Acknowledgment. This research is supported by the Bundesministerium für Forschung und Technologie (BMFT), Germany, and The Israeli Council for Research and Development, Ministry of Science and Technology, Israel.

Mediated Electron Transfer in Glutathione Reductase Organized in Self-Assembled Monolayers on Au Electrodes

Itamar Willner,* Eugenii Katz,† Azalia Riklin, and Ron Kasher

Institute of Chemistry
The Hebrew University of Jerusalem
Jerusalem 91904, Israel

Received August 20, 1992

Electrical communication of enzyme redox sites and electrodes is the basis for numerous amperometric biosensor devices.[1,2] Immobilization of redox enzymes in functionalized redox polymers[3] or chemical modification of proteins with electron-transfer mediators[4] provides general means to establish electrical interactions between insulated enzyme redox centers and electrodes. Most of the presently developed, electrically communicated protein assemblies were utilized in an oxidative pathway. We have recently shown that immobilization of enzymes in bipyridinium-functionalized polymers[3d,5] or functionalization of proteins by bipyridinium components[6] establishes electrically wired biocatalytic assemblies in reductive routes. Here we wish to report on the novel development of electrically communicated enzyme electrodes by organization of monolayer redox-functionalized enzymes on Au electrodes. We describe the stepwise construction of these electrodes and reveal that the spatial structure of the redox functionalization controls the effectiveness of the electron-transfer-mediated process.

The enzyme glutathione reductase (EC 1.6.4.2) has been covalently attached[7,8] to a cysteic acid active ester monolayer that is chemisorbed to an Au electrode, according to Scheme I. The resulting electrode-immobilized protein has been treated by *N*-methyl-*N'*-(carboxyalkyl)-4,4'-bipyridinium (**1**) in the presence of urea[9] to yield an electron relay modified enzyme exhibiting electrical communication with the electrode.

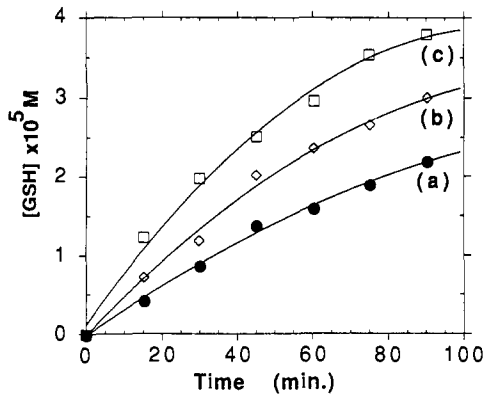
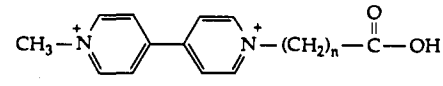


Figure 1. Rate of reduced glutathione, GSH, formation using the monolayer enzyme electrodes with different bipyridinium relays. The different curves correspond to bipyridinium units anchored by various spacers to the enzyme: (a) **1a**; (b) **1b**; (c) **1c**. All experiments were performed in a phosphate buffer, pH 7.2, and the applied potential on the working modified electrode was $E^0 = -0.72$ vs SCE; [GSSG] = 1×10^{-2} M.



(1)

(a) n=2
(b) n=5
(c) n=11

The density of active ester spacer groups associated with the Au electrode monolayer formed after step i (Scheme I) was determined to be 8×10^{-11} mol cm^{-2} by derivatization of the modified electrode with aminonaphthoquinone.[10] The density of enzyme molecules associated with the electrode was determined to be 2×10^{-11} mol cm^{-2} by immobilization of radioactive labeled glutathione reductase[11] to the electrode. The activities of bipyridinium-modified glutathione reductase ((**1a-c**)-gluthathione reductase) and of nonmodified enzyme in similar monolayer configurations on Au electrodes are identical, as revealed by the similar performances of all electrodes in the presence of solubilized methyl viologen radical as reductant. Glutathione reductase modified by **1b** or **1c** exhibits reversible cyclic voltammograms[12] ($E^0 = -0.58$ V vs SCE; $\Delta E_p = 30$ mV at $\nu < 1$ V s^{-1}). By assuming that all bipyridinium units anchored to the protein backbone are reduced in the voltammetric cycle, the charge passed in the reduction wave allows us to determine the average loading degree of the protein backbone by the redox component to be ca. 8. Namely, out of the 39 lysine residues present in the glutathione reductase backbone,[13] 8 are modified by the bipyridinium redox groups. The rate constants[14] for electron transfer from the electrode to **1b** or **1c** units anchored to the protein are similar, $k_{et} = 130$ s^{-1}.

