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Direct Electrical Communication between Chemically Modified Enzymes and Metal Electrodes. 2. Methods for Bonding Electron-Transfer Relays to Glucose Oxidase and D-Amino-Acid Oxidase

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Abstract: Flavoenzymes such as glucose oxidase and D-amino-acid oxidase do not directly transfer electrons to conventional electrodes, because the distance between their redox centers and the electrodes' surface exceeds, even on closest approach, the distance across which electrons are transferred at sufficient rates. After chemical bonding of an average of one electron-transfer relay to each 12 000–75 000 Da of enzyme, the substrate-reduced enzymes are, however, directly oxidized at gold, platinum, or carbon electrodes. Fast redox couples, having redox potentials 0.07–0.55 V positive of the redox potentials of the enzyme-bound $FAD/FADH_2$ ($E° = -0.05$ V vs SHE), are effective relays when covalently or coordinatively bound to the enzyme's proteins. Examples of relays include (a) *amides* formed between (I) ferrocylacetic acid and enzyme amines ($E° = 0.35$ V vs SHE), (II) ferrocenecarboxylic acid and enzyme amines ($E° = 0.5$ V vs SHE), and (III) ruthenium pentaammine complexes of isonicotinic acid and enzyme amines ($E° = 0.35$ V vs SHE); (b) *azo compounds* made by reacting the diazonium salt of 4-aminopyridine with an enzyme, followed by complexing of the enzyme-bound pyridine functions with ruthenium pentaammine ($E° = 0.4$ V vs SHE); and (c) ruthenium pentaammine, bound coordinatively to the protein ($E° = 0.02$ V vs SHE). After most chemical modifications, the enzymes retain 25–75% of their normal catalytic activity of accelerating the reaction of glucose and oxygen, to form gluconolactone and hydrogen peroxide, or of D-alanine and oxygen, to form ammonium pyruvate and hydrogen peroxide. One modification [example (b) above] increases the activity of glucose oxidase by a factor of 3.

Electrical communication between enzymes and metal electrodes is relevant to the electrochemical assay of biochemicals, where a substrate is selectively oxidized in an enzyme-catalyzed process. In such a process, the enzyme is first reduced by the substrate and then is reoxidized either directly at an electrode[1] or indirectly by a diffusing redox mediator.[2-12] Electrochemical or chemical reoxidations of mediators serve, respectively, in amperometric[2-12] or colorimetric[13,14] assays of substrates such as glucose or cholesterol.

In a preceding paper[1] we showed that although glucose-reduced natural glucose oxidase [E.C. 1.1.3.4] cannot be directly reoxidized at gold, platinum, or carbon electrodes, one can chemically modify the enzyme, through attaching electron relays, so that its direct electrochemical reoxidation becomes possible (Figure 1). The modification that we described involved the covalent bonding of 12 ferrocene units to the protein part of the enzyme, through amide links between ferrocenecarboxylic acid and "free" enzyme amines (such as those of lysine). In electrodes made with chemically modified glucose oxidase, the faradaic currents associated with the direct electrochemical reoxidation of the glucose-reduced modified enzyme increase with glucose concentration.

In this paper we discuss the chemical modification and properties of glucose oxidase and D-amino-acid oxidase. We show that direct electrical communication between the $FAD/FADH_2$ centers of the enzymes and metal or carbon electrodes can be established through bonding any of five different electron-transer relays. The relays can be bound to lysine, tyrosine, or histidine sites of the protein.

Experimental Section

Chemicals. Glucose oxidase [E.C. 1.1.3.4.] type X and D-amino-acid oxidase [E.C. 1.4.3.3.] type I were purchased from Sigma; Na-HEPES (sodium 4-(2-hydroxyethyl)-1-piperazinethanesulfonate), DEC [1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride], ferrocenecarboxylic acid, isonicotinoyl chloride hydrochloride, and 4-aminopyridine from Aldrich; and Sephadex G-15 and Sephadex C-25 from Pharmacia Fine Chemicals. $[Ru(NH_3)_5Cl]Cl_2$ was prepared from $[Ru(NH_3)_6]Cl_3$ (Johnson Matthey).[15] Ferrocylacetic acid was made from [(*N,N*-dimethylaminomethyl]ferrocene (Aldrich) by a reported procedure.[16]

Preparation of Modified Enzymes. Amides between Ferrocylacetic Acid and Glucose Oxidase Amines. The procedure followed is outlined in Scheme I. Ferrocylacetic acid (60 mg), Na-HEPES (200 mg), and urea (480 mg) were dissolved in water (4 mL). The solution was adjusted to pH 7.0 by adding 1.0 M HCl dropwise. Glucose oxidase (100 mg) was then added and dissolved, followed by DEC (50 mg). The solution was readjusted to pH 7.0 and left to react overnight (16 h) in an ice bath. The chemically modified enzyme was separated from the reaction mixture by gel filtration chromatography on Sephadex G-15.

