Electron-Transfer Communication in Glutathione Reductase Assemblies: Electrocatalytic, Photocatalytic, and Catalytic Systems for the Reduction of Oxidized Glutathione

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Abstract: Glutathione reductase, GR, is electrically communicated with its environment in electrochemical, photochemical, and chemical assemblies. Electron-transfer communication between the protein redox site and its surroundings is achieved either by covalent attachment of electron relays to the protein or by using redox copolymers as electron mediators. GR is covalently attached to self-assembled monolayers of the *N*-hydroxysuccinimide ester of cysteic acid formed by chemisorption of the respective disulfide, **1**, onto Au electrodes. The resulting GR monolayer electrode is derivatized by *N*-methyl-*N'*-(carboxyalkyl)-4,4'-bipyridinium (**2**) in the presence of urea. The relay-modified GR electrode exhibits electrical communication that leads to bioelectrocatalyzed reduction of oxidized glutathione, GSSG, to GSH upon application of a negative potential, $E = -0.72$ V vs SCE on the electrode. The rate of GSH formation is enhanced as the chain length linking the bipyridinium groups to the protein is increased. This enhancement in GSH formation is attributed to improved electrical communication with the enzyme active site. Photosensitized reduction of GSSG is achieved in a photosystem composed of Ru(II) tris(bipyridine), $Ru(bpy)_3^{2+}$, the protein glutathione reductase that is chemically derivatized by *N,N'*-bis(carboxyethyl)-4,4'-bipyridinium (**3**), PAV^+-GR, and EDTA as sacrificial electron donor. The formation of GSH in the photosystem is controlled by the electron-transfer quenching rate of the excited state. The electron relay units linked to the protein act in the system as quenchers of the excited state and as electron mediators for electron transport to the protein active site. PAV^+-GR was immobilized in the cross-linked redox copolymer, **8**, composed of *N*-methyl-*N'*-(3-acrylamidopropyl)-4,4'-bipyridinium (**4**) and acrylamide. The resulting protein–copolymer assembly affects the efficient photoinduced reduction of GSSG in the presence of $Ru(bpy)_3^{2+}$ as photosensitizer and EDTA as sacrificial electron donor. In this system, vectorial electron transfer from the excited state to the protein redox site proceeds across the polymer backbone and the protein shell. Photosensitized reduction of GSSG by native GR has also been accomplished by using *N*-methyl-*N'*-(carboxyalkyl)-4,4'-bipyridinium poly(L-lysine), PL-C_nV^{2+} (**9**), as electron relay, $Ru(bpy)_3^{2+}$ as photosensitizer, and EDTA as electron donor. The rate of GSH formation is controlled by the tether length linking the redox units to the polymer backbone. Time-resolved laser flash photolysis experiments reveal that the rate of electron transfer from the reduced polymer, PL-$C_nV^{\bullet+}$, to the enzyme redox site are controlled by the length of the tethers linking the redox units to the polymer. With long chains, the electron mediator penetrates the protein backbone and attains short distances in respect to the protein redox center, resulting in enhanced electron transfer. The rate constants for electron transfer from a series of redox polymers of varying spacer lengths to the protein redox center obey Marcus theory. Reduction of GSSG to GSH is also achieved by PAV^+-GR using a Pt colloid and gaseous hydrogen as reducing agent. In this system, Pt catalyzes the reduction of protein-bound bipyridinium units by H_2. The reduced electron relay, $PAV^{\bullet}$-GR, mediates the electron transport to the protein active center, where reduction of GSSG occurs.

Introduction

Long-range electron-transfer reactions in proteins are extensively studied from basic and practical points of interest. The parameters and mechanisms controlling long-range electron transfer from the macroscopic environment to the protein-shielded redox sites are of fundamental importance for understanding biochemical redox processes.[1-3] Marcus theory, as well as other studies, provides theoretical grounds for long-range electron transfer in proteins and for the influence of the protein backbone on these processes.[4,5] Marcus theory predicts that the rate of electron transfer between a donor–acceptor pair embedded in proteins is given by eq 1, where ΔG° and λ are the free energy

$$k_{ET} \propto e^{-\beta(d-d_0)}\, e^{-(\Delta G^\circ+\lambda)^2/4RT\lambda} \qquad (1)$$

change and reorganization energy associated with the process, respectively, while d is the distance separating the donor–acceptor pair. Recent studies on redox proteins modified by electron-mediating probes reveal that various effects control long-range electron transfer in proteins: spatial separation of the electron mediator from the active site (distances),[6,7] the ordering of the amino acid in the protein, intraprotein hydrogen bonds separating the donor–acceptor pair,[8] and conformational dynamics of the

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electron mediator in respect to the redox center[9] are characteristic factors that control long-range electron transfer in proteins.

Redox centers of proteins could, in principle, communicate with electron (or "hole") sources that are present in their macroscopic environment: these include electrodes, excited species (photosensitizers or semiconductors), and chemical reducing agents (or oxidants). Electrical communication of redox enzymes and their environment has important practical implications.[10,11] Electrical interaction of redox enzymes with electrodes is the basis for the development of a variety of amperometric biosensor devices[10] and electrobiocatalyzed chemical transformations.[11] Electron transfer between photoexcited species and redox enzymes is the basis for photosynthetic processes and provides means to artificially mimic natural photosynthesis.[12] Also, electron-transfer communication between enzyme redox centers and abundant reducing (or oxidizing) agents provides an important route for developing new biotechnological processes. Nevertheless, redox proteins usually do not electrically communicate with their macroscopic surroundings, as the active center is shielded by the protein backbone. In fact, the protein acts as an insulating shell where the active redox center is spatially separated (distance separation) from the external electron sources (or holes). Nature has developed means to electrically communicate protein redox centers and their environment. Communication is achieved by protein-associated cofactors, such as FAD, or diffusional cofactors that penetrate the protein and attain close distances relative to the active site. These short distances between the cofactors and enzyme redox centers facilitate mediated electron transfer. The $NAD(P)^+/NAD(P)H$ couple represents a diffusional cofactor that mediates interprotein electron-transfer processes in native systems. Accordingly, substantial efforts are directed towards the development of electrochemical,[13] photochemical,[14] and chemical[15] means for the regeneration of $NAD(P)^+/NAD(P)H$ cofactors and their use in biocatalyzed transformations. Similarly, the application of artificial electron mediators that are recognized by native redox enzymes is of great interest as a means to substitute natural cofactors. Biocompatibility, adequate size and hydrophobicity to allow penetration into the protein backbone, and proper redox potentials are essential factors that have to be met by such artificial electron carriers. Various redox compounds, such as *N,N'*-dialkyl-4,4'-bipyridinium salts,[16] ferrocene,[17] quinones,[18] and tungsten or molybdenum octacyanides,[19] have been applied as artificial electron mediators that communicate with redox proteins by a diffusional pathway in electrochemical, photochemical, and chemical assemblies.

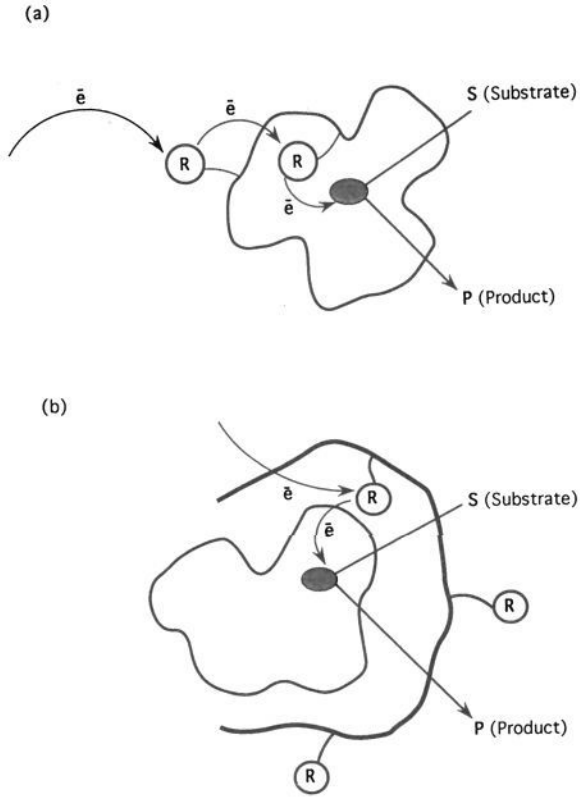

Figure 1. Electron-transfer communication of a redox protein with its environment. (a) By chemical modification of the enzyme with redox relay groups. (b) By immobilization of the enzyme in a redox-tethered polymer.

It is, however, of broad interest, especially for practical reasons, to develop methods for mediated electron transfer in redox proteins by nondiffusional pathways. Based on our understanding of the steric and spatial limitations for electron transfer in proteins, one could envisage two general methods to establish electron-transfer communication between enzyme redox centers and their environment. One method would involve chemical modification of the protein by electron mediators, Figure 1a. Here, the shielded enzyme redox center electrically communicates with its surroundings by a series of electron relay units R associated with the protein. A second approach to attain electron transfer between the enzyme redox center and its environment could involve the application of polymers functionalized by redox relay components, Figure 1b. Here the enzyme and polymer backbone form a supramolecular assembly by mutual recognition elements (such as electrostatic attractions or hydrogen bonds). The redox relay groups tethered to the polymer exhibit conformational flexibility that allows the penetration of the redox mediator into the protein backbone. As a result, close distances of the relay, relative to the enzyme redox site, are attained, and hence effective mediated electron transfer is anticipated.

These two methods provide general approaches to electrically couple redox enzymes with their environment. The environment could, in principle, consist of an electrode interface, an excited species, or a chemical redox reagent, as schematically presented

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in Figure 1. In fact, these methods to effect mediated electron transfer in redox enzymes mimic native pathways. Protein-bound electron relays facilitate electron transport similarly to enzyme-associated cofactors, while interaction of enzymes with redox polymers simulates interprotein electron-transfer processes.

Chemical modification of redox proteins by electron mediators has been successfully applied to electrically communicate enzymes with electrode surfaces in oxidative pathways,[20] i.e., glucose oxidase and lactate oxidase. Similarly, immobilization of proteins in functionalized redox polymers associated with electrodes proved to be an effective route to establish mediated electron transfer.[21,22] For example, immobilization of glucose oxidase or nitrate reductase onto electrodes by means of redox polymers results in electrical communication in oxidative and reductive routes, respectively. Also, nitrate reductase immobilized in a bipyridinium-modified acrylamide copolymer leads to electrical communication of the protein with photoexcited species and to the photobiocatalyzed reduction of nitrate to nitrite.[23]

Here we present a comprehensive study on mediated electron transfer in assemblies comprising the enzyme glutathione reductase. We examine the direct modification of the protein by relay units and its conjugation to redox polymers as methods to effect mediated electron transfer with the macroscopic environment. We use these methods to couple the enzyme in electrochemical,[24] photochemical,[25] and chemical[26] assemblies.

Experimental Section

Absorption spectra were recorded on Uvikon-860 (Kontron) spectrophotometer. In the appropriate experiments, a thermostated cell holder was used. Continuous illumination experiments were performed with a 150-W Xenon arc lamp (Oriel). The light was filtered through a $CuSO_4$ solution and a cutoff filter GG-420 (Schott). The laser flash photolysis system included a Nd:Yag laser (Laser Photonics MY 34–10) coupled to a dye laser (Laser Photonics DL-18) as light pulse sources (pulse width < 5 ns). The detection system included a monochromator and a photomultiplier (Applied Photophysics K-347) linked to a digital oscilloscope (Tektronics 2430 A) and a computer for data recording and analysis. Electrochemical measurements were performed with a cyclic voltammetry apparatus (BAS CV-27). The electrochemical cell consisted of three electrodes where the chemically modified electrode acted as working electrode, a glassy carbon was the auxiliary electrode, isolated by a frit, and a saturated calomel electrode (SCE), connected to the working volume by a Luggin capillary, was used as reference electrode. The cell was thermostated during the measurements (27 °C).

The synthetic procedures used in the study are outlined below or detailed in the supplementary material. All compounds were characterized by ^1H NMR and gave appropriate elementary analyses. All other materials were of commercial source.

