and for the back electron transfer from Q^- to $Zncyt^+$, k_4, are listed in Table 1.[53]

The quenching of ^{3}Zncyt by both $FeEDTA^-$ and $Ru(NH_3)_6^{3+}$ in solution and by $FeEDTA^-$ in sol−gel glass is monoexponential. The quenching of ^{3}Zncyt by $Ru(NH_3)_6^{3+}$ in sol−gel glass is best described by a stretched-exponential function (eq 16).[54] In eq 16, the quenching rate constant k_q is the most probable value within the family of reactive species; k_d is the intrinsic rate constant (in our case, natural decay of ^{3}Zncyt); and *n* is the distribution parameter. The nonexponential kinetics observed with chromophores encapsulated in sol−gel glasses reflects the microheterogeneous environment in the silica pores.[5,6] The qualitative difference in the kinetic behavior of the two quenchers, $FeEDTA^-$ and $Ru(NH_3)_6^{3+}$, is consistent with their interactions with sol−gel glass being essentially masked for the former quencher and still being prominent for the latter.

$$\Delta A = A_0 \exp\{(-k_d t) - (k_q t)^n\} + b \tag{16}$$

Because the glass monoliths doped with Zncyt are equilibrated with the quencher Q prior to photoexcitation of Zncyt, quenching in sol−gel glass is not limited by transfer of Q from the bulk solution to the glass interior, which is slow. The photoinduced reaction in eq 3 *within the glass pores* is relatively fast, although slower than that in solution. As Table 1 shows, for both quenchers $FeEDTA^-$ and $Ru(NH_3)_6^{3+}$, the bimolecular rate constant k_3 is ca. 5 times smaller in sol−gel glass than in solution, presumably because trapping of zinc cytochrome *c* lessens the probability of collisions with the quencher. In addition, the movement of even small molecules may be retarded by the glass matrix.[6] Because the pores in a sol−gel glass are convoluted, species diffusing in confined liquids cover a longer distance than they would in free liquids.[55]

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(53) The reported self-exchange rate constants for the complexes $FeEDTA^{-/2-}$ and $Ru(NH_3)_6^{3+/2+}$ are 3×10^4 and $(8 \pm 1) \times 10^2$ M^{-1} s^{-1}, respectively.[47] These reactions are too slow to influence the effective donor−acceptor distance.
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Table 2. Yield of the Electron-Transfer Products, Zncyt$^+$ and Q$^-$, from the Bimolecular Quenching of ^{3}Zncyt by the Oxidant Q[a,b]

	Y_{ET}	
quencher, Q	in solution	in sol–gel silica glass
$FeEDTA^-$	0.38 ± 0.09	0.33 ± 0.09
$Ru(NH_3)_6^{3+}$	0.37 ± 0.11[c]	0.25 ± 0.10

[a] The solvent was a sodium cacodylate buffer at pH 6.0 and ionic strength of 1.0 M. [b] Estimated according to eq 14. [c] Previously reported value of Y_{ET} for this reaction in solution is 0.4 (ref 21)

The rate constant for the back reaction, k_4, is also smaller in sol−gel glass than in solution; see Table 1. The representative traces of Zncyt$^+$ in solution and in sol−gel glass are shown in Figure S3 in the Supporting Information. The relatively small retardation of the back reaction in sol−gel can be explained as was done above for the forward reaction. Moreover, a fraction of the photogenerated Q$^-$ may diffuse away from the immobilized Zncyt$^+$ into the glass pores, thus slowing the back reaction.