Amides between Ferrocenecarboxylic Acid and Glucose Oxidase Amines. These were formed by the procedure reported in our earlier paper.[1]

Amides between Ferrocenecarboxylic Acid and D-Amino-Acid Oxidase Amines. The procedure used to form the ferrocenecarboxamides of glucose oxidase[1] was followed.

Amides between the Ruthenium Pentaammine Complex of Isonicotinic Acid and Glucose Oxidase Amines. The modified enzyme was formed by the reaction sequence outlined in Scheme II. Glucose oxidase (100 mg), Na-HEPES (500 mg), and urea (240 mg) were dissolved in 2 mL of water. Solid isonicotinoyl chloride hydrochloride (50 mg) was added to the stirred solution. The resulting solution was kept at room temperature for 1 h. The isonicotinoyl-modified glucose oxidase was separated from the reaction mixture by gel filtration chromatography (Sephadex G-15). $[Ru(NH_3)_5Cl]Cl_2$ (20 mg) was added to the yellow enzyme containing fraction, followed by glucose (20 mg). The solution was stirred under

(1) Degani, Y.; Heller, A. *J. Phys. Chem.* **1987**, *91*, 1285.
(2) Cass, A. E. G.; David, G.; Francis, G. D.; Hill, H. A. O.; Aston, W. J.; Higgins, J. I.; Plotkin, E. V.; Scott, L. D. L.; Turner, A. P. F. *Anal. Chem.* **1984**, *56*, 667.
(3) Crumbliss, A. L.; Hill, H. A.; Page, D. J. *J. Electroanal. Chem. Interfacial Electrochem.* **1986**, *206*, 327.
(4) Kulys, J. T.; Cenas, N. K. *Biochim. Biophys. Acta* **1983**, *744*, 57.
(5) Albery, W. J.; Bartlett, D. N. *J. Electroanal. Chem. Interfacial Electrochem.* **1985**, *194*, 211.
(6) Albery, W. J.; Bartlett, D. N.; Craston, D. H. *J. Electroanal. Chem. Interfacial Electrochem.* **1985**, *194*, 223.
(7) Gough, D. A.; Lucisano, J. Y.; Tse, P. H. S. *Anal. Chem.* **1985**, *57*, 2351.
(8) Narashimhan, K.; Wingard, L. B., Jr. *Anal. Chem.* **1986**, *58*, 2984.
(9) Claremont, D. J.; Penton, C.; Pickup, J. C. *J. Biomed. Eng.* **1986**, *8*, 272.
(10) Turner, A. P. F. *World Biotech. Rep.* **1985**, *1*, 181.
(11) Gorton, L. O.; Scheller, F.; Johansson, G. *Stud. Biophys.* **1985**, *109*, 199.
(12) Chua, K. S.; Tan, I. K. *Clin. Chem. (Winston-Salem, NC)* 1978, *24*, 150.
(13) Shirey, T. L. *Clin. Biochem. (Ottawa)* **1983**, *16*, 147.
(14) Curme, H. G.; et al. *Clin. Chem. (Winston-Salem, NC)* **1978**, *24*, 1335.
(15) Vogt, L. H.; Katz, J. L.; Wiberley, S. E. *Inorg. Chem.* **1965**, *4*, 1157.
(16) Lednicer, D.; Lindsey, J. K.; Hauser, C. R. *J. Org. Chem.* **1958**, *23*, 653.

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Scheme I

Scheme II

Scheme III

Ar until the $[Ru(NH_3)_5Cl]Cl_2$ dissolved, and then an amount of urea sufficient to yield a 2 M solution was added. The resulting solution was allowed to react at room temperature for 4 h. As the complex formed, the color of the solution changed from yellow to deep red (λ_{max} 490 nm), a color typical of the $[Ru(NH_3)_5(\text{isonicotinamide})]^{2+}$ ion.[17] The $(NH_3)_5RuNC_5H_4CONH$-protein-modified glucose oxidase was then separated from the reaction mixture on a 15-cm length, 2-cm diameter column of Sephadex C-25 cation-exchange resin, equilibrated with 0.1 M phosphate buffer (pH 7.0). This buffer was used also as eluent.