N-Methyl-*N*'-(carboxyalkyl)-4,4'-bipyridinium compounds **2b–2d** were prepared according to a slight modification of the reported procedures.[27]

N-Methyl-*N*'-(carboxyethyl)-4,4'-bipyridinium dichloride (**2a**) was prepared by reacting 4-(*N*-methylpyridinium)pyridine iodide, (2 g, 7 mmol) with 15 mL (0.21 mmol) of acrylic acid in a mixture of chloroform (15 mL) and acetonitrile (5 mL). The mixture was stirred at room temperature for 48 h, and the resulting precipitate was washed with chloroform. The product was recrystallized from a methanol–chloroform mixture. Exchange to the dichloride salt was performed by solubilizing the solid in concentrated HCl, followed by precipitation with acetone. The dichloride of *N*,*N*'-bis(carboxyethyl)-4,4'-bipyridinium (**3**) was prepared by reaction of 4,4'-bipyridine (2.0 g, 15 mmol) with acrylic acid (30 mL, 50-fold molar excess) in 25 mL of chloroform (36 h, room temperature). A solution of acetone–HCl (6:1, 10 mL) was added to the reaction mixture, and the resulting precipitate was filtered and recrystallized from methanol–acetone.

N-Methyl-*N*'-(3-acrylamidopropyl)-4,4'-bipyridinium (**4**) was prepared by adding the active ester *N*-(acryloxy)succinimide (**7**),[28] (27 mg, 0.17 mmol) dissolved in 0.25 mL of DMSO, to a solution of *N*-methyl-*N*'-(3-aminopropyl)-4,4'-bipyridinium trichloride (**5**)[29] (53.5 mg, 0.17 mmol) dissolved in 0.6 M HEPES buffer solution, pH = 7.5 (0.25 mL). The resulting solution was stirred at room temperature for 1.5 h under nitrogen and was used for the preparation of polymer **8**.

Preparation of *N*-Methyl-*N*'-(carboxyalkyl)-4,4'-bipyridinium-Modified Polylysine, PL-C_nV^{2+} (9). Polylysine, PL, MW = 43 500 (187 mg, 0.0043 mmol), the respective *N*-methyl-*N*'-alkylcarboxylic acid dichloride (**2**) (0.056 mmol), and 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid sodium salt (208 mg) were dissolved in 4.2 mL of water, and the solution was adjusted to pH = 7.6. To the cooled solution (0 °C) were added *N*-hydroxy-3-sulfosuccinimide sodium salt, NHS-SO_3Na, (11 mg) and *N*-(3-(dimethylamino)propyl)-*N*'-ethylcarbodiimide hydrochloride, EDC·HCl (10 mg). The resulting solution was stirred and allowed to warm up to room temperature within 1 h and stirred for 44 h. The resulting polymer solution was dialyzed seven times with a cutoff membrane (MW 10 000) using 1 L of distilled water each time. The resulting polymer solution was lyophilized to yield a powder. The loading of the PL-C_nV^{2+} polymer by bipyridinium units was determined spectroscopically. The polymer (3–10 mg) was dissolved in 1.97 mL of 0.1 M Tris buffer solution, pH = 8.0, and the solution was inserted into a cuvette and deaerated with Ar. Thirty microliters of a deaerated sodium dithionite, 0.005 M, in 0.1 M Tris·HCl buffer solution was added to the cuvette solution and heated to 42 °C. The absorbance at λ = 602 nm was measured every 6 s for 1 min and extrapolated to $t = 0$. The loading was determined from the absorbance of PL-$C_nV^{\bullet+}$ (λ = 602 nm, ϵ = 12 500 $M^{-1}\cdot cm^{-1}$).

The Pt colloid was prepared by the citrate reduction method.[30] The concentration of Pt was determined by atomic absorption to be 120 $mg\cdot L^{-1}$.

Preparation of Self-Assembled GR Enzyme Electrodes. A clean Au electrode (0.2 cm^2 area) was immersed for 2 h at room temperature in a 0.02 M DMSO solution of **1**, the bis(N-hydroxysuccinimide ester) of 3,3'-dithiopropionic acid (Fluka). The resulting active ester self-assembled monolayer-modified electrode was washed with DMSO and water. The monolayer electrode was then incubated overnight in 1 mL of 0.1 M phosphate buffer solution, pH = 7.3, that included GR, EC 1.6.4.2 (0.18 mg, 100 units) (Sigma). The resulting enzyme-modified monolayer electrode was rinsed several times with the same buffer solution and subsequently derivatized by the respective *N*-methyl-*N*'-(carboxyalkyl)-4,4'-bipyridinium (**2**) dichloride salt. The monolayer enzyme electrode was introduced into 1 mL of 0.1 M HEPES buffer solution, pH = 7.3, that included **2** (1×10^{-2} M), urea (1 M), 1-ethyl-3-[3-(dimethylamino)-propyl]carbodiimide (EDC, 4 mg), and *N*-hydroxysulfosuccinimide sodium salt (1.5 mg). Coupling of **2** occurred within 20 h of incubation at 4 °C. The resulting electrode was washed with the HEPES buffer solution and subsequently used in the electrochemical experiments.

Preparation of PAV$^+$-GR. The commercial GR enzyme solution was dialyzed two times against 500 mL of 0.05 M HEPES buffer solution, pH = 7.5. To generate a loading of 4 of PAV$^+$-GR, a stock solution of *N*,*N*'-bis(carboxyethyl)-4,4'-bipyridinium dichloride (**3**, 23 mg), Na-HEPES (80 mg), and urea (184 mg) dissolved in 1.6 mL of water and adjusted to pH = 7.2 with 1 M HCl was prepared. Coupling of PAV was achieved by adding 1 mL of GR solution (8 mg) to 1 mL of the PAV stock solution, cooling the mixture to 4 °C, and adding *N*-hydroxysulfosuccinimide sodium salt (5.5 mg) and 1-ethyl-3-[3-(dimethylamino)-propyl]carbodiimide (12 mg). The resulting solution was stirred overnight at 4 °C. The solution was then dialyzed four times against 0.1 M phosphate buffer, pH = 7.5 (each time 1 L for 48 h). The dialysis solution was checked for free bipyridinium salt by addition of $Na_2S_2O_4$. No free **3** could be detected in the third and fourth dialyses. This procedure leads to a protein loading of 4. By changing the PAV content and appropriate

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EDC amount, different loadings were obtained. The loading of PAV^+-GR was determined spectroscopically. A volume of the PAV^+-GR was taken, and the content of protein in the sample was determined by the Lowry method.[31] The molar concentration of the enzyme was calculated using its known molecular weight, MW = 118 000. The concentration of PAV^+ associated with the protein was determined by taking a 200-μL sample of the enzyme into 800 μL of 0.1 M phosphate buffer solution, pH = 7.5. The solution was added to a cuvette that contained $Na_2S_2O_4$ under Ar. The spectrum of the resulting $PAV^{\bullet}$-GR was recorded. The solution was then flushed with oxygen, and the baseline spectrum was subtracted from the previous spectrum. The concentration of $PAV^{\bullet}$-GR was calculated from the absorbance at $\lambda = 602$ nm ($\epsilon = 11\,800\ M^{-1}\cdot cm^{-1}$).

Immobilization of PAV^+-GR in Acrylamide Redox Copolymer 8. Acrylamide (187.5 mg, 2.6 mmol) and methylenebis(acrylamide) (10 mg, 0.07 mmol) were dissolved in cold water (0.5 mL, 4 °C). The solution was mixed with 0.5 mL of a freshly prepared solution of *N*-methyl-*N*′-(3-acrylamidopropyl)-4,4′-bipyridinium (**4**). Solutions of 0.05 M GSSG (0.1 mL) and PAV^+-GR (1.2 mg of protein in 0.3 mL of 0.1 M phosphate buffer, pH = 7.5) were added to the monomer solution. The resulting solution was allowed to reach room temperature under nitrogen, and polymerization was initiated by adding 125 μL of 50% (dimethylamino)-propionitrile in water and 0.25 mL of 1% $K_2S_2O_8$. Polymerization was continued for 1 h, and the resulting polymer gel was cut into pieces and immersed into 0.1 M phosphate buffer, pH = 7.5. The ground polymer was repeatedly washed by a buffer solution and centrifuged until no free bipyridinium salt could be detected in the solution, using $Na_2S_2O_4$ as reducing agent.

The following photochemical, electrochemical, and chemical procedures were employed for the biocatalyzed reduction of GSSG and for characterization of mechanistic aspects involved in the biotransformation.

Photochemical Reduction of GSSG by PAV^+-GR. The system consisted of 3 mL of 0.1 M phosphate buffer, pH = 7.5, that contained $Ru(bpy)_3^{2+}$ (6.8×10^{-5} M), Na_2EDTA (0.01 M), and PAV^+-GR (4 mg). The solution was introduced into a cuvette equipped with a stopper and a magnetic stirrer. The deaerated solution was illuminated, $\lambda > 420$ nm, and the concentration of GSH was followed at time intervals of illumination. The concentration of GSH was determined by the Ellman method.[32] Samples of the illuminated solution, 100 μL, were taken out at time intervals of illumination and diluted with 0.1 M phosphate buffer, pH = 7.6 (1.9 mL). To the analyzed samples was added 0.1 mL of a 5,5′-dithiobis-(2-nitrobenzoic acid) solution (1.6 mg·mL^{-1} in 0.1 M phosphate buffer), and the absorbance at $\lambda = 412$ nm ($\epsilon = 13\,600\ M^{-1}\cdot cm^{-1}$) was measured after 2 min of incubation.

Photochemical assay of the activities of PAV^+-GR and native GR was performed by the introduction of PAV^+-GR or GR (1.85 mg) into a photosystem that consisted of 3 mL of 0.1 M phosphate buffer, $Ru(bpy)_3^{2+}$ (6.8×10^{-5} M), Na_2EDTA (6.7×10^{-3} M), MV^{2+} (1×10^{-2} M), and GSSG (1×10^{-2} M). The resulting solution was placed into a cuvette, deaerated, and illuminated, $\lambda > 420$ nm. The rate of GSH formation at time intervals of illumination was determined by withdrawing 100-mL samples from the illuminated solution and analyzing GSH as described earlier. Flash photolysis experiments with PAV^+-GR were performed in photosystems that included 0.75 mL of phosphate buffer, pH = 7.5, $Ru(bpy)_3^{2+}$ (1×10^{-4} M), and PAV^+-GR (loading of 4 mol of bipyridinium per mol of protein). The quenching rate constant was derived by following the shortening of $Ru(bpy)_3^{2+}$ lifetime at different concentrations of bipyridinium: 1.4×10^{-4} M (4 mg of protein), 8.6×10^{-5} M (2.3 mg of protein), and 6.8×10^{-5} M (1.9 mg of protein). The lifetime was followed at $\lambda = 610$ nm, $\lambda_{excitation} = 532$ nm. A cuvette in which the excitation path length was 0.5 cm and the analyzing path length was 1 cm was used in these experiments.

Photochemical Reduction of GSSG by PAV^+-GR Immobilized in the Bipyridinium Acrylamide Copolymer 8. Polymer pieces, 1.6 g, that included the protein PAV^+-GR (0.2 mg of protein per 1 g of polymer gel), were introduced to a photosystem composed of 3 mL of 0.12 M phosphate buffer, pH = 7.5, $Ru(bpy)_3^{2+}$ (6.8×10^{-5} M), Na_2EDTA (0.01 M), and GSSG (0.01 M). The mixture was placed in a cuvette equipped with a magnetic stirrer and a serum stopper. The deaerated system was illuminated, $\lambda > 420$ nm, and GSH formation was followed as described above.

Chemical Reduction of GSSG by H_2 Using PAV^+-GR and Pt as Catalysts. The system consisted of 1.7 mL of phosphate buffer, pH = 7.5, the Pt colloid (1 mL; 120 mg·L^{-1} Pt), that included PAV^+-GR (3.1 mg of protein of loading 4), and the substrate GSSG (1.18×10^{-2} M). The system was placed in a glass tube equipped with a stopper, deaerated, and bubbled with H_2 for 5 min. Hydrogen pressure was maintained at 1 atm throughout the reaction. The rate of GSH evolution as a function of time was followed as described above.

Bioelectrocatalyzed reduction of GSSG: The monolayer GR-modified Au electrodes that included covalently attached bipyridinium units were used as working electrodes. The electrolyte solution of the cell was composed of 2 mL of phosphate buffer solution, pH = 7.5, and included GSSG (1×10^{-2} M). Electrolysis was conducted by applying a constant potential corresponding to E = –0.72 V vs SCE onto the electrode. GSH formation at time intervals of electrolysis was followed spectroscopically.