In studies of the pyrene−methyl viologen assemblies, Py−MV^{2+}, the back reaction was considerably slower in sol−gel glass than in solution.[4] For that system, adsorption of the photogenerated cation radical MV$^{\bullet+}$ by the sol−gel matrix at relatively low ionic strength may contribute to the stabilization of the charge-separated pair. In other systems, the back reaction had very similar rate constants in solution and in sol−gel glass, and it was suggested that low yields of cage escape were responsible.[4,56]

For both $FeEDTA^-$ and $Ru(NH_3)_6^{3+}$, the products from the redox quenching of ^{3}Zncyt are formed with similar yields, Y_{ET}, in solution and in sol−gel glass; see Table 2. This similarity and a relatively small difference in the rate constants are consistent with the fact that interactions between ionic complexes and the sol−gel glass are minimized at high ionic strength. These results may also suggest that the redox chemistry occurs primarily in the solvent-like porous regions of the glass.[56] Indeed, our gel materials were not dried and remained "solvent-rich", fully compatible with the native conformations of the entrapped proteins.[1]

To summarize, the reactivity of the quenchers $FeEDTA^-$ and $Ru(NH_3)_6^{3+}$ with the triplet ^{3}Zncyt is minimally affected by the interior of the sol−gel glass. Having demonstrated this, we turn to the role of these complexes as mediators of interprotein communications.

III. Observation of the Long-Lived Cation Radical Zncyt$^+$ in the Zncyt/Q/pc(II) Glasses. A very different behavior of the cation radical Zncyt$^+$ was observed in glasses containing both proteins, Zncyt and pc(II), and a charge carrier Q. As shown in Figure 6 and Figures S4 and S5 in the Supporting Information, the lifetime of Zncyt$^+$ is significantly longer in the "diprotein" than in the "monoprotein"glasses. The decay of Zncyt$^+$ appears to occur on two time scales. The faster and major component of this decay in the Zncyt/Q/pc(II) glasses is analogous to the only mode of the decay in the Zncyt/Q glasses and is a second-order process. This process is attributed to the back electron transfer from Q$^-$ to Zncyt$^+$ according to eq 4 and its second-order rate constant matches the rate constant k_4 in Table 1. Between 25 and 40% of the initially formed Zncyt$^+$ lives longer ($k_{slow} = 1/\tau_{slow} = 1.1 \pm 0.2$ s^{-1}) and decays by a first-order

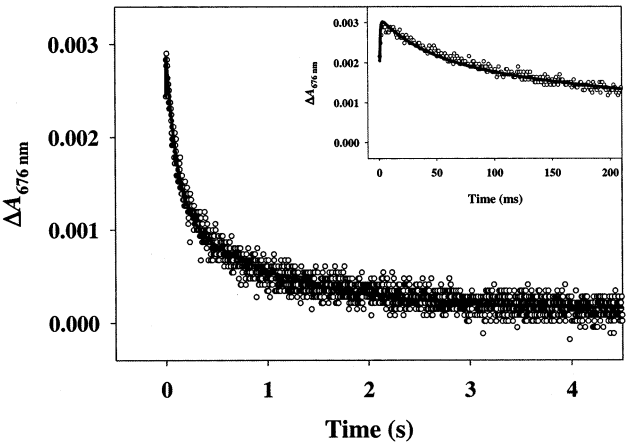

Figure 6. Appearance and disappearance of the cation radical Zncyt$^+$, monitored at 676 nm, in the sol−gel glass doped with zinc cytochrome *c* and cupriplastocyanin, in the presence of 100 μM $FeEDTA^-$ as the quencher Q of ^{3}Zncyt. The concentrations of the encapsulated proteins are 10 μM zinc cytochrome *c* and 20 μM cupriplastocyanin. (Inset) The same kinetic trace, but at a shorter time scale. The solvent is a sodium cacodylate buffer at pH 6.0 and ionic strength of 1.00 M; temperature is 25 °C.

process. This behavior persists in glasses containing different concentrations of the quencher (from 10 to 100 μM) and cupriplastocyanin (20 and 40 μM); see Figure S6 in the Supporting Information.

In solution, the thermal back reaction in eq 4 occurs rapidly. In the sol−gel glasses containing pc(II), a fraction of the photogenerated Q$^-$ is able to escape into the pores and react with the immobilized pc(II) according to eq 5. Therefore, diminished amounts of Q$^-$ would remain available for the reaction in eq 4, and a fraction of the formed Zncyt$^+$ may decay by a different mechanism.