Pyridyl Azo Binding of $Ru(NH_3)_5$ to Glucose Oxidase. The reaction sequence through which multiple pyridyl azo groups, complexed with $Ru(NH_3)_5$, were bound to the enzyme is shown in Scheme III. Glucose oxidase (100 mg) was dissolved in 2 mL of carbonate buffer (0.5 M, pH 10.5) and the resultant mixture chilled in an ice bath. A 94-mg aliquot of 4-aminopyridine was dissolved simultaneously in 1 mL of HCl (4 M) and also chilled in an ice bath. A 100-μL aliquot of 10 M sodium nitrite was then added to the 4-aminopyridine solution. The solution turned orange-yellow promptly, and gas evolution was observed. A total of 100 mL of this solution was added dropwise to the vigorously stirred glucose oxidase solution, which immediately turned red. The modified enzyme was separated from the reaction mixture by gel filtration chromatography (Sephadex G-15) and was subsequently coupled to $[Ru(NH_3)_5(H_2O)]^{2+}$, following the procedure described for the isonicotinoyl-modified enzyme. The blue-green enzyme solution shows absorption maxima at 460 and 610 nm.

Complexes of Ruthenium Pentaammine and Glucose Oxidase. The reaction sequence for this enzyme modification is based on reported procedures[18] for binding of ruthenium pentaammine to ferricytochrome *c* and is outlined in Scheme IV. Freshly prepared amalgamated Zn (1 g) was added to a vigorously stirred suspension of $[Ru(NH_3)_5Cl]Cl_2$ (200 mg) in 5 mL of phosphate buffer (0.1 M, pH 7.0). The suspension was stirred under Ar for 15 min and then filtered (0.2-μm filter) to yield a deep yellow solution of $[Ru(NH_3)_5(H_2O)]^{2+}$. Glucose oxidase (100 mg) and urea (480 mg) were dissolved in the $[Ru(NH_3)_5(H_2O)]^{2+}$ solution, and the resulting solution was kept under Ar in an ice bath for 48 h. Subsequently, O_2 was bubbled through the solution for 1 h. The ruthenium pentaammine complex modified glucose oxidase was then separated from the reaction mixture by cation-exchange chromatography (Sephadex C-25).

Scheme IV

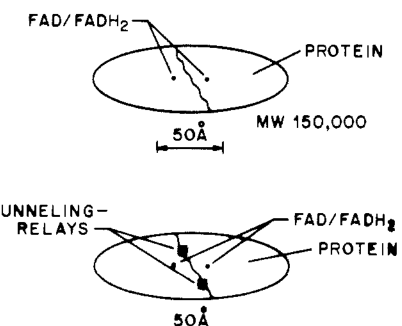


Figure 1. Schematic diagram showing a section along the long axis of glucose oxidase before (top) and after (bottom) chemical bonding of electron-transfer relays to the protein.

Assay of the Iron and Ruthenium Contents of the Chemically Modified Enzymes. To determine the average number of electron-transfer relays bound to each enzyme molecule, the iron or ruthenium content of the enzyme-containing solutions was assayed by atomic absorption spectroscopy. Determination of the number of relays per enzyme molecule was made possible by quantitative recovery of the enzymes in the gel filtration chromatography or cation-exchange steps, where they were quantitatively separated from low molecular weight complexes of the metals. Quantitative enzyme recovery was established by measuring the enzyme activity prior to and after gel filtration or cation exchange. Quantitative separation from low molecular weight mediators was established by passing solutions containing both the unmodified enzyme and diffusing mediators through the columns. Although glucose concentration dependent faradaic currents were observed prior to passage, none were observed following passage through the columns.

Electrochemical Measurements. These were performed with the earlier described cells, electrodes, apparatus, and procedures.[1] In this work, the cyclic voltammograms of the native and modified enzymes were obtained at 25 units/mL (25 mg/mL) for both native and modified D-amino-acid oxidase and at 1200 units/mL (10 mg/mL) for both native and modified glucose oxidase. The solutions of D-amino-acid oxidase were buffered with 0.1 M HEPES (pH 8.2) and those of glucose oxidase, with 0.1 M phosphate (pH 7.0). All solutions contained 3000 units/mL

(17) Ford, P.; Rudd, D. F. P.; Gaunder, R.; Taube, H. *J. Am. Chem. Soc.* **1968**, *90*, 1187.