Photochemical Reduction of GSSG Using GR and Bipyridinium-Modified Polylysine, PL-C_nV^{2+} (9), as Electron Mediators. The photosystems consisted of 3 mL of a 0.1 M Tris buffer solution, pH = 7.0, that included GR (50 units, EC 1.6.4.2), $Ru(bpy)_3^{2+}$ (7.5×10^{-5} M), Na_2EDTA (1×10^{-2} M), GSSG (1×10^{-2} M), and 10 mg of the respective PL-C_nV^{2+} polymer (**9**). The deaerated solution was illuminated, $\lambda > 420$ nm, and the formation of GSH at time intervals of illumination was determined spectroscopically.

The electron-transfer quenching rate constants and recombination rates of the intermediate charge-separated photoproducts were followed by laser flash photolysis. The electron-transfer quenching rate constants were derived by following the lifetime of $Ru(bpy)_3^{2+}$ in photosystems that included a Tris buffer solution, pH = 7.0, $Ru(bpy)_3^{2+}$ (3.75×10^{-4} M), and different concentrations of the respective polymers, PL-C_nV^{2+} (**9**). Lifetimes were determined in deaerated solutions at $\lambda = 610$ nm and $\lambda_{exc} = 532$ nm. The recombination rate constants of the charge-separated photoproducts were derived by following the transient decay of the flash-generated PL-$C_nV^{\bullet+}$ at $\lambda = 602$ nm ($\epsilon = 12\,600\ M^{-1}\cdot cm^{-1}$). Electron-transfer rates from PL-$C_nV^{\bullet+}$ to the GR redox center were followed in photosystems that included 3 mL of a 0.1 M Tris buffer solution, $Ru(bpy)_3^{2+}$ (3.75×10^{-4} M), Na_2EDTA (1×10^{-2} M), GSSG (1×10^{-2} M), GR (50–500 units), and the respective PL-C_nV^{2+} polymer (15.6 mg). The system was deaerated and excited by laser flashes, $\lambda_{exc} = 532$ nm. The transient decay of photogenerated PL-$C_nV^{\bullet+}$ was followed spectroscopically, $\lambda = 602$ nm ($\epsilon = 12\,600\ M^{-1}\cdot cm^{-1}$).

Results and Discussion

Glutathione (γ-L-glutamyl-L-cysteinylglycine), GSH, is a well-known biologically active tripeptide that participates in various redox systems[33] and acts as coenzyme for ion-channel proteins.[34] The enzyme glutathione reductase, GR, is a NADPH-dependent protein and mediates the reduction of oxidized glutathione, GSSG, to GSH, eq 2. The amino acid sequence constituting the enzyme

$$GSSG + NADPH + H^+ \xrightarrow{GR} 2GSH + NADP^+ \quad (2)$$

from various sources is established,[35] and the X-ray structure of the enzyme isolated from human erythrocyte cells has been elucidated.[36] The protein is composed of two polypeptide chains linked by a disulfide bond. Each part of the enzyme contains a molecule of the flavin dinucleotide cofactor (FAD). The active site structure of the enzyme comprises isoalloxazine units, which are a part of the FAD cofactor, and the disulfide bridge linking the two polypeptide chains. The X-ray structure of the enzyme indicates that the protein backbone effectively shields the active site. The standard reduction potential of GR has been estimated to be $E^\circ = -0.255$ V at pH = 7.0.[37] Previous studies[38] indicated that *N*,*N*′-dimethyl-4,4′-bipyridinium radical cation, $MV^{\bullet+}$, generated by chemical, photochemical, or electrochemical means mediates the reduction of GSSG in the presence of GR, implying

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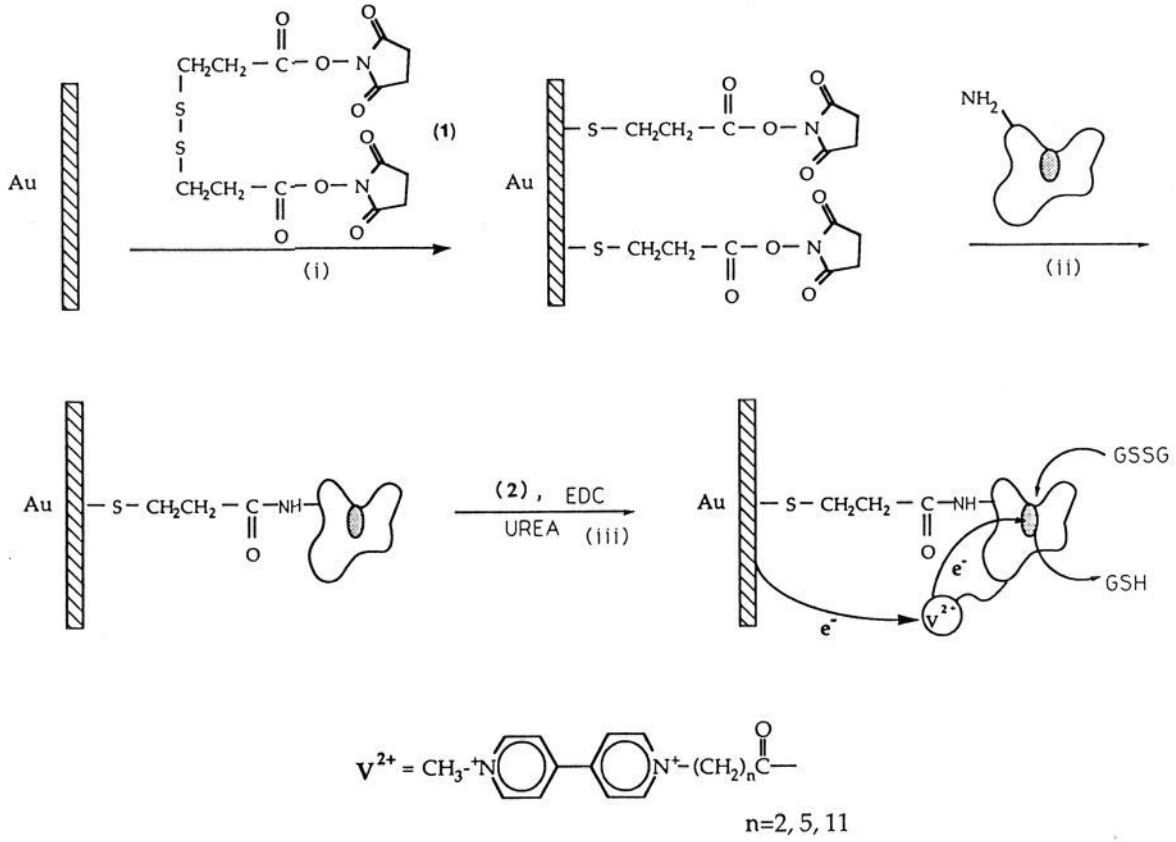
Scheme 1. Sequence of Reactions for Assembling the Glutathione Reductase Monolayer Exhibiting Electrical Communication on a Au Electrode



$V^{2+} = CH_3\text{-}{}^{+}N$ ⟨⟩ $N^{+}\text{-}(CH_2)_n C(=O)-$

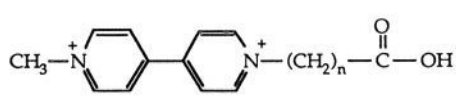
n=2, 5, 11

that electron-transfer communication between the protein active site and the electron mediator is attained by a diffusional mechanism. Accordingly, we have initiated an extensive study to establish electron-transfer communication between the enzyme GR and its macroscopic environment using 4,4′-bipyridinium units covalently attached to the protein or as a part of a redox-functionalized copolymer. In this context, we examine the effects of the bridging chain lengths linking the bipyridinium units to the protein or the polymer on electron-transfer communication and discuss the methodologies to attain electrical interactions of GR with its surroundings in electrochemical, photochemical, and chemical systems.

Electron-Transfer Communication of GR in Electrocatalyzed Systems. In order to develop a bioelectrocatalyzed system involving GR, it is essential to tailor an enzyme electrode, where the enzyme is immobilized onto the electrode surface with minimum degradation of its bioactivity as compared to the native protein. As thiols (or disulfides) form self-assembled monolayers on gold (or other metal) surfaces,[39-41] we decided to utilize this property to construct the monolayer enzyme electrode. Scheme 1 outlines the stepwise construction of the GR monolayer electrode.

Chemisorption of *bis*(*N*-hydroxysuccinimidyl) 3,3′-dithiopropionate (**1**), onto a Au electrode creates the respective self-assembled monolayer composed of the *N*-hydroxysuccinimide ester of cysteic acid. The density of the monolayer array is estimated[42] by its derivatization with aminonaphthoquinone and by consecutively following the charge associated with the reduction (or oxidation) of the quinone-modified electrode. Assuming a complete derivatization of the surface by the quinone, the active ester monomer density comprising the monolayer corresponds to 8×10^{-11} mol·cm^{-2}. This value should be considered, however, as a lower limit due to the stated assumption. The active ester self-assembled monolayer is subsequently reacted with the enzyme GR to yield the covalent attachment (amide bond) of the protein to the monolayer through the lysine residues. The density of the covalently linked GR to the monolayer has been estimated to be 2×10^{-11} mol·cm^{-2} by modification of the monolayer with tritium-labeled GR.[43]

The GR-modified monolayer assembled onto the Au electrode was subsequently modified by a series of *N*-methyl-*N*′-(carboxyalkyl)-4,4′-bipyiridinium salts (**2**) acting as electron-transfer mediators, Scheme 1. It should be noted that modification of the



2a, $n = 2$
2b, $n = 5$
2c, $n = 7$
2d, $n = 11$

GR monolayer by **2** in the presence of urea is essential to obtain the electron-transfer communication between the enzyme and

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(43) [^{3}H]Iodoacetic acid was anchored to cysteine residues of glutathione reductase by reacting the enzyme with an excess of the radioactive label in phosphate buffer, pH 7.3, at room temperature for 1 h, followed by Sephadex G-25 separation of the enzyme.

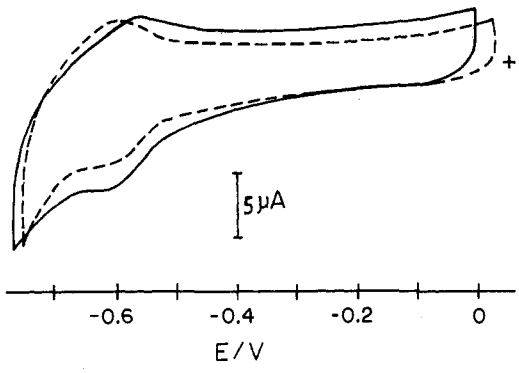

Figure 2. Cyclic voltammograms of bipyridinium-tethered GR of monolayer-modified electrodes: (- - -) of **2b**-modified GR and (—) of **2d**-modified GR.

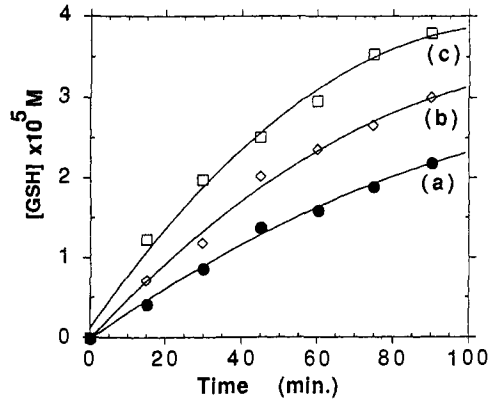

Figure 3. Rate of reduced glutathione (GSH) formation using GR monolayer electrodes with different bipyridinium tethers: (a) **2a**, (b) **2b**, and (c) **2d**. In all experiments, [GSSG] = 1×10^{-2} M, and the applied potential corresponds to E = –0.72 V vs SCE.

the electrode. As urea causes the unfolding of proteins,[44] it enables the modification of intraprotein lysine residues by the electron mediator **2** (vide infra). The cyclic voltammograms of **2**-modified GR monolayers are characteristic of 4,4′-bipyridinium salts. The cyclic voltammograms of **2b**- and **2d**-modified GR are displayed in Figure 2. These cyclic voltammograms reveal the reversible reduction–oxidation wave, at $v < 1$ V·s^{-1}, corresponding to the reduction of the 4,4′-bipyridinium relays to their respective radical cations and their reoxidation. The separation between the cathodic and anodic peaks is $\Delta E_p = 30$ mV, a value that is lower than the peak separation of a diffusional reversible couple. This is consistent with the fact that the redox components are adsorbed onto the electrode in a monolayer configuration. The reduction potential of the different bipyridinium salts linked to GR is similar and is estimated to be –0.58 V vs SCE. This reduction potential is ca. 100 mV more positive than that of free **2b** and **2d**, ($E°$ = –0.68 V vs SCE). Similar changes in the reduction potential of bipyridinium derivatives were reported by Schlereth et al.[45] with *N*-propyl-*N*′-(carboxypropyl)-4,4′-bipyridinium bound to bovine serum albumin. Assuming that all bipyridinium units linked to the GR monolayer are reduced (and subsequently oxidized) in the cyclic voltammetry experiments, one can estimate the loading degree of the protein by the redox-active groups by integrating the reduction (or oxidation) peaks. We find that the modification of the GR monolayer by **2b** or **2d** leads to a similar loading, and that eight out of 39 lysine residues that make up the protein are derivatized by the redox-active group. The electron-transfer rate constants from the electrode to **2b**- and **2d**-tethered GR monolayer were derived from the dependence of the respective cathodic and anodic peak separations as a function of scan rates using Laviron's method.[46] We find that the electron-transfer rate constants to **2b**- and **2d**-modified GR are similar[47] and exhibit the value k_{ET} = 130 s^{-1}. It should be noted, however, that while **2b** and **2d** reveal the reversible electroresponse in the cyclic voltammograms, modification of the GR monolayer by **2a** results in a protein assembly lacking any electrochemical response. Nevertheless, we find that modification of the GR monolayer Au electrode by **2a** takes place since the resulting electrode exhibits bioelectrocatalytic activity toward reduction of GSSG. The lack of a resolved cyclic voltammogram for the **2a**-tethered GR monolayer is at present unknown. A possible origin for the lack of the electrical response of the **2a**-modified GR monolayer electrode could be the short chains linking the redox units to the protein backbone. Such short links could result in a distribution of redox units in substantially different chemical environments apart from the electrode surface. As a result, the electron-transfer rates from the electrode to the relay units could be quite low and distributed over a broad region. This would effect a broad electrochemical response that is difficult to detect.