In a previous study with small molecules, similar experimental design has led to long-lived separation of charges.[7] The immobilized donor and acceptor were separated by a long distance, and the slow phase of the decay of the electron-transfer intermediate was attributed to recombination of such charge-separated pair. By analogy, we can envision the formation of the charge-separated pair Zncyt$^+$−pc(I) in our system.

The slower phase of the Zncyt$^+$ decay, however, cannot be attributed to recombination of the Zncyt$^+$−pc(I) pairs. In contrast to the previous system,[7] the slower process of the Zncyt$^+$ decay in our Zncyt/Q/pc(II) glasses is independent of the concentration of pc(II). For the charge-recombination process, a change in the concentration of the immobilized electron acceptor is expected to affect the average spatial separation between the immobilized donor and acceptor and thus alter the rate of the process. We did not observe such an effect in our system.

As discussion below will show, reduction of plastocyanin by photogenerated Q$^-$ in sol−gel glass occurs on the time scale of hours. At no time did we observe the presence of the Zncyt$^+$ at these time scales. The visible spectrum of the glass sample taken within 1 min after laser excitation did not show any characteristic features associated with Zncyt$^+$.[21] These observations argue against the slow recombination of Zncyt$^+$−pc(I) pairs in our system and suggest that there are other routes leading to rereduction of Zncyt$^+$.

Other researchers noticed the intrinsic instability of Zncyt$^+$, which appears to decompose in less than 1 s.[21] Indeed, the slow process of the Zncyt$^+$ decay in our system occurs on a similar

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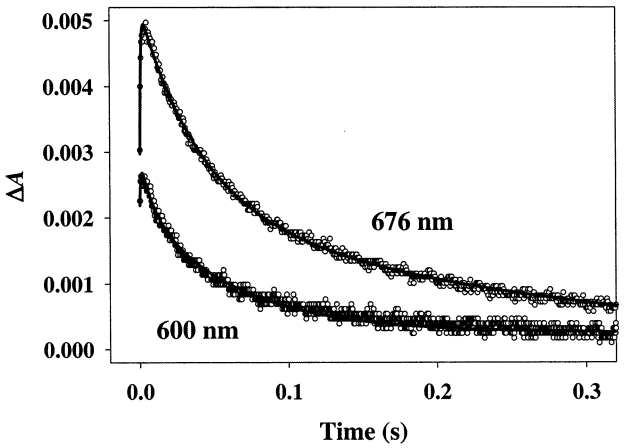

Figure 7. Changes in absorbance at 600 nm (lower trace) and 676 nm (upper trace) of the sol−gel glass Zncyt/FeEDTA$^-$/pc(II), doped with 10 μM zinc cytochrome *c* and 20 μM cupriplastocyanin, in the presence of 100 μM FeEDTA$^-$ as the quencher Q of ^{3}Zncyt. The solvent is a sodium cacodylate buffer at pH 6.0 and ionic strength of 1.00 M; temperature is 25 °C.

time scale. The exact nature of this process remains unknown. The cation radicals of zinc porphyrins, ZnP$^+$, are relatively short-lived.[57] In studies of zinc-substituted proteins, the radical transfer from ZnP$^+$ to some aromatic residues has been suggested.[20,58] Other studies implicated water as a reductant of ZnP$^+$.[59] The detailed mechanism of rereduction of Zncyt$^+$ is beyond the scope of this study. This rereduction is not a part of the biomimetic electron-transport chain under investigation here.

IV. Delivering the Electron to Cupriplastocyanin: Reduction of Cupriplastocyanin by Photogenerated Q$^-$. The qualitative difference between the decay of Zncyt$^+$ in the "monoprotein" Zncyt/Q and "diprotein" Zncyt/Q/pc(II) glasses gave the first indication of the reaction in eq 5. The experiments described below provide additional evidence for this process, the last step in our vectorial electron-transport chain.

Wavelengths around 600 nm are best suited for monitoring the redox state of copper in plastocyanin. Unfortunately, the triplet ^{3}Zncyt and the cation radical Zncyt$^+$ also contribute to the absorbance changes around 600 nm. As shown in Figure 7, in the Zncyt/Q/pc(II) glass the transient absorbances at 600 and 676 nm evolve differently. At 600 nm, the slow decay of the cation radical Zncyt$^+$ is accompanied by a faster process, different from the decay of the triplet ^{3}Zncyt.