(18) Yocom, K. M.; Shelton, J. B.; Shelton, J. R.; Schroeder, W. A.; Worosila, G.; Isied, S. S.; Bordignon, E.; Gray, H. B. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79*, 7052.

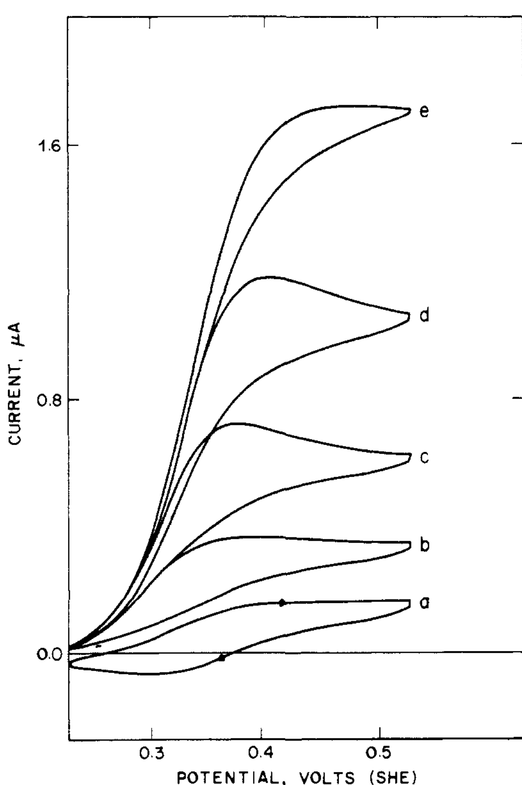


Figure 2. Cyclic voltammograms obtained with glucose oxidase modified with 13 ± 1 ferrocylacetamide functions at different glucose concentrations; 3-mm-diameter glassy carbon electrodes; scan rate, 2 mV/s. Glucose (mM): a, 0; b, 0.8; c, 2; d, 4; e, 8.

catalase to decompose any hydrogen peroxide that might be formed in the presence of traces of oxygen.

Measurement of the Diffusion Coefficients of the Native and Chemically Modified Enzymes. The coefficients were measured by dynamic light scattering as described by Wiltzius, in his apparatus.[19]

Measurement of the Stability of the Chemically Modified Enzymes and of the Enzyme Electrode. The enzymatic and electrochemical activity of the native or modified enzymes as a function of time was determined by incubating these in 30 mM glucose. Periodically retrieved samples were subjected to gel filtration chromatography, in order to eliminate all low molecular weight decomposition products. Following such separation, the enzymes were assayed for their iron or ruthenium content. The enzymatic activity of the samples was determined by measuring the time required to bleach a blue (oxidized) indophenol solution. The rate of decrease of the electrochemical activity, i.e., the drop in the faradaic current, at 30 mM glucose concentration, was then measured by cyclic voltammetry.

Results

Glucose oxidase and D-amino-acid oxidase can be chemically modified in both the presence or the absence of 2 M urea. Because at a 2 M concentration urea reversibly opens the structure of proteins, modification in the presence of 2 M urea resulted in both better reproducibility of properties and superior electrochemical characteristics. At a higher (6 M) concentration urea alters the enzymes drastically and often irreversibly.[20,21]

The modified enzymes, irrespective of the chemical changes in their structure, continue to catalyze their normal reactions (reactions 1 and 2). The changes in enzymatic activity upon chemical modification are summarized in Table I.

$$\text{glucose} + O_2 \xrightarrow{\text{glucose oxidase}} \text{gluconolactone} + H_2O_2 \quad (1)$$

$$\text{D-alanine} + O_2 + H_2O \xrightarrow{\text{D-amino-acid oxidase}} \text{ammonium pyruvate} + H_2O_2 \quad (2)$$