The bipyridinium-derivatized GR monolayer electrodes are catalytically active in the electroreduction of GSSG to GSH. Application of the potential E = –0.72 V vs SCE on the monolayer-assembled electrodes in the presence of GSSG affords the bioelectrocatalyzed formation of GSH. Figure 3 shows the rate of formation of GSH in the presence of the various bipyridinium-modified enzyme electrodes. All GR electrodes exhibit catalytic activities toward reduction of GSSG, and thus electron-transfer communication between the enzyme active site and the electrode is attained. Furthermore, it is evident that as the tether linking the bipyridinium relays to the protein is lengthened, the rate of GSH reduction is enhanced. The rates of GSH formation in the presence of **2b**- and **2d**-derivatized GR monolayer electrodes are 1.5- and 2.2-fold higher, respectively, than that in the presence of **2a**-modified enzyme electrode. Control experiments reveal that the unmodified GR monolayer electrode does not show any biocatalytic activity toward GSSG reduction by itself, yet it affects the bioelectrocatalyzed reduction of GSSG in the presence of the diffusional electron mediator *N*,*N*′-dimethyl-4,4′-bipyridinium, MV^{2+}. It is also important to note that the **2a**–GR, **2b**–GR, and **2d**–GR monolayer electrodes exhibit similar activities in the presence of the diffusional electron mediator, MV^{2+}. Thus, the series of enzymes **2**–GR, immobilized onto the electrodes, exhibit similar intrinsic protein activities.

The effect of added urea during the modification of the GR monolayer electrode by **2** on the electron-transfer properties of the resulting biocatalytic assembly requires specific attention. We find that chemical modification of the GR monolayer electrode by **2**, in the absence of urea, yields enzyme electrodes that reveal the similar cyclic voltammograms as described earlier (i.e., Figure 2), but the enzyme electrodes lack the activities to reduce GSSG. The resulting **2**-modified enzyme electrodes, in the absence of added urea, include, however, the GR protein in a bioactive state: addition of the diffusional electron-transfer mediator MV^{2+} to the inactive **2**-derivatized GR electrodes (prepared in the absence of urea) leads to electrobiocatalyzed reduction of GSSG to GSH. Thus, we conclude that chemical modification of the GR monolayer enzyme electrode in the absence of urea yields assemblies that lack electron-transfer communication between the enzyme active site and the electrode, although the protein is present in a biologically active state. Addition of urea during the modification of GR by **2** unfolds the protein backbone and allows the covalent linkage of the electron relay units to intraprotein lysine positions that attain short distances in respect to the active site.

The experimental results presented for the bioelectrocata-

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(47) The similar electron-transfer rate constants for electrode processes (k_{ET}) for **2b**– and **2c**–GR monolayer electrodes suggest that the two tethers attain similar distances with respect to the electrode surface within the monolayer array.

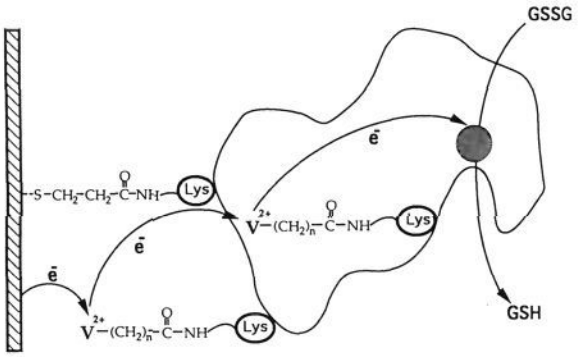


Figure 4. Electron-transfer communication of GR in the monolayer enzyme electrode.

lyzed reduction of GSSG to GSH by the bipyridinium-derivatized glutathione reductase monolayer Au electrodes enable us to reveal the important parameters that control electron-transfer communication between the enzyme active site and the electrode in the self-assembled monolayer assembly, Figure 4. Covalent linkage of **2** to the GR monolayer electrode, in the presence of urea, leads to modification of lysine residues located at the protein exterior as well as at intraprotein positions, close to the shielded redox center of the enzyme. The externally positioned bipyridinium electron relay units electrically communicate with the electrode but cannot induce direct electron transfer to the protein active site due to distance limitations (as evidenced by the system constructed in the absence of urea). Stepwise electron transfer from the peripherially attached bipyridinium units to the intraprotein transplanted relay components shortens the intraprotein electron-transfer distances and allows charge transport to the active site. Thus, by introduction of redox units at intraprotein positions, the enzyme is converted into an "electrically wired" matrix, where vectorial electron transfer proceeds effectively from the electrode to the active site across the series of relay units. These observations are consistent with the distance dependence of electron transfer predicted by Marcus theory (see Introduction). Transplanting relay units at intraprotein positions shortens the respective electron-transfer distances and allows electrical communication between the enzyme redox center and its macroscopic environment. The distance effects on electron transfer in the GR monolayer electrodes are further emphasized by examining the electrical communication in the **2**–GR electrodes that include different chain lengths linking the electron mediators to the protein. The electrochemical experiments reveal that electron transfer from the electrode to the relay units is independent of the tether length, $k_{ET} = 130\ s^{-1}$ for the **2b**– and **2d**–GR electrodes, yet the rate of GSSG reduction differs substantially, and the rate of GSH formation is ca. 1.4-fold faster with the **2d**–GR electrode than with the **2b**–GR electrode. These results indicate that the rate-limiting step in the reduction of GSSG involves the intraprotein electron transfer from the peripheral electron relays to the active site. With long tethered relays, the electron transport units can attain shorter distances with respect to the peripheral electron-donating groups as well as the electron-accepting active site of the enzyme. Consequently, the long-chain, protein-anchored redox units reveal improved electrical communication within the enzyme monolayer.

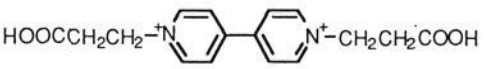
The primary modification of the Au electrode by **1** generates a stable self-assembled monolayer that is not substituted or exchanged by GSSG. Immobilization of GR to this monolayer with subsequent attachment of the respective redox relays generates a stable bioactive assembly for the electrobiocatalyzed reduction of GSSG.

Electron-Transfer Communication in GR Photocatalytic Assemblies. Electron transfer (or hole transfer) between an enzyme redox site and an external excited photosensitizer depends on electrical communication across the protein and concomitant participation of the redox relay units as electron-transfer quenchers of the excited species. We examined two different approaches to effect electrical communication in photochemical assemblies involving glutathione reductase as redox enzyme, Figure 5.

(i) The first method involves the modification of the protein by electron relay groups that provide the electrical channel for electron transport across the protein.[25a] The relay units linked to protein periphery also participate in electron-transfer quenching of the excited photosensitizer that results in charge separation. Thus, the assembly provides the basis for the vectorial electron transport from the excited state across the protein backbone to the active site where the photosynthetic process takes place (Figure 5a).

(ii) By the second approach, a redox-active copolymer is used as an interface to establish electron-transfer communication, Figure 5b. By this approach, the redox copolymer attracts the protein and forms a supramacromolecular assembly.[25b] Electron-transfer quenching of the excited photosensitizer by the redox copolymer is followed by charge separation and subsequent electron transport to the protein active site. As bipyridinium salts act as electron mediators for GR and since they act effectively in electron-transfer quenchers of excited species, we have based the development of these two approaches on this relay unit.

The redox relay *N*,*N*′-bis(carboxyethyl)-4,4′-bipyridinium, PAV (**3**), was covalently attached to GR in the presence of urea.



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The loading of PAV^+ units attached to the protein lysine residues was calculated as the molar ratio between bipyridinium groups and the enzyme. It was determined by reducing the PAV^+-modified protein with dithionite and spectroscopic analysis of the resulting $PAV^{\bullet}$ protein ($\epsilon = 12\ 000\ M^{-1}\cdot cm^{-1}$). The loading of the protein by PAV^+ relay units can be controlled by the amount of **3** employed in the chemical modification step, and proteins exhibiting different loadings were accordingly prepared.

A further aspect to consider is the activity of PAV^+-modified GR as compared to the native enzyme. The assay of the native enzyme involves the spectroscopic analysis of NADPH consumption upon reduction of GSSG by the biocatalyst. Interestingly, the PAV^+-derivatized GR does not recognize the natural cofactor NADPH, and no GSH is produced in the presence of the native cofactor. The relay-modified enzyme, however, exhibits bioactivity toward reduction of GSSG: in the presence of photogenerated *N*,*N*′-dimethyl-4,4′-bipyridinium radical cation, $MV^{\bullet+}$, the PAV^+-modified enzyme acts as biocatalyst for reduction of GSSG to GSH. Thus, in order to compare the activity of the chemically modified PAV^+-GR enzyme to that of the native protein, a new assay (eliminating NADPH) is essential. As the native protein and PAV^+-GR communicate with $MV^{\bullet+}$, the assay consists of the photosensitizer Ru(II) tris(bipyridine), $Ru(bpy)_3^{2+}$, MV^{2+} as diffusional electron acceptor and electron mediator, and EDTA as sacrificial electron donor. The electron relay MV^{2+} is applied at a relatively high concentration, 0.01 M, and thus quenching of the excited state proceeds essentially by MV^{2+} with no interference of PAV^+ units linked to the protein (vide infra). The activity of the PAV^+-modified GR toward GSSG reduction in the presence of the photogenerated diffusional electron carrier, $MV^{\bullet+}$, is then compared to that of native GR. We find that the PAV^+-modified GR (at all loadings) retains ca. 70% of the activity of the native enzyme. Thus, PAV^+-modified glutathione reductase does not communicate with the native NADPH cofactor, although the active site structure for the reduction of GSSG in the protein backbone is retained. We assume that covalent linkage of PAV^+ units to the protein perturbs its structure to the extent that it