This faster component likely arises from reduction of pc(II) in the close vicinity of Zncyt$^+$, according to eq 5. We do not, however, rely on these results alone to prove that this "shuttling" electron-transfer step occurs in our system. Multiphasic kinetics due to several absorbing species and the small magnitude of the signal complicate the analysis of the transient spectra. To explore further the reaction in eq 5, we followed it on a longer time scale.

The redox reactions in eq 5 between pc(II) and FeEDTA^{2-} and between pc(II) and Ru(NH$_3$)$_6^{2+}$ are relatively fast in solution; the reported second-order rate constants fall in the range 10^4-10^6 M^{-1} s^{-1}.[47,60,61] In our experiments, the species Q$^-$ are generated in the vicinity of Zncyt, according to eq 3. Because the proteins Zncyt and pc(II) are spatially separated from each other by the sol−gel glass, the rate of the reaction in eq 5 is *limited by diffusion of the photogenerated Q$^-$ in the porous glass network*. The uptake experiments indicate that such diffusion is a slow process, happening on the time scale of several hours.

Having generated Q$^-$, we expect to observe photoinduced reduction of pc(II) in the Zncyt/Q/pc(II) glasses. To produce appreciable amounts of Q$^-$, the glass monoliths were exposed to 200 laser pulses, as explained in Experimental Procedures. The irradiated sample was transferred into the UV−visible spectrophotometer within 1 min, and reduction of plastocyanin was investigated by following the visible spectra of the glass monolith.

Upon irradiation, the protein Zncyt remained stable for days; the visible spectra showed no detectable photodegradation.[17] As mentioned before, the decay of the cation radical Zncyt$^+$ occurs on the time scale of the laser flash photolysis experiments. In many experiments on the longer time scale, we never observed the cation radical Zncyt$^+$.

As for plastocyanin, a slow reduction of pc(II) was observed in the irradiated Zncyt/Q/pc(II) glasses, taking place over a time period similar to that for the uptake of Q. The reduction was evident from the increased bleaching of the absorbance in the 500−700 nm region. The recorded changes corresponded to the simple pc(I) − pc(II) difference spectrum with a characteristic minimum at 597 nm; see Figure 8a. Figure 8b shows the photoinduced changes in the absorbance at 597 nm as a function of time. Compete reoxidation of the copper site was observed upon addition of $K_3Fe(CN)_6$; see Figure 8c. Evidently, the process is simply reduction of the copper site and not removal of copper ions or denaturation of the protein.

Many attempts to observe the reduction of pc(II) on the long time scale in the Zncyt/Q/pc(II) glasses were frustrated by poor reproducibility of these experiments. In several instances, a pronounced bleaching around 597 nm was observed even in nonirradiated samples. The tendency of plastocyanin, the redox protein with the relatively high redox potential, to autoreduce is well-known.[62] Anaerobic conditions in our experiments may favor this side reaction. The erratic reproducibility of the experiments is likely caused by small differences in the deaeration of the glass samples. Because the autoreduction of plastocyanin occurs on the time scale of hours and even days,[62] the processes observed on the shorter time scale in our laser flash photolysis experiments should not be affected by this reaction.

Although it may be tempting to speculate that the "shuttling" electron-transfer step in eq 5 occurs in the Zncyt/Q/pc(II) glasses, uncertainties associated with autoreduction of plastocyanin preclude us from a definitive conclusion. Replacement of pc(II) with cyt(III) as the ultimate electron acceptor in our model system eliminated these uncertainties; see Scheme 1. The additional experiments described below provide the clear