The bipyridinium-modified enzyme monolayer electrodes were examined as biocatalytic redox assemblies for electroreduction of oxidized glutathione, GSSG (Figure 1). The active site of glutathione reductase consists of a disulfide bond formed by two cysteine residues. Its reduction potential corresponded to E^0 =

* Author to whom correspondence should be addressed.

† Postdoctoral fellow from the Institute of Soil Science and Photosynthesis, USSR Academy of Science, Pushchino, Moscow Region, Russia.

(1) Heller, A. *Acc. Chem. Res.* **1990**, *23*, 128-134.

(2) Wilson, G. In *Biosensors: Fundamentals and Applications*; Turner, A., Karube, I., Wilson, G., Eds.; Oxford University Press: New York, 1987.

(3) (a) Degani, Y.; Heller, A. *J. Am. Chem. Soc.* **1989**, *111*, 2357-2358. (b) Gorton, L.; Karan, H.; Hale, P.; Inagaki, T.; Okamoto, Y.; Skotheim, T. *Anal. Chem. Acta* **1990**, *228*, 23-30. (c) Foulds, N.; Lowe, C. *Anal. Chem.* **1988**, *60*, 2473-2478. (d) Willner, I.; Katz, E.; Lapidot, N.; Bäuerle, P. *Bioelectrochem. Bioenerg.*, in press.

(4) Degani, Y.; Heller, A. *J. Am. Chem. Soc.* **1988**, *110*, 2615-2620.

(5) Willner, I.; Riklin, A.; Lapidot, N. *J. Am. Chem. Soc.* **1990**, *112*, 6438-6439.

(6) Willner, I.; Lapidot, N. *J. Am. Chem. Soc.* **1991**, *113*, 3625-3626.

(7) For extensive studies on chemisorption of thiol and disulfide compounds see: (a) Nuzzo, R. G.; Dubois, L. H.; Allara, D. L. *J. Am. Chem. Soc.* **1990**, *112*, 558-569. (b) Chidsey, C. E. D.; Bertozzi, C. R.; Putvinski, T. M.; Mujsce, A. M. *J. Am. Chem. Soc.* **1990**, *112*, 4301-4306. (c) De Long, H. C.; Buttry, D. A. *Langmuir* **1990**, *6*, 1319-1322. (d) Bain, C. D.; Whitesides, G. M. *Angew. Chem.* **1989**, *101*, 522-528. (e) Steinberg, S.; Tor, Y.; Sabatani, E.; Rubinstein, I. *J. Am. Chem. Soc.* **1991**, *113*, 5176-5182. (f) Collard, D. M.; Fox, M. A. *Langmuir* **1991**, *7*, 1192-1197. (g) Rowe, G. K.; Creager, S. E. *Langmuir* **1991**, *7*, 2307-2312. (h) Uosaki, K.; Sato, Y.; Kita, H. *Langmuir* **1991**, *7*, 1510-1514. (i) Lee, K. A. B. *Langmuir* **1990**, *6*, 709-712. (j) Bain, C. D.; Biebuyck, H. A.; Whitesides, G. M. *Langmuir* **1989**, *5*, 723-727. (k) Nuzzo, R. G.; Fusco, F. A.; Allara, D. L. *J. Am. Chem. Soc.* **1987**, *109*, 2358-2368. (l) Katz, E. Yu.; Solov'ev, A. A. *J. Electroanal. Chem.* **1990**, *291*, 171-186.

(8) Experimental details on the organization of the enzyme electrode according to Scheme I are provided as supplementary material.