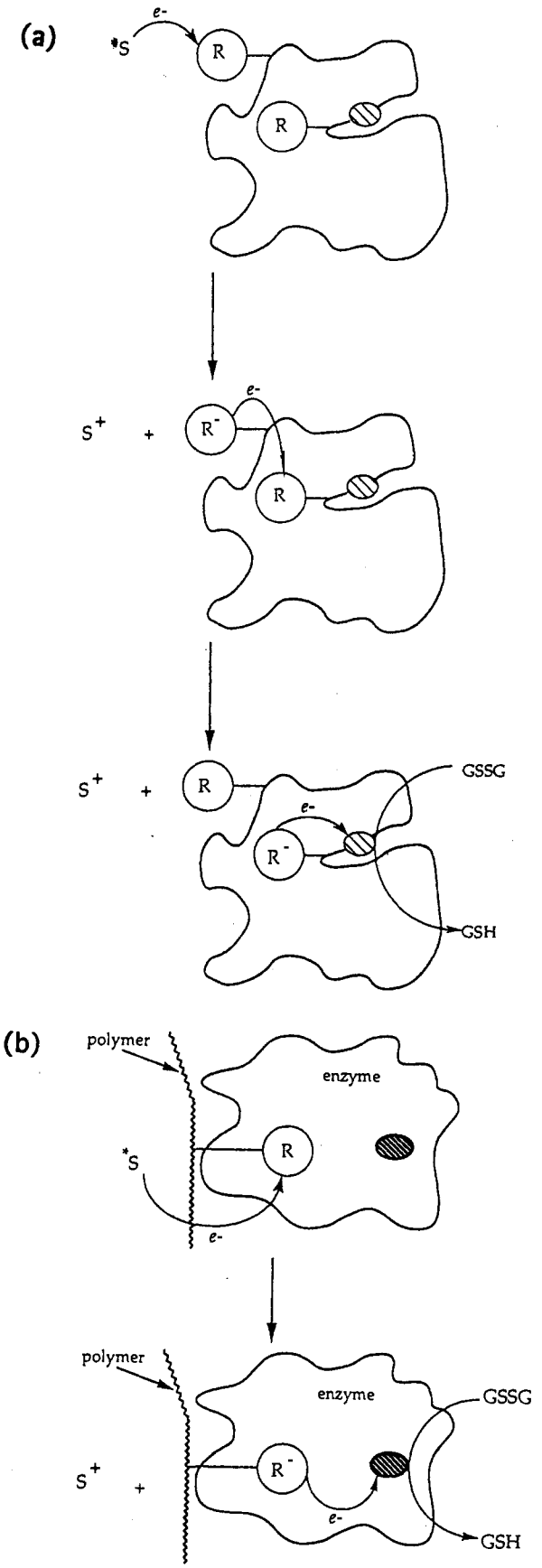


Figure 5. Electron-transfer communication between an enzyme and an excited species. (a) Charge separation and electron transfer by an electron relay-modified protein. (b) Charge separation and electron transfer by a redox tethered polymer.

eliminates the intraprotein association of NADPH but still retains the active site configuration. Consequently, electron transfer

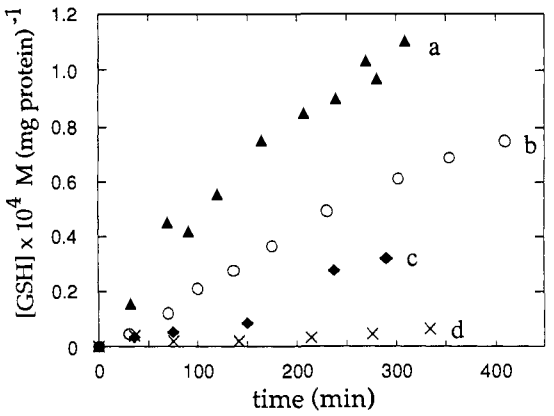


Figure 6. Rates of reduced glutathione (GSH) formation as a function of illumination time ($\lambda > 420$ nm) using GR of different loadings by PAV^+ units. Loading (mol/mol) corresponds to (a) 3.9, (b) 1.8, (c) 1.4, and (d) 0.5. In all experiments, systems consist of a 0.1 M phosphate buffer solution, pH = 7.5, [GSSG] = 1×10^{-2} M, [EDTA] = 1×10^{-2} M, and $[Ru(bpy)_3^{2+}] = 6.8 \times 10^{-5}$ M.

from NADPH to the protein is eliminated, but the reduction ability of the active center toward GSSG is preserved.

The PAV^+-modified GR was introduced into a photosystem that contained $Ru(bpy)_3^{2+}$ as photosensitizer, EDTA as a sacrificial electron donor, and the substrate GSSG (phosphate buffer, pH = 7.5). Various photosystems consisting of the enzyme at different PAV^+ loadings were employed. Illumination of the systems ($\lambda > 420$ nm) results in the reduction of GSSG to GSH. The rates of GSH formation in the different assemblies are displayed in Figure 6. It is evident that the higher the loading of GR by PAV^+ units, the more rapid is the reduction rate of GSSG. Control experiments reveal that the reduction of GSSG does not occur in the dark, nor does it proceed in the absence of any of the components included in the photosystems. Furthermore, GSSG is not photoreduced when the modified enzyme is replaced by native GR, unless the diffusional electron mediator, MV^{2+}, is also included. Thus, the PAV^+ units covalently attached to GR are essential to enable the photoreduction of GSSG. Moreover, these groups participate in a light-induced process involving $Ru(bpy)_3^{2+}$ as photosensitizer and provide the relay units for electrical communication between the enzyme redox site and the external light-harvesting compound.

Laser flash photolysis experiments allow us to evaluate the primary electron-transfer rate constant from the excited species, $*Ru(bpy)_3^{2+}$, to protein-bound bipyridinium units, eq 3. The

$$*Ru(bpy)_3^{2+} + PAV^+\text{-}GR \xrightarrow{k_q} Ru(bpy)_3^{3+} + PAV^{\bullet}\text{-}GR \quad (3)$$

fluorescence decay lifetimes of $*Ru(bpy)_3^{2+}$ ($\lambda = 610$ nm) in the absence and presence of different concentrations of PAV^+-GR have been determined. The derived electron-transfer quenching rate constant corresponds to $k_q = 5.6 \times 10^9$ $M^{-1}\cdot s^{-1}$, indicating that the modified enzyme effectively quenches the excited species. The primary photogenerated photoproducts subsequently mediate the reduction of GSSG under steady-state illumination. That is, the oxidized photoproduct, $Ru(bpy)_3^{3+}$, is reduced by EDTA, thereby recycling the light-harvesting compound, while $PAV^{\bullet}$ units attached to the protein provide the electron-transport channel to the protein active site, where GSSG is reduced. Thus, PAV^+ units attached to GR provide two complementary functions in electrically communicating the enzyme redox site with the photoexcited species: they quench the excited state and generate the primary charge-separated intermediates, and they act as the relay units that mediate electron transport across the protein.

An interesting question that should be addressed is the origin of the marked influence of the enzyme loading by PAV^+ units

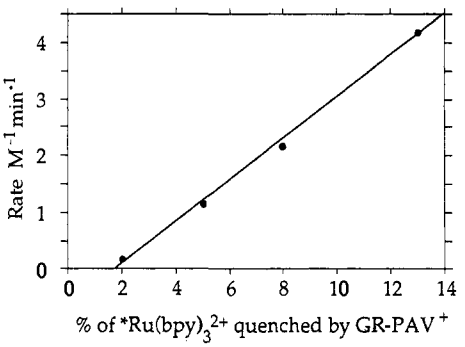
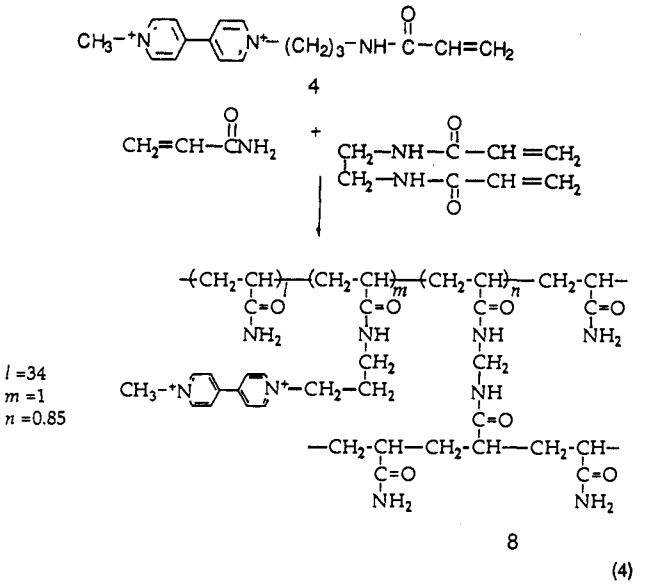


Figure 7. Rates of GSH formation in the photochemical system as a function of the fraction of excited photosensitizer quenched by PAV^+-GR. The rate corresponds to the initial slopes of Figure 6.

on the observed reduction rates of GSSG. As the bipyridinium groups participate in the quenching process of the excited state and in the electron transport across the protein, any of these processes might be rate determining and accordingly should be affected by the concentration of the relay units. Since the quenching rate constant (k_q) of the excited photosensitizer by protein-bound PAV^+ units is known, it is possible to estimate the fraction of excited species that decays through the electron-transfer quenching reaction at various degrees of bipyridinium loadings. The correlation between the fraction of excited species which is quenched by the proteins of different loading degrees and the overall reduction rate of GSSG by the respective enzymes is given in Figure 7. The linear relationship that is obtained implies that the rate-limiting step in the photoreduction of GSSG in these assemblies involves the primary electron-transfer quenching of the excited photosensitizer by the protein-anchored bipyridinium groups.

The conclusion that photoreduction of GSSG in the PAV^+-GR photosystems is controlled by the electron-transfer quenching of the excited species suggests that enhancement of the overall reduction rate of GSSG could be achieved by improving this step. The number of bipyridinium units that can be attached to the protein is, however, limited by the number of available lysine residues. Attempts to increase the loading of GR by PAV^+ units beyond the value described here were unsuccessful. On the other hand, a large number of bipyridinium groups could be introduced by immobilization of PAV^+-GR into a bipyridinium-functionalized redox polymer. Under these conditions, the electron-transfer quenching of the excited state would proceed effectively by the polymer, and the rate of PAV^+-GR formation is independent of the photoinduced electron transfer.

We have recently shown that immobilization of the enzyme nitrate reductase in a cross-linked copolymer (**8**) composed of the monomers *N*-methyl-*N'*-(3-acrylamidopropyl)-4,4'-bipyridinium (**4**) and acrylamide yields a biocatalytic assembly (eq 4), exhibiting electrical communication toward the photochemical reduction of nitrate.[23] Immobilization of GR in the same copolymer does not yield, however, electrical communication. That is, illumination of a photosystem that includes GR immobilized in the cross-linked bipyridinium acrylamide copolymer, **8**, $Ru(bpy)_3^{2+}$ as photosensitizer, EDTA as sacrificial electron donor, and GSSG as substrate does not yield GSH, although the polymer is effectively photoreduced to the respective bipyridinium radical cation acrylamide copolymer. This lack of electrical communication in the GR–polymer assembly presumably originates from the rigidity of the electron relay groups in the polymer matrix and short tethers linking the bipyridinium redox units to the polymer. The cross-linked bipyridinium–acrylamide copolymer, **8**, may provide, however, a dense interface of electron-acceptor units that could effectively quench the excited photosensitizer, $Ru(bpy)_3^{2+}$. Accordingly, immobilization of the



(4)

PAV^+-modified GR in this polymer could then provide an organized assembly for effective electrical communication, Figure 8.

A photosystem composed of $Ru(bpy)_3^{2+}$ as photosensitizer, PAV^+-GR immobilized in the acrylamide bipyridinium redox polymer, **8**, EDTA as sacrificial electron donor, and the substrate, GSSG, was illuminated (pH = 7.5, $\lambda > 420$ nm). The system exhibits high photobiocatalytic activity, and GSH is formed effectively. Figure 9 shows the rate of GSH formation as a function of illumination time. GSH is formed with an initial rate corresponding to 3.3×10^{-8} mol·min^{-1}. For comparison, the rate of GSH formation in the most effective PAV^+-GR assembly in the absence of the polymer (highest loading) is also shown. Evidently, the rate of GSH formation in the presence of PAV^+-GR immobilized in the redox polymer is 25-fold higher than the reduction rate of GSSG by the nonpolymerized PAV^+-GR. Furthermore, we realize that immobilization of PAV^+-GR in the redox assembly stabilizes the biocatalyst, as compared to the homogeneous PAV^+-GR photosystem. With PAV^+-GR in a nonimmobilized state, GSH formation levels off after ca. 3 h of illumination, implying that the enzyme is deactivated. In turn, PAV^+-GR immobilized in the redox copolymer reveals bioactivity even after 7 h of illumination. The efficient reduction of GSSG by PAV^+-GR immobilized in the redox acrylamide copolymer **8** is attributed to the vectorial electron transfer outlined in Figure 8. The polymer-anchored bipyridinium units act as efficient quenchers of the excited state and provide an electron pool for protein-bound PAV^+ units. The latter components mediate electron transfer to the enzyme redox site.