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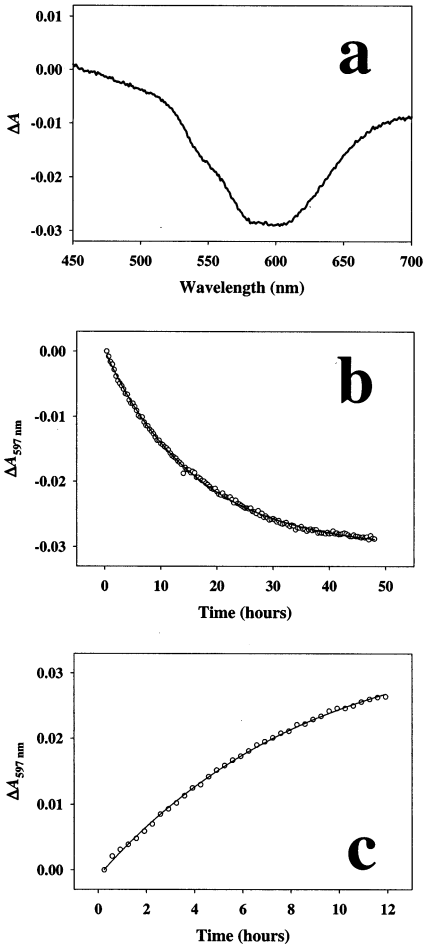

Figure 8. (a) Difference spectra after exposure of the glass sample Zncyt/$Ru(NH_3)_6^{3+}$/pc(II) to 200 laser pulses, recorded 48 h after the irradiation. (b) Decrease in the absorbance at 597 nm of the glass sample after laser irradiation. In 48 h after irradiation, about 5.9 μM plastocyanin was reduced. In the control "dark" sample, only 0.9 μM plastocyanin was reduced over the same time period. (c) Reoxidation of photoreduced pc(I) with an excess of $K_3Fe(CN)_6$. The concentrations of the encapsulated proteins are 10 μM zinc cytochrome *c* and 20 μM cupriplastocyanin, and the concentration of $Ru(NH_3)_6^{3+}$ in the external solution is 100 μM. The solvent is a sodium cacodylate buffer at pH 6.0 and ionic strength of 1.00 M; temperature is 25 °C.

evidence for both electron-transfer steps that mediate the interprotein reaction in our rudimentary electron-transport chains.

Electron Transfer between Immobilized ^{3}Zncyt and Ferricytochrome *c*. We studied the electron-transfer reactions in Zncyt/Q/cyt(III) glasses for three reasons. First, to eliminate the uncertainties caused by autoreduction of plastocyanin. Second, to increase the sensitivity of the experiments on the long time scale. Reduction of cytochrome *c* causes larger changes in the visible spectrum than reduction of plastocyanin does: $\Delta\epsilon_{550} = 1.9 \times 10^4$ M^{-1} cm^{-1} for cytochrome *c*[35] and $\Delta\epsilon_{597} = 4.7 \times 10^3$ M^{-1} cm^{-1} for plastocyanin.[34] Third, to verify that the observed shuttling reactions are a general phenomenon and are not restricted to the Zncyt/Q/pc(II) system.

In solution, ferricytochrome *c* quenches the triplet ^{3}Zncyt by electron transfer.[63] The cation radical $Zncyt^+$ can be observed directly; see Figure S7 in the Supporting Information. The

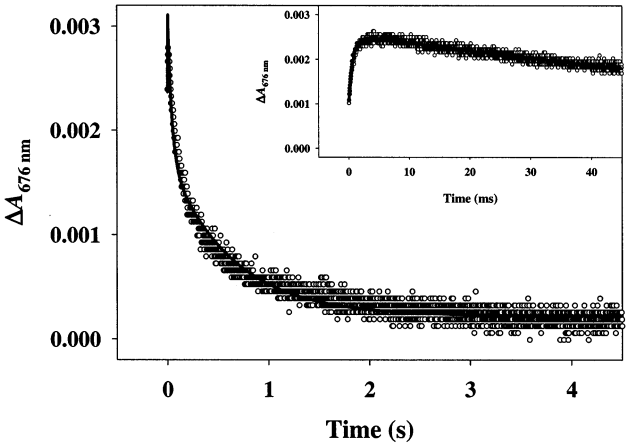

Figure 9. Appearance and disappearance of the cation radical $Zncyt^+$, monitored at 676 nm, in the sol−gel glass doped with zinc cytochrome *c* and ferricytochrome *c*, in the presence of 100 μM $FeEDTA^-$ as the quencher Q of ^{3}Zncyt. The concentrations of the encapsulated proteins are 10 μM zinc cytochrome *c* and 30 μM ferricytochrome *c*. (Inset) The same kinetic trace, but at a shorter time scale. The solvent is a sodium cacodylate buffer at pH 6.0 and ionic strength of 1.00 M; temperature is 25 °C.