Table I. Activity of the Chemically Modified Enzymes

RELAY/ENZYME	NUMBER OF RELAYS PER ENZYME MOLECULE	ACTIVITY OF MODIFIED ENZYME / ACTIVITY OF NATIVE ENZYME
(ferrocene: C_5H_5–Fe–C_5H_4)–CO–NH–GLUCOSE OXIDASE	12 ± 1	0.60 ± 0.05
(ferrocene: C_5H_5–Fe–C_5H_4)–CH_2CO–NH–GLUCOSE OXIDASE	13 ± 1	0.6 ± 0.1
$(NH_3)_5$Ru N(pyridine)–CO–NH–GLUCOSE OXIDASE	6 ± 1	0.75 ± 0.05
$(NH_3)_5$Ru N(pyridine)–N=N–GLUCOSE OXIDASE	2 ± 0.3	2.7 ± 0.3
$(NH_3)_5$Ru–GLUCOSE OXIDASE	14 ± 1	0.70 ± 0.05
(ferrocene: C_5H_5–Fe–C_5H_4)–CO–NH–D–AMINO–ACID OXIDASE	3 ± 1	0.27 ± 0.02

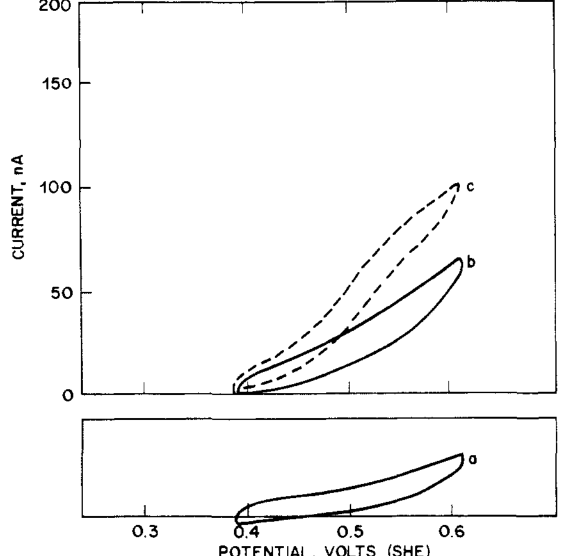


Figure 3. Cyclic voltammograms obtained with native D-amino-acid oxidase without D-alanine (curve a) or with D-alanine (curve a) and with the same enzyme after attachment of 3 ± 1 ferrocenecarboxamide functions, without D-alanine (curve b) and at 5 mM D-alanine (curve c); 1.5-mm-diameter gold disk electodes; scan rate, 1 mV/s.

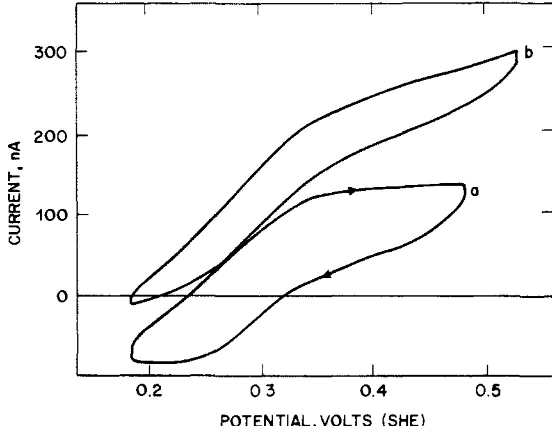


Figure 4. Cyclic voltammograms obtained with glucose oxidase modified with 6.5 ± 0.5 enzyme-bound ruthenium pentaammine isonicotinamide function without glucose (curve a) and at 20 mM glucose (curve b); 3-mm-diameter glassy carbon disk electrodes; scan rate 1 mV/s.

The cyclic voltammograms (Figures 2–6), combined with those published in our earlier paper,[1] show that while the enzymes do not communicate prior to modification with metal or carbon electrodes, they do so after electron-transfer relays are chemically bound to their proteins. Direct electrical communication is established with gold, platinum, glassy carbon, or graphite electrodes aftr binding to the flavoenzymes any of a series of fast redox couples, having redox potentials 0.07–0.55 V positive of the en-

(19) Wiltzius, P. *Phys. Rev. Lett.* **1987**, *58*, 710.
(20) Sugiura, N.; Ohama, H.; Kotaki, A.; Yagi, K. *J. Biochem.* (*Tokyo*) **1973**, *73*, 901.
(21) Bogdanov, V. P.; Morozkin, A. D.; Abalikhina, T. A. *Mol. Biol.* (*Kiev*) **1974**, *n10*, 14.