An alternative methodology for establishing electrical communication between a redox polymer and GR might involve the application of flexible polymers that include long, tethered redox relay groups. For this goal we have utilized poly(L-lysine), PL (MW = 43 500) as the basic polymer backbone for attachment of bipyridinium redox units. This polymer has several advantages for our purpose: it is positively charged at the pH range 7.0–8.0 and is expected to associate by electrostatic interactions the negatively charged GR ($pK_i = 6.4$).[48] Thus, bipyridinium groups linked to the polymer could interact with the protein active site. Furthermore, the solubility of PL in aqueous media facilitates mechanistic analysis of electron transfer from the polymer to the enzyme redox site. *N*-Methyl-*N'*-(carboxyalkyl)-4,4'-bipyridinium (**2**) was covalently linked to PL to yield *N*-methyl-N'-(carboxyalkyl)-4,4'-bipyridinium–poly(L-lysine) (**9**), PL-C_nV^{2+}, eq 5. A series of polymers **9a–9d** consisting of the bipyridinium

(48) Eriksson, S.; Askelof, P.; Axelsson, K.; Carlberg, I.; Guthenberg, C.; Mannervic, B. *Acta Chem. Scand.* **1974**, *B28*, 922.

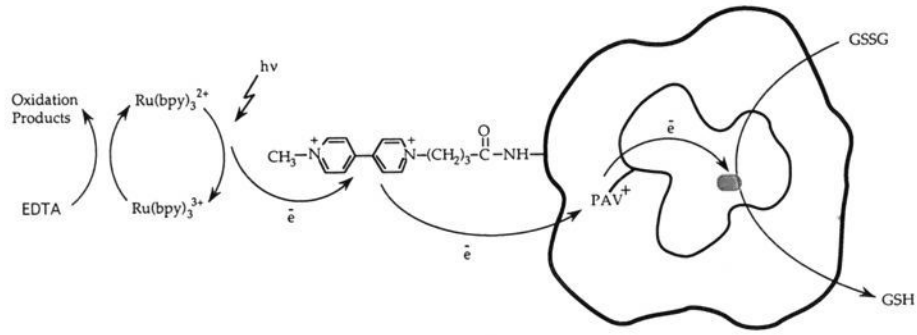


Figure 8. Photosensitized electron-transfer reactions involved in the reduction of GSSG using PAV^+-GR enzyme immobilized in the redox copolymer **8**.

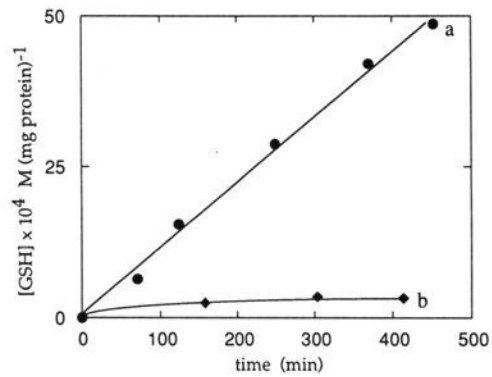
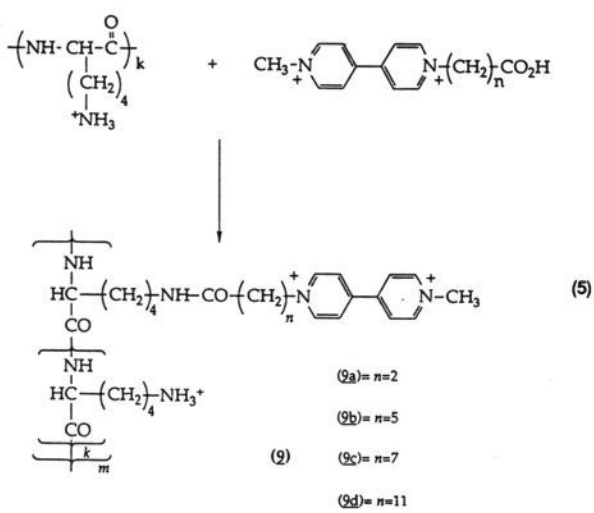


Figure 9. Rate of reduced glutathione (GSH) formation as a function of illumination time using (a) PAV^+-GR immobilized in the redox copolymer **8** and (b) PAV^+-GR in the absence of polymer.

redox relay attached to PL by different bridging chain lengths was prepared.



The loading of the bipyridinium relay units on PL was verified by chemical reduction of PL-C_nV^{2+} with sodium dithionite at 42 °C and spectroscopic analysis of the respective monomer bipyridinium radical cation (λ_{max} = 392 and 602 nm). The loadings of the various polymers **9a**–**9d** are summarized in Table 1. It should be noted that spectral analysis of the derived PL-$C_nV^{\bullet+}$ should be performed at 42 °C (or higher). At room temperature, the polymer-bound bipyridinium radical cations, PL-$C_nV^{\bullet+}$, tend to form dimer aggregates (detected by an additional absorption band at λ = 370 nm and a wide absorption band at 490–580 nm). The formation of bipyridinium radical cation aggregates is well established.[49] Dimer formation (in equilibrium with the monomer form) is affected by high radical cation concentrations as well as by the hydrophobicity of the alkyl chains attached to the radical cation.[50] Thus, aggregation of the bipyridinium units in PL-$C_nV^{\bullet+}$ polymers is attributed to the high local concentration of the radical on the polymer backbone and the hydrophobicity of the linking alkyl chains. At elevated temperatures, the monomer–dimer equilibrium shifts to the monomer form. Thus, to estimate the loading of PL-C_nV^{2+}, the spectra of PL-$C_nV^{\bullet+}$ were recorded at 42 °C, where the radical exists in the monomer form.

Table 1. Rates of Photosensitized GSH Formation in Photosystems That Include the PL-C_nV^{2+} Polymers[a]

polymer	loading[b] (±0.2)	reaction rate of GSH formation (M·min^{-1})	relative rate of GSH formation
PL-C_2V^{2+} (**9a**)	2.5	1.9×10^{-6}	1
PL-C_5V^{2+} (**9b**)	2.9	2.6×10^{-6}	1.4
PL-$C_{11}V^{2+}$ (**9d**)	2.8	7.3×10^{-6}	3.9

[a] All photosystems consist of $Ru(bpy)_3^{2+}$ (7.5×10^{-5} M), PL = C_nV^{2+} (5.2 mg·mL^{-1}), Na_2EDTA (1×10^{-2} M), GR (50 units), and GSSG (1×10^{-2} M). [b] Defined as moles of bipyridinium units per mole of polymer (MW of PL = 43 500).

Photoreduction of GSSG was examined in photosystems comprising $Ru(bpy)_3^{2+}$ as photosensitizer, EDTA as a sacrificial electron donor, each one of the bipyridinium-modified polylysines PL-C_nV^{2+}, **9a**, **9b**, and **9d**, and glutathione reductase. Illumination of these photosystems, $\lambda > 420$ nm, in the presence of GSSG results in the formation of GSH, Figure 10. We see that the rate of GSH evolution varies with the length of the alkyl chain connecting the bipyridinium groups to PL. The reaction rate is ca. 4 times faster with **9d** than with **9a**. The reaction rates of GSH formation, as well as the relative rates, are also given in Table 1. We note that during illumination of the various photosystems, the solution is colored blue and shows the typical spectrum of the bipyridinium radical cation. This observation indicates that photogeneration of the radical cation PL-$C_nV^{\bullet+}$ is faster than its consumption by the subsequent electron transfer to the enzyme. Thus a steady-state concentration of the cation radical is obtained during illumination. Control experiments reveal that the reaction does not occur in the dark nor in the absence of any of the stated components. Illumination of the various photosystems in the absence of GR but in the presence of GSSG affords the bipyridinium radical cation, as evidenced by the blue coloration of the solution and its characteristic spectrum, but no GSH is produced. On the basis of these control experiments, we conclude that electron-transfer communication between the redox polymer, PL-$C_nV^{\bullet+}$, and the native (nonmodified) GR is attained. The effectiveness of electrical commu-

(49) Kosower, E. M.; Cotter, J. L. *J. Am. Chem. Soc.* **1964**, *86*, 5524.
(50) It should be noted that unbound bipyridinium salts **2** do not form dimers upon reduction with $Na_2S_2O_4$ at 2×10^{-3} M concentration. This concentration is higher than the bulk concentration of PL-$C_nV^{\bullet+}$ solutions.

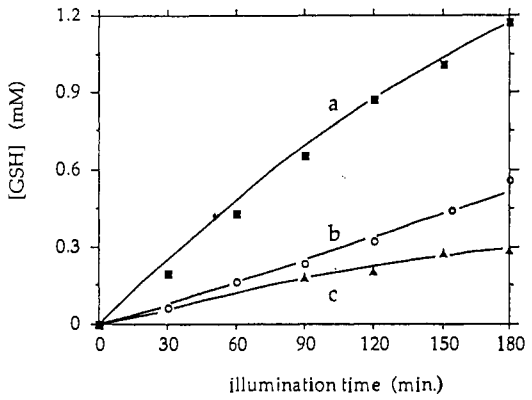


Figure 10. Rates of reduced glutathione (GSH) formation as a function of illumination time using the different PL-C_nV^{2+} polymers: (a) **9d**, (b) **9b**, and (c) **9a**. All photosystems consist of 3 mL of buffer solution, pH = 7.0, that includes $[Ru(bpy)_3^{2+}] = 7.5 \times 10^{-5}$ M, [EDTA] = 10^{-2} M, [GSSG] = 1×10^{-2} M, and GR = 50 units. Illumination is conducted under Ar, $\lambda > 420$ nm.

nication is presumably controlled by the lengths of the tethers linking the redox relays to PL (vide infra). Accordingly, we suggest that reduction of GSSG to GSH in these photosystems proceeds by the sequence of electron-transfer processes summarized in eqs 6–10. The polymer-bound bipyridinium groups act

$$*Ru(bpy)_3^{2+} + PL\text{-}C_nV^{2+} \xrightarrow{k_q} Ru(bpy)_3^{3+} + PL\text{-}C_nV^{\bullet+} \quad (6)$$

$$Ru(bpy)_3^{3+} + PL\text{-}C_nV^{\bullet+} \xrightarrow{k_b} Ru(bpy)_3^{2+} + PL\text{-}C_nV^{2+} \quad (7)$$

$$Ru(bpy)_3^{3+} + EDTA \rightarrow Ru(bpy)_3^{2+} + \underset{\text{oxidation products}}{EDTA^{\bullet+}} \quad (8)$$

$$PL\text{-}C_nV^{\bullet+}\ (E_{ox}) \xrightarrow{k_{ET}} PL\text{-}C_nV^{2+}\ (E_{red}) \quad (9)$$

$$E_{red} + {}^1/_2GSSG + H^+ \rightarrow E_{ox} + GSH \quad (10)$$

as oxidative quenchers of the excited state of the photosensitizer, $*Ru(bpy)_3^{2+}$ (eq 6). The intermediate photoproducts, $Ru(bpy)_3^{3+}$ and PL-$C_nV^{\bullet+}$, may either recombine, eq 7, or mediate subsequent electron transfer. That is, EDTA is irreversibly oxidized by $Ru(bpy)_3^{3+}$, thereby recycling the photosensitizer (eq 8). The reduced relay groups PL-$C_nV^{\bullet+}$ penetrate the protein backbone and reach appropriate distances that allow electrical communication with the redox site. Electron transfer to the active site, eq 9, is then accompanied by the reduction of the substrate, eq 10. To this end, two control experiments should be specifically detailed. Illumination of a photosystem composed of $Ru(bpy)_3^{2+}$, PL, and *N*,*N*′-dimethyl-4,4′-bipyridinium, MV^{2+}, does not yield any photoreduced bipyridinium radical cation, $MV^{\bullet+}$. Similarly, illumination of a photosystem that includes $Ru(bpy)_3^{2+}$ and PL-C_nV^{2+}, $\lambda > 420$ nm, for more than 4 h, does not yield any detectable reduced product, PL-$C_nV^{\bullet+}$, whose information is observed only upon addition of the sacrificial electron donor, Na_2EDTA. These control experiments clearly indicate that PL does not act as electron donor in the photosystems. This conclusion is of critical importance, as any participation of PL-C_nV^{2+} as a sacrificial electron donor could yield low molecular weight bipyridinium radical cation fragments that might operate in the reduction of GSSG by a diffusional route.