second-order rate constants k_1, for electron transfer from ^{3}Zncyt to cyt(III), and k_2, for the back electron transfer from cyt(II) to $Zncyt^+$, are shown in Table 1.

In sol−gel glass, there is no electron transfer from ^{3}Zncyt to cyt(III) in the absence of a mobile redox mediator. As Figure S7 in the Supporting Information shows, the only observed process is the natural decay of the triplet state ^{3}Zncyt. As with Zncyt and pc(II), the two proteins Zncyt and cyt(III) do not interact with each other because they are spatially separated by the sol−gel matrix.

In the Zncyt/Q/cyt(III) glasses, a mobile redox mediator, $FeEDTA^-$ or $Ru(NH_3)_6^{3+}$, oxidatively quenches the triplet ^{3}Zncyt according to eq 3. As in the Zncyt/Q/pc(II) glasses, the cation radical $Zncyt^+$ is long-lived; see Figure 9 and Figures S8 and S9 in the Supporting Information. Again, the decay of $Zncyt^+$ occurs on two time scales. The rate constant for the slower process of the $Zncyt^+$ decay, k_{slow}, is 1.0 ± 0.2 s^{-1}, the same as that for the Zncyt/Q/pc(II) glasses. The longevity of the cation radical $Zncyt^+$ is consistent with the partial escape of Q^- into the glass pores and the subsequent reaction in eq 5′. The similarity in the values of k_{slow} for Zncyt/Q/pc(II) and Zncyt/Q/cyt(III) glasses support our previous conclusion about alternative routes for rereduction of $Zncyt^+$, which do not involve slow recombination of $Zncyt^+$−pc(I) or $Zncyt^+$−cyt(II) pairs.

Figure 10a shows the absorption difference spectrum of the Zncyt/$FeEDTA^-$/cyt(III) glass sample, which was recorded 1 min after exposure of this sample to 200 laser shots. The spectrum is a simple cyt(II) − cyt(III) difference spectrum,[35] a strong evidence for the conversion of cyt(III) into cyt(II) according to eq 5′. No formation of cyt(II) was detected in the nonirradiated samples.

The initial amount of cyt(II), detected right after laser excitation, is assigned to a faster component of the reaction in eq 5′, in the vicinity of Zncyt. The absorption spectra recorded later showed the increase in the concentration of cyt(II); see Figure 10b. The rate of reduction of cyt(III) is limited by diffusion of photogenerated Q^- in the glass matrix and is similar to the rate of uptake of Q ions by the sol−gel glass, determined earlier.[64]

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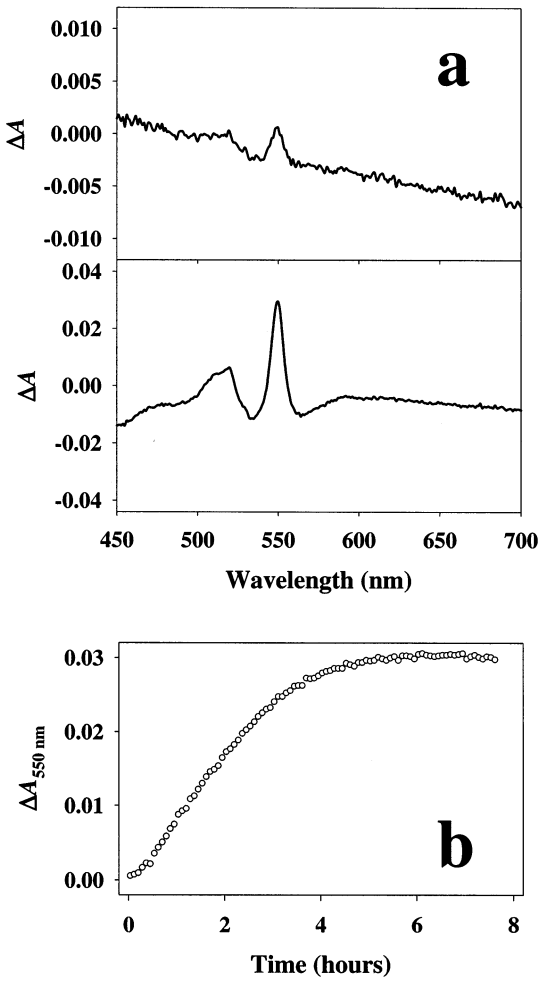