(9) (a) Colman, R.; Black, S. *J. Biol. Chem.* **1965**, *240*, 1796-1803. (b) Degani, Y.; Heller, A. *J. Phys. Chem.* **1987**, *91*, 1285.

(10) Katz, E. Yu. *J. Electroanal. Chem.* **1990**, *291*, 257-260.

(11) [^{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.

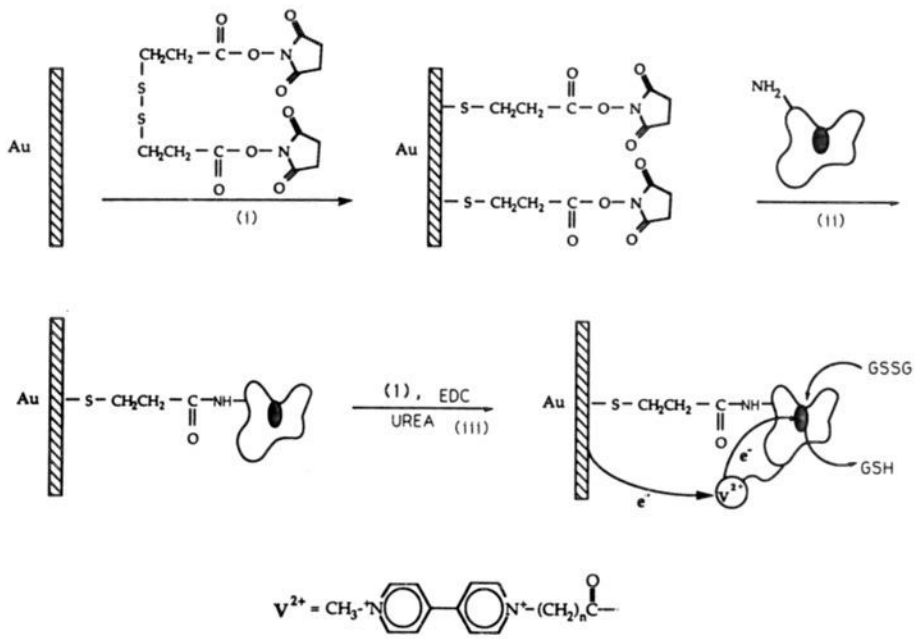
(12) No electrochemical response in the cyclic voltammogram of the **1a**-enzyme-modified electrode is observed. However, **1a** linkage to the enzyme is detectable by the blue coloration of the Au electrode upon application of negative potential. Modification of the enzyme with **1a** is also essential to electrically wire the protein toward the reduction process.

(13) (a) Charles, H.; Williams, J. R. In *The Enzymes*; Boyer, P., Ed.; Academic Press: New York, 1976; Vol. 13. (b) Pai, F.; Schulz, G. *J. Biol. Chem.* **1983**, *258*, 1752-1757.

(14) Laviron, E. *J. Electroanal. Chem.* **1979**, *101*, 19-28.

0002-7863/92/1514-10965$03.00/0

Scheme I. Sequence for Assembling the Monolayer of Glutathione Reductase Exhibiting Electrical Communication



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

n=2, 5, 11

−0.255 V at pH 7.0,[15] and thus the bipyridinium units are thermodynamically capable of mediating electron transfer to the active site.

It can be seen that electroreduction of GSSG proceeds with all bipyridinium-functionalized enzyme assemblies. Nevertheless, the rates of GSH formation depend on the alkyl chain length bridging the bipyridinium component with the protein backbone: As the chain length is increased, the electroreduction of GSSG is improved. Control experiments reveal that in the absence of functionalization of the protein by bipyridinium components no reduction of GSSG occurs. Similarly, when the modification of glutathione reductase by the bipyridinium components is performed without unfolding of the protein with urea, an electrically inactive protein assembly toward reduction of GSSG is formed, although the bipyridinium units are electroactive. These results clearly indicate that the bipyridinium units covalently linked to glutathione reductase mediate electron transfer from the Au electrode to the enzyme redox site. Unfolding of the protein by urea[9] during the redox modification is essential to substitute inner shell lysine residues with bipyridinium components acting as electron-transfer-mediating stations for electron tunneling to the active site. The improved electrical communication upon lengthening the alkyl chain bridges is attributed to enhanced intraprotein electron-transfer rates as a result of electron-donor distance shortening.[16] Namely, the flexible alkyl bridging "arms" generate shorter intraprotein electron-transfer distances, resulting in enhanced electrical communication (Scheme I). The fact that the electron-transfer rate from the electrode to the bipyridinium components is similar for **1b** and **1c** implies that the effectiveness of the electrical communication in the protein assemblies is controlled by the intraprotein electron-transfer process from the reduced anchored relays to the enzyme active site.