Laser flash photolysis experiments were used to analyze the rate constants of the respective electron-transfer steps that lead to the reduction of GSSG to GSH, eqs 6–10. Photosystems comprising the photosensitizer, $Ru(bpy)_3^{2+}$, and the bipyridinium-modified polymers, PL-C_nV^{2+}, were employed. The rate constants of the oxidative electron-transfer quenching of the excited photosensitizer by PL-C_nV^{2+}, eq 6, were determined by following the fluorescence decay lifetimes of the excited photosensitizer in the absence and presence of different PL-C_nV^{2+} concentrations. The quenching rate constants, k_q, of the excited photosensitizer by PL-C_nV^{2+} are summarized in Table 2. For comparison, the quenching rate constant of $*Ru(bpy)_3^{2+}$ by MV^{2+} is $k_q = 2.0 \times 10^9$ $M^{-1}\cdot s^{-1}$. The quenching rate constants of the photosensitizer by PL-C_nV^{2+} are lower than the value found for MV^{2+}. This result is reasonable, since the polymer backbone is positively charged (protonation of lysine residues) and thus it electrostatically repels the positively charged excited photosensitizer, $*Ru(bpy)_3^{2+}$. Such electrostatic effects on the quenching efficiency of excited states have been observed with other charged interfaces.[51,52]

Table 2. Electron-Transfer Quenching Rate Constants of $Ru(bpy)_3^{2+}$ by PL-C_nV^{2+} Polymers and Electron-Transfer Rates from PL-$C_nV^{\bullet+}$ to GR Active Site

polymer	k_q ($M^{-1}\cdot s^{-1}$)[a]	k_{ET} (s^{-1})[b]
PL-C_2V^{2+} (**9a**)	$(1.3 \pm 0.2) \times 10^9$	490
PL-C_5V^{2+} (**9b**)	$(4.2 \pm 0.4) \times 10^8$	1090
PL-C_7V^{2+} (**9c**)	*c*	1700
PL-$C_{11}V^{2+}$ (**9d**)	$(8.5 \pm 0.9) \times 10^8$	4350

[a] Electron-transfer quenching rate constant, eq 6. [b] Electron-transfer rate constant from PL-$C_nV^{\bullet+}$ to the redox site of GR. [c] Not determined.

The subsequent back electron transfer processes of the photogenerated redox products, eq 7, were analyzed by following the absorption transient decays of PL-$C_nV^{\bullet+}$ in these photosystems ($\lambda_{detection}$ = 602 nm). The recombination rate constants of the various PL-$C_nV^{\bullet+}$ are very similar and correspond to $k_b = (2\text{–}3) \times 10^8$ $M^{-1}\cdot s^{-1}$. These back electron transfer rates are ca. 10-fold retarded as compared to the $MV^{\bullet+}$ system, where $k_b = 3.5 \times 10^9$ $M^{-1}\cdot s^{-1}$. The retardation of the back electron transfer rates in the presence of PL-$C_nV^{\bullet+}$ is also attributed to the electrostatic interactions exerted by the polymer.[53] The positively charged polymer interface repels the oxidized photoproduct, $Ru(bpy)_3^{3+}$, and thus retards its recombination with the polymer-bound bipyridinium radical cation.

The electron-transfer rate constants (k_{ET}) from the reduced polymer, PL-$C_nV^{\bullet+}$, to the enzyme redox site, corresponding to the electrical communication step, eq 9, were analyzed. Here two situations may occur: (i) $k_b \gg k_{ET}$, and then it would be impossible to measure k_{ET} under conditions where the back electron transfer is dominant; (ii) $k_b \approx k_{ET}$, and then the two processes would compete one with the other. As a result, k_{ET} could be extracted by following the decay transients of PL-$C_nV^{\bullet+}$ in the photosystems that include GR, knowing k_b from the system without the enzyme. To verify the relative magnitudes of the two rate constants (k_b and k_{ET}), the transient decays of PL-$C_nV^{\bullet+}$ generated upon flashing the system $Ru(bpy)_3^{2+}$ and PL-C_nV^{2+} in the absence or presence of GR and GSSG were analyzed. We find that the decay transients of PL-$C_nV^{\bullet+}$ in both systems are identical. We thus conclude that $k_b \gg k_{ET}$ and that the recombination process is the dominant route in the disappearance of PL-$C_nV^{\bullet+}$. Hence, it is impossible to estimate k_{ET} in this configuration of the photosystems. It is thus required to introduce a sacrificial electron donor into the systems which will function as a scavenger of the oxidized species, $Ru(bpy)_3^{3+}$. Under these conditions, the recombination process is eliminated and PL-$C_nV^{\bullet+}$

(51) Wolff, C.; Grätzel, M. *Chem. Phys. Lett.* **1977**, *52*, 542.
(52) Katusin-Razen, B.; Wong, M.; Thomas, J. K. *J. Am. Chem. Soc.* **1978**, *100*, 1679.
(53) (a) Degani, Y.; Willner, I. *J. Chem. Soc., Chem. Commun.* **1982**, 1249. (b) Degani, Y.; Willner, I. *J. Am. Chem. Soc.* **1983**, *105*, 6228. (c) Sassoon, R. E.; Rabani, J. *J. Phys. Chem.* **1980**, *84*, 1319.

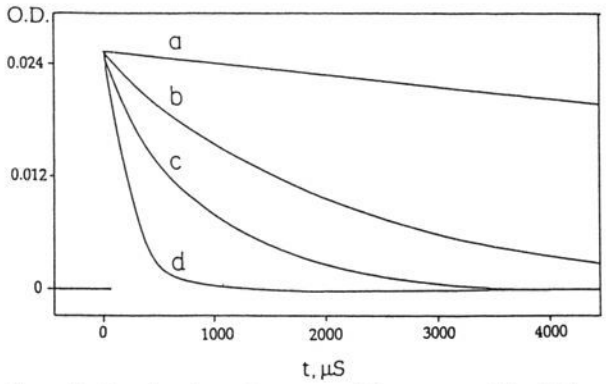


Figure 11. Transient absorption spectra of photogenerated bipyridinium radical cations anchored to poly(L-lysine), PL-$C_nV^{\bullet+}$ (λ = 602 nm), in the absence and in the presence of the enzyme glutathione reductase. (a) PL-$C_{11}V^{2+}$ (**9d**) without GR. (b) PL-C_2V^{2+} (**9a**) and GR, 500 units. (c) PL-C_5V^{2+} (**9b**) and GR, 500 units. (d) PL-$C_{11}V^{2+}$ (**9d**) and GR, 500 units. In all systems, $[Ru(bpy)_3^{2+}] = 3.75 \times 10^{-4}$ M, [EDTA] = 10^{-2} M, [GSSG] = 1×10^{-2} M, and polymer = 5.2 mg·mL^{-1}.

is accumulated. In the presence of the enzyme GR and the substrate GSSG, the decay of photoinduced accumulated PL-$C_nV^{\bullet+}$ corresponds to the electron-transfer rate from PL-$C_nV^{\bullet+}$ to the enzyme redox site. Thus, the photosystems in which k_{ET} was determined included $Ru(bpy)_3^{2+}$ as photosensitizer, PL-C_nV^{2+} as different electron acceptors and charge mediators, EDTA as sacrificial electron donor, the enzyme GR, and its substrate GSSG. Flash photolysis of the different photosystems which include PL-C_nV^{2+} was performed by excitation of the photosensitizers at λ = 532 nm, following the decay of PL-$C_nV^{\bullet+}$ at λ = 602 nm. Figure 11 shows the decay curves of PL-$C_nV^{\bullet+}$ in the different systems. For comparison, the accumulated PL-$C_nV^{\bullet+}$ in the photosystem that includes polymer **9d** but excludes the enzyme GR is also provided in Figure 11 (curve a). We see that while PL-$C_nV^{\bullet+}$ decays very slowly in the absence of the enzyme, presumably due to oxygen leakage, the photogenerated PL-$C_nV^{\bullet+}$ decays effectively in the presence of GR and GSSG. Furthermore, it is evident that the various systems reveal significant differences in the decay rates of PL-$C_nV^{\bullet+}$: the longer the chain length connecting the bipyridinium relay to the polymer backbone, the faster is the decay of bipyridinium radical. The electron-transfer rate constant from PL-$C_nV^{\bullet+}$ to the enzyme redox site, k_{ET}, eq 9, can be evaluated from these transients. We find that the decay curves fit best a first-order kinetics, suggesting that a supramolecular assembly is formed between the negatively charged enzyme and the positively charged polymer. Electron transfer then proceeds within this united supramolecular assembly. This assumption is supported by the fact that the decay processes are independent of the enzyme concentration in the range 50–500 U (5.6×10^{-7}–5.6×10^{-6} M). The values of k_{ET} for the various systems that include PL-C_nV^{2+} with varying tether lengths are given in Table 2. It is clear that k_{ET} increases as the length of the tether linking the relay to PL is longer and follows the order **9d > 9c > 9b > 9a**. For example, with PL-$C_{11}V^{2+}$ as polymer interface (bridging chain composed of 11 methylene units), k_{ET} is ca. 9-fold higher than the respective value of PL-C_2V^{2+} (bridging chain includes two methylene units). These results are in accordance with the rates of GSH formation under steady-state illumination as described above, where the rate of GSH production is most effective with the long-chain anchored bipyridinium polymer.[54]

The results of the flash photolysis experiments, as well as the steady-state illumination experiments, clearly reveal that the rates of electron transfer from the polymer-anchored bipyridinium radical cation units to the enzyme active site are controlled by the spacer length linking the electron mediator to the polymer. Since the PL polymer is positively charged, the bipyridinium

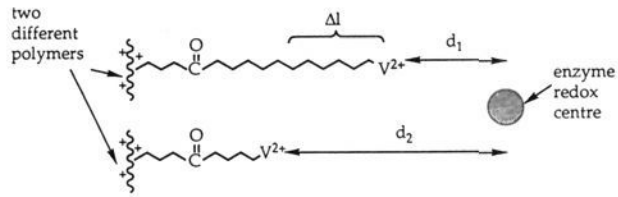


Figure 12. Schematic model for distance dependence of electron transfer from PL-$C_nV^{\bullet+}$ to redox center of GR enzyme.

groups are expected to be repelled by it and to attain an averaged stretched configuration. We surmise that the enzyme and polymer, being oppositely charged, are held together by multiple electrostatic interactions. The formation of this supermacromolecular structure is indeed supported by the first-order kinetics of the electron transfer within the assembly. Furthermore, since the methylene chain linking the bipyridinium units to PL is hydrophobic, the bipyridinium radical cations are likely to penetrate the protein. Thus, the longer the spacer chain length, the shorter the distances of the electron mediator in respect to the active site will be obtained, and electron transfer will be enhanced. This model is schematically depicted in Figure 12.

According to Marcus theory, eq 1, and assuming that $\Delta G°$ and λ are identical for the various polymers, PL-$C_nV^{\bullet+}$–enzyme assemblies, the ratio of two electron-transfer rate constants, k_1 and k_2, from two different polymers that position the electron mediator at average distances from the active site of the enzyme, d_1 and d_2, respectively, is given by eq 11,

$$k_1/k_2 = \exp[-\beta(d_1 - d_0)]/\exp[-\beta(d_2 - d_0)] \qquad (11)$$

where d_0 is the van der Waals distance for electron transfer between the donor–acceptor pair. By application of the model shown in Figure 12, eq 11 can be formulated in the form of eq 12,

$$\ln(k_1/k_2) = \beta(d_2 - d_1) = \beta\Delta l \qquad (12)$$

where Δl is the difference between the chain lengths of two alkyl spacers of two PL-$C_nV^{\bullet+}$. A plot of the ratios of rate constants against the distance differences[55] gives a linear relationship, as shown in Figure 13. The derived β value (corresponding to the slope) is 0.15 Å^{-1}. This value should be, however, regarded as a lower limit. The model assumes that the alkyl chains are fully stretched and retain a perpendicular position with respect to the polymer interface. However, if the chains are only partially stretched or if a tilt angle between the polymer chain and the relay tether exists, then Δl should be smaller than the values used in this analysis, and accordingly β would be higher. Previous researchers have found values in the range 0.8–1.2 Å^{-1} for electron transfer in proteins that contain donor–acceptor pairs attached at fixed orientations.[56] To our knowledge, there are no other

(54) The ratio of k_{ET} of the various relay-tethered polymers (see Table 2) is, however, larger than the ratio of k_{obs} values observed under steady-state illumination. For example, while k_{ET} is 9-fold faster for PL-$C_{11}V^{2+}$ than for PL-C_2V^{2+}, the rate of GSH formation under steady-state illumination in the system composed of PL-$C_{11}V^{2+}$ is 4-fold higher than that of formation in the system with PL-C_2V^{2+}. This difference is attributed to the fact that GSH, being photogenerated under steady-state illumination, acts as an inhibitor for GR upon accumulation [Scott, E. M.; Duncan, I. W.; Ekstrand, V. *J. Biol. Chem.* **1963**, *238*, 3928] as well as the fact that GSH acts as an electron donor for $Ru(bpy)_3^{3+}$. Thus, the product, GSH, is partially consumed under steady-state illumination. Also, the enzyme GR is inhibited to some extent. Both effects result in lower GSH amounts under steady-state illumination than expected on the basis of the kinetic analysis and k_{ET} values.