Figure 10. (a) Difference spectra after exposure of the glass sample Zncyt/FeEDTA$^-$/cyt(III) to 200 laser pulses, recorded 1 min (upper panel) and 5 h (lower panel) after the irradiation. The concentrations are 10 μM zinc cytochrome *c*, 30 μM ferricytochrome *c*, and 100 μM FeEDTA$^-$. The spectra show reduction of ferricytochrome *c* according to eq 5′. (b) Growth in the absorbance at 550 nm, indicative of slow reduction of ferricytochrome *c*. The solvent is a sodium cacodylate buffer at pH 6.0 and ionic strength of 1.00 M; temperature is 25 °C.

The experiments with Zncyt/Q/cyt(III) glasses clearly demonstrate the occurrence of the shuttling electron-transfer step, the reaction in eq 5′. The large absorbance changes associated with reduction of cytochrome *c* allowed us to observe this reaction almost immediately after photoexcitation. We conclude that *stepwise electron transfer takes place in our model electron-transport chain*.

Biomimetic Electron-Transport Chain in Sol−Gel Glass: Significance of This Study. Photoinduced electron transfer within solid materials is the area of intense research interest. The donor and acceptor molecules have been variously assembled in polymers,[65−67] zeolites,[68,69] clays,[70] sol−gel glasses,[3,5,7] lipids,[71] and self-assembled films.[72,73]

In sol−gel glass, electron transfer is possible between the molecules immobilized in the glass matrix and mobile species in the intrapore solution.[3,4,7] This property of the sol−gel glasses makes them well suited for the assembly of the biomimetic electron-transport chain. In our studies, we followed stepwise electron transfer between two encapsulated redox proteins: from zinc cytochrome *c* to cupriplastocyanin and from zinc cytochrome *c* to ferricytochrome *c*.

Excitation of Zncyt with visible light triggers the electron-transfer processes shown in Scheme 1. Chemical communication between the two immobilized proteins is mediated by the diffusive shuttling of the redox mediator Q/Q$^-$ in the solvent-filled pore. The observed stepwise electron transfer between immobilized proteins is a general phenomenon; it is independent of the specific mediator, FeEDTA$^{-/2-}$ or $Ru(NH_3)_6^{3+/2+}$, and the ultimate electron acceptor, cupriplastocyanin or ferricytochrome *c*.

The experimental design appreciably extends the lifetime of the cation radical Zncyt$^+$. Because of the intrinsic reactivity of Zncyt$^+$, long-lived Zncyt$^+$−pc(I) or Zncyt$^+$−cyt(II) pairs are not formed in our model system. Instead, we observe *photo-induced reduction* of cupriplastocyanin and ferricytochrome *c*.

The later phenomenon is a remarkable one. The observed photoinduced reduction is not only relevant to biomimetic studies of rudimentary electron-transport chain but also bears on the general problem of light-energy conversion. Previous attempts at using visible light to form long-lived charge-separated pairs in sol−gel glass were unsuccessful.[5,56] Our system also does not support long-lived charge-separated Zncyt$^+$−pc(I) or Zncyt$^+$−cyt(II) pairs. We, however, succeeded in slowing the back reaction between Zncyt$^+$ and Q$^-$ (eq 4) in the "diprotein" glasses, Zncyt/Q/pc(II) and Zncyt/Q/cyt(III). In our biomimetic system, photoexcitation of one protein, Zncyt, leads to ultimate reduction of another protein, pc(II) or cyt-(III). These results demonstrate the potential for integrating proteins, with their optimally adjusted redox sites, in photo-catalytic materials.