We thus conclude that tailored monolayer assemblies composed of redox-modified proteins linked to thiol-derivatized Au electrodes provide novel configurations for mediated electron transfer in enzymes. We find that other functionalized disulfides such as dimethyl 3,3′-dithiobis(propionimidate) can be applied as the monolayer microstructuring component for attachment of the enzyme to Au electrodes.

Further experiments to apply this concept to other redox enzymes and to design polymeric enzyme layers associated with thiolate monolayer structures are underway in our laboratory.

Acknowledgment. This research is supported by the Bundesministerium für Forschung und Technologie, Germany, and the Israeli Council for Research and Development, Ministry of Science and Technology, Israel.

Supplementary Material Available: Experimental procedure for the preparation of Au electrodes comprising self-assembled thiolated monolayers and glutathione reductase modified by bipyridinium tethers as electron-transfer mediators (1 page). Ordering information is given on any current masthead page.

(15) Rakauskienė, G.; Čėnas, N.; Kulys, J. *FEBS Lett.* **1989**, *243*, 33-36.
(16) For other examples involving long-chain-mediated electron transfer in proteins, see: (a) Schmidt, H.-L.; Grenner, G. *Eur. J. Biochem.* **1976**, *67*, 295. (b) Schuhmann, W.; Ohara, T. Y.; Schmidt, H.-L.; Heller, A. *J. Am. Chem. Soc.* **1991**, *113*, 1394.

Effects of Hydration on the Claisen Rearrangement of Allyl Vinyl Ether from Computer Simulations

Daniel L. Severance and William L. Jorgensen*

Department of Chemistry, Yale University
New Haven, Connecticut 06511-8118
Received August 4, 1992

The synthetic importance of the Claisen rearrangement in organic chemistry and biochemistry has stimulated numerous mechanistic studies.[1-6] A fundamental issue is the extent of dipolarity in the transition state (TS).[3-6] Substantial rate increases are observed in protic solvents for substituted cases that enhance putative enolate/allyl cation character; however, solvent effects on Claisen rearrangements in general and especially for the parent allyl vinyl ether (AVE) are often thought to be modest.[1,3,7] The present results challenge this notion and the importance of ion-pair development. Enhanced hydrogen bonding that accompanies more subtle polarization appears as the alternative explanation, and a desirable feature for catalysts is noted.

(1) For a review, see: Ziegler, F. E. *Chem. Rev.* **1988**, *88*, 1423.
(2) Burrows, C. J.; Carpenter, B. K. *J. Am. Chem. Soc.* **1981**, *103*, 6983.
(3) Coates, R. M.; Rogers, B. D.; Hobbs, S. J.; Peck, D. R.; Curran, D. P. *J. Am. Chem. Soc.* **1987**, *109*, 1160.
(4) Gajewski, J. J.; Jurayj, J.; Kimbrough, D. R.; Gande, M. E.; Ganem, B.; Carpenter, B. K. *J. Am. Chem. Soc.* **1987**, *109*, 1170.
(5) Brandes, E.; Grieco, P. A.; Gajewski, J. J. *J. Org. Chem.* **1989**, *54*, 515.
(6) White, W. N.; Wolfarth, E. F. *J. Org. Chem.* **1970**, *35*, 3585.
(7) Dewar, M. J. S.; Jie, C. *J. Am. Chem. Soc.* **1989**, *111*, 511.

0002-7863/92/1514-10966$03.00/0