(55) To calculate the difference in chain lengths, it is assumed that each CH_2 group lengthens the tether by 1.6 Å.

(56) (a) Li, T. T.-T.; Weaver, M. J. *J. Am. Chem. Soc.* **1984**, *106*, 6107. (b) Li, T. T.-T.; Weaver, M. J. *J. Electroanal. Chem. Interfacial Electrochem.* **1985**, *188*, 121. (c) Weaver, M. J.; Li, T. T.-T. *J. Phys. Chem.* **1986**, *90*, 3823. (d) Wasielewski, M. R. In *Photoinduced Electron Transfer*; Fox, M. A., Chanon, M., Eds.; Elsevier: Amsterdam, 1988; Part A, p 161. (e) Oevering, H.; Verhoeven, J. W.; Paddon-Row, M. N.; Warman, J. M. *Tetrahedron* **1989**, *45*, 4751. (f) Hong, H.-G.; Mallouk, T. E. *Langmuir* **1991**, *7*, 2362. (g) Chidsey, C. E. D. *Science* **1991**, *251*, 919.

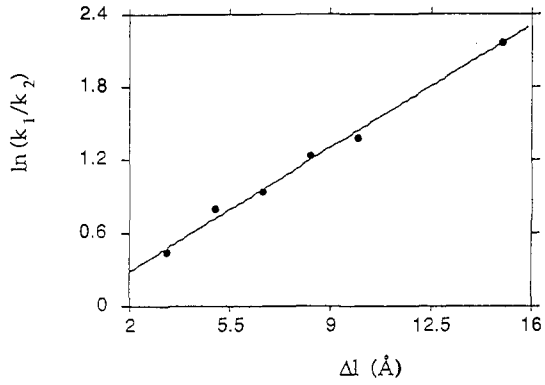


Figure 13. Kinetic analysis of electron-transfer rate constants as a function of the difference in chain lengths, Δl, of bipyridinium components linked to the polylysine.

examples of less rigid systems like the one described here, where the force holding together the redox polymer and the enzyme is exerted by electrostatic attractions and the electron-transfer active groups attain flexibility in respect to distance and orientation. To this extent, it is even surprising that such flexible spacers obey such a distance relationship.

Electrical Communication in GR Catalytic Systems. Redox enzymes are of specific interest as biocatalysts for biotransformations and thus have substantial biotechnological potential. Nevertheless, many redox enzymes require for their activity the participation of electron relay cofactors such as $NAD(P)^+$/NAD(P)H (in oxidative and reductive pathways, respectively). This requirement poses a serious obstacle from a practical view, since continuous regeneration of the cofactor is needed.[13-15] The multicomponent nature of these $NAD(P)^+$/NAD(P)H regeneration systems and the difficulties in immobilizing the low molecular weight cofactors in rigid polymer matrices limit the broad applicability of redox enzymes in biotechnological processes. Enzymes modified by electron relay groups that mediate electrical communication between the active site and their macroscopic environment could resolve these difficulties and eliminate the need for the native cofactor. For example, the enzyme glutathione reductase modified by bipyridinium relays exhibits electrical communication with its environment (electrodes, photosystems), and the need for the native enzyme cofactor, NADPH, is eliminated. We even noticed with this chemically modified enzyme that the biocatalyst did not recognize its biological cofactor. We thus aimed to show that the bipyridinium-modified GR could also be applied as a biocatalyst for "dark" biotransformations. For this purpose, electrical communication of the bipyridinium-modified GR, PAV^+-GR, with a chemical reducing agent acting as electron source is required. Molecular hydrogen is an attractive reducing agent for deriving biotransformations, as it is biocompatible and is a relatively mild reducing agent ($E°$ = –0.42 V at pH = 7.0).

Utilization of H_2 as reducing agent in biotransformations requires the introduction of catalysts for the activation of molecular hydrogen. The enzyme hydrogenase[57] was previously used to activate hydrogen and to stimulate regeneration of NAD(P)H cofactors together with the diffusional electron mediator, *N,N'*-dimethyl-4,4'-bipyridinium, MV^{2+}. However, the participation of a second protein in addition to the electrically wired enzyme is problematic, especially because of the sensitivity of the enzyme hydrogenase. Artificial heterogeneous catalysts such as Pt or Pd are known to catalyze the reversible electron

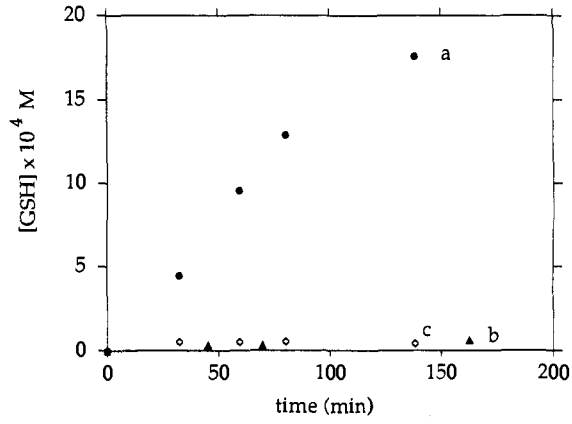


Figure 14. Rate of GSSG reduction to GSH by H_2. In all systems, [GSSG] = 1.18×10^{-2} M, 1 atm H_2, and Pt content = 70.6 mg·L^{-1}. (a) In the presence of PAV^+-GR (1.5 mg, loading of 4 mol/mol); (b) in the presence of GR (1.5 mg) and [PAV] = 9.4×10^{-3} M; and (c) in the presence of GR (1.5 mg) and [MV^{2+}] = 9.4×10^{-3} M.

transfer between H_2 and MV^{2+}. For example, Wrighton et al.[58] used Pt embedded in a bipyridinium polymer as a catalytic interface for mediated electron transfer to cytochrome *c* and other small redox proteins, using H_2 as electron source. Nevertheless, these proteins can be directly reduced on a Pt electrode albeit at lower rates, and being small proteins, they establish electrical communication without any electron mediator attached to the protein.

To develop our approach, we have used a Pt colloid and the bipyridinium-modified enzyme PAV^+-GR as multicatalyst assembly for the reduction of GSSG by molecular hydrogen. It is well known that Pt colloids adsorb strongly to various functional groups of proteins, such as quaternary ammonium ions.[59] In fact, such adsorption of metal particles on proteins could have adverse effects on the catalytic properties of the metal clusters or the biocatalyst. Yet, if both the heterogeneous catalysts and enzyme preserve their activities, it is anticipated that effective electrical communication will take place in this microheterogeneous protein assembly.

The bipyridinium-modified enzyme PAV^+-GR and the Pt colloid were introduced into an aqueous buffer solution, pH ≈ 7.5, that contained the substrate GSSG. GSSG is quantitatively reduced to GSH in this system, under an atmosphere of H_2 (1 atm), at ambient temperature. The rate of GSH formation in the system is shown in Figure 14. Formation of GSH levels off after ca. 20 h, and the conversion yield of the substrate to GSH corresponds to 100%. During the reaction, the solution shows the characteristic spectrum of the bipyridinium radical cation, implying that the protein-bound bipyridinium relay groups are reduced by H_2. Control experiments reveal that all of the components are essential to drive the reduction of GSSG. Exclusion of the Pt colloid or application of native GR instead of the bipyridinium-modified protein, PAV^+-GR, does not lead to the formation of GSH. These results clearly indicate that the Pt catalyst and the bipyridinium units that are covalently attached to the protein are essential components to facilitate electron flow from hydrogen to the protein redox site. Interestingly, upon addition of the bipyridinium salt, MV^{2+}, to a system composed of the Pt colloid, native GR, and GSSG, no GSH is formed and no bipyridinium radical cation, $MV^{\bullet+}$, could be detected. These observations need an explanation since MV^{2+} is reduced to $MV^{\bullet+}$ by hydrogen in the presence of the Pt colloid and in the absence of the enzyme and since native GR electrically communicates with photogenerated or electrogenerated $MV^{\bullet+}$ by a diffusional

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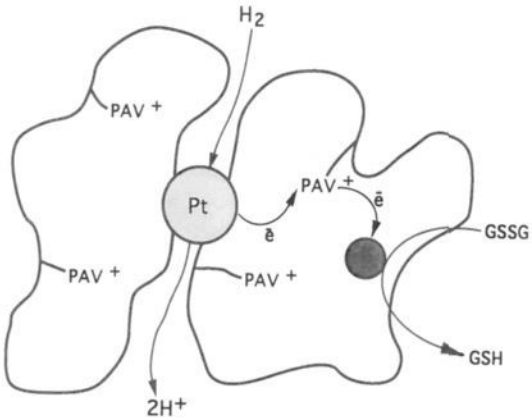

Figure 15. Electron-transfer processes involved in the reduction of GSSG by molecular hydrogen.

electron transfer. A possible explanation is that the enzyme backbone is strongly adsorbed onto the Pt clusters, generating a dense network that prevents (inhibits) the diffusion of MV^{2+} to the metal surface. In contrast, when the bipyridinium units are covalently bound to the protein structure, the relay units are an intact part of the protein which is adsorbed onto the Pt colloid. As a result, the redox PAV^{+}-GR groups are effectively reduced by H_2. Figure 15 outlines schematically the electron transport steps involved in the reduction of GSSG by molecular hydrogen, eq 13. Molecular hydrogen reduces protein-bound bipyridinium

$$GSSG + H_2 \xrightarrow[2.\ V^{2+}\text{-GR}]{1.\ Pt} 2GSH \qquad (13)$$

units at the Pt surface. The reduced relay groups then mediate charge transport to the active site, where reduction of GSSG occurs. This semiartificial bioactive redox assembly mimics the functions of many redox systems in nature.[60] In native systems, two or more proteins can be held together by interprotein interactions such as electrostatic interactions and/or hydrogen bonds. In these assemblies, protein-associated cofactors such as FAD/FADH mediate electron transfer from one redox protein to the other (for oxidation and reduction of the specific substrates of the linked enzymes). In our system, the Pt clusters provide one catalytic center for accumulation of the electrons, while the protein-linked bipyridinium units duplicate the functions of the native cofactor and communicate the heterogeneous catalyst site with the protein redox site.

Conclusions

The subject of electron-transfer communication of enzyme redox sites with their macroscopic environment is of broad interest as a means of developing novel biocatalytic, electrobiocatalytic, and photobiocatalytic assemblies. In the present study, we described a comprehensive study of electron-transfer communication of glutathione reductase in such systems.

We have followed two general strategies to attain electrical communication of GR with external electron sources: one approach involved chemical modification of GR by redox components, thereby converting the protein into an electron-transferable assembly, the second methodology involved interaction of GR with redox polymers, resulting in a supramacromolecular assembly where electron transfer is feasible.

We have exemplified a novel method of constructing an enzyme electrode comprising GR covalently linked to a self-assembled monolayer of a functionalized thiol on a Au electrode. The resulting GR electrode was modified by bipyridinium relay groups that facilitate electron transfer from the electrode to the protein active site. This approach seems to be of broad applicability in designing enzyme electrodes of other redox proteins and could be of practical use in tailoring a variety of amperometric biosensor devices. Similarly, we have shown that relay-modified GR can be coupled to external photoexcited species and reducing agents. Such electron relay-modified enzymes could be effective catalysts for photosynthetic transformations.

Application of a similar methodology for enzymes such as hydrogenase or formate dehydrogenase could establish novel photobiocatalysts for hydrogen evolution and CO_2 fixation. The direct electrical communication of bipyridinium-modified GR with molecular hydrogen represents a new approach to circumvent the need for NAD(P)H cofactors regeneration and provides a methodology for developing new catalysts for biotechnological transformations. In this context, it is important to note that the bipyridinium-modified enzyme, PAV^{+}-GR, does not recognize its natural cofactor, NADPH. Thus, chemical modification of GR yields a new semisynthetic enzyme. It attains the active site structure for the reduction of GSSG but is structurally perturbed to the extent that the native electron carrier is not recognized. The development of other electron relay units and their coupling to various redox proteins is certainly a challenge for developing novel biosensors, photosynthetic systems, and biocatalyzed transformations.

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Supplementary Material Available: Detailed synthetic procedure for the preparation of **2b–2d** and **5**, plots of peak separations as a function of scan rates for **2b** and **2d**–GR monolayer electrodes, and absorption spectra of PL-$C_nV^{\bullet+}$ at 42 °C and 25 °C (4 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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