There is still another advantage of proteins over small molecules in materials based on sol−gel glass. One of the problems of encapsulating small molecules in porous glass is their tendency to leach out by diffusion through the pores.[25] By contrast, the size of proteins prevents their leaching from the sol−gel matrix. While the protein remains entrapped, many small reagents can diffuse through the pores, preserving communications between the proteins.

Conclusions

Our studies show that interprotein electron transfer between compartmentalized redox proteins is feasible. The rigid sol−gel matrix spatially separates encapsulated proteins from each

(64) Consistent with the uptake experiments, photoinduced reduction of plastocyanin and cytochrome *c* is slower when mediated by $Ru(NH_3)_6^{3+/2+}$ than when mediated by FeEDTA$^{-/2-}$ and occurs on the time scale of days; see Figure 8 and Figure S2 in the Supporting Information.
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other. The photoinduced electron transfer between encapsulated redox proteins zinc cytochrome *c* and cupriplastocyanin or zinc cytochrome *c* and ferricytochrome *c* is possible only in the presence of mobile charge carriers. The mobile inorganic complexes $FeEDTA^{-/2-}$ and $Ru(NH_3)_6^{3+/2+}$ diffuse in the pores of the sol−gel glass and react with the encapsulated protein molecules. These processes ultimately lead to stepwise electron transfer between immobilized proteins, resembling the events in biological electron-transport chains.

The experimental design appreciably extends the lifetime of the cation radical $Zncyt^+$. The energy of visible light is converted to reducing equivalents for plastocyanin and cytochrome *c*. These findings demonstrate the potential for integrating proteins in photocatalytic materials.

Acknowledgment. This study was supported by the U.S. National Science Foundation through Grant MCB-9808392. We thank Dr. Mikael Ejdebäck, Ms. Hanna Jansson and Dr. Örjan Hansson, of Göteborg University, for recombinant spinach plastocyanin. E.V.P. thanks Dow Chemical Company for the 1999 and 2000 Fellowships awarded by the Department of Chemistry and the National Honor Society for Women in Chemistry Iota Sigma Pi for the 2001 Anna Louise Hoffman Award.

Supporting Information Available: Nine figures showing visible spectra of cupriplastocyanin in solution and in sol−gel glass; uptake of $FeEDTA^-$ and $Ru(NH_3)_6^{3+}$ by undoped (protein-free) glass; the kinetic traces of $Zncyt^+$ in solution and in the Zncyt/Q glasses with $FeEDTA^-$ and $Ru(NH_3)_6^{3+}$ as the quencher Q of ^{3}Zncyt; comparison of the kinetic traces of $Zncyt^+$ in the Zncyt/Q and Zncyt/Q/pc(II) glasses, with $FeEDTA^-$ as the quencher Q of ^{3}Zncyt; the kinetic trace of $Zncyt^+$ in the Zncyt/Q/pc(II) glasses with $Ru(NH_3)_6^{3+}$ as the quencher Q of ^{3}Zncyt; the kinetic traces of $Zncyt^+$ in the Zncyt/Q/pc(II) glasses with different concentrations of pc(II); changes in transient absorbance of the solution of Zncyt and cyt(III) and of the glass sample Zncyt/cyt(III); comparison of the kinetic traces of $Zncyt^+$ in the Zncyt/Q and Zncyt/Q/cyt(III) glasses, with $FeEDTA^-$ as the quencher Q of ^{3}Zncyt; and the kinetic trace of $Zncyt^+$ in the Zncyt/Q/cyt(III) glasses with $Ru(NH_3)_6^{3+}$ as the quencher Q of ^{3}Zncyt (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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