Table II. Electron-Transfer Relays in Flavoenzymes

Relay	Probable amino-acids bound to the relay	Relays per enzyme molecule	Enzyme daltons per relay	E^0 of enzyme-bound relay, volts (SHE)	$E^0_{relay} - E^0_{enzyme}$ volts at pH 7	Period to 10% loss in current
(C_5H_4)–CO–NH– $Fe^{2+/3+}$ (C_5H_5)	Lysines of glucose oxidase	13 ± 1	11,500 ± 1,000	0.5	0.55	2 ± 0.5 h
(C_5H_4)–CO–NH– $Fe^{2+/3+}$ (C_5H_5)	Lysines of D-amino-acid oxidase	3.5 ± 0.5	11,000 ± 2,000	0.5	0.55	2 ± 0.5 h
(C_5H_4)–CH_2–CO–NH– $Fe^{2+/3+}$ (C_5H_5)	Lysines of glucose oxidase	13 ± 1	11,500 ± 1,000	0.35	0.4	2 ± 0.5 h
$(NH_3)_5Ru^{2+/3+}$ N(pyridyl)–CO–NH–	Lysines of glucose oxidase	6 ± 1	25,000 ± 5,000	0.3	0.35	10 ± 2 h
$(NH_3)_5Ru^{2+/3+}$ N(pyridyl)–N=N–(HO-phenyl)–	Tyrosines or tryptophanes of glucose oxidase	2 ± 0.3	75,000	0.4	0.45	10 ± 2 h
$(NH_3)_5Ru^{2+/3+}$ N(imidazole)N–H	Histidines of glucose oxidase	14 ± 1	11,000 ± 1,000	0.02	0.07	os Ru^{3+} > 1 week os Ru^{2+} < 5 min

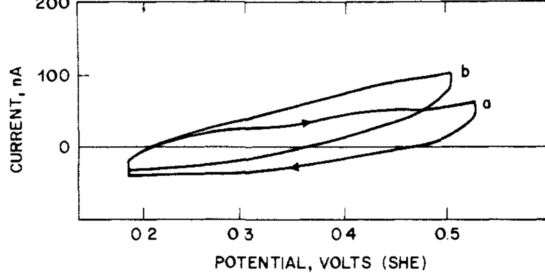


Figure 5. Cyclic voltammograms obtained with glucose oxidase modified with ~2 enzyme-bound ruthenium pentaammine pyridyl azo functions without glucose (curve a) and at 20 mM glucose (curve b); 3-mm-diameter glassy carbon disk electrodes; scan rate, 1 mV/s.

zymes' redox potential (−0.05 V vs SHE at pH 7).[22] Relays attached through amide links to protein amines (usually lysines) through azo bonds to the protein' activated aromatic rings (such as those of tyrosine or tryptophan) or through coordination to the proteins' heterocyclic rings (imidazole rings of histidine) are all effective. Any one of these is sufficient to alter the electrochemical characteristics of the enzyme electrodes that are, prior to enzyme modification, insensitive to change in the concentration, or even to the very presence of substrate. The relay-modified enzyme electrodes sense the presence of their enzyme's substrate and show faradaic currents that increase, across a defined concentration range, with substrate concentration. One thus observes, at potentials equaling or exceeding the redox potentials of the electron relays, glucose concentration dependent currents in the case of modified glucose oxidase and response to D-alanine in the case of modified D-amino-acid oxidase. Fast redox couples, with potentials reducing with respect to those of glucose oxidase or D-amino-acid oxidase, such as cresyl violet (−0.12 V vs SHE) or methyl viologen (−0.42 V vs SHE), do not mediate electron transfer from either enzyme to electrodes even when they are unbound, i.e., free to diffuse. The limiting currents, i.e., the currents observed at sufficiently oxidizing potentials and at high substrate concentrations, increase, in general, with the number of relays per enzyme molecule (Figures 2–6 and Table I, column 2).

The number of relays per enzyme molecule, the number of enzyme daltons per relay, the observed redox potentials of the enzyme-bound relays, the potential difference between the flavoenzymes $FAD/FADH_2$ centers and those of the bound relays, and the period to 10% loss in substrate-dependent current 100 mV positive of the bound relay's reversible redox potential are summarized in Table II. The glucose concentration dependence of the current of a relay-modified glucose oxidase solution at 0.43 V (vs SHE), derived from the data in Figure 2, is shown in Figure 7. The enzyme used in this measurement was modified by forming amides between 13 ± 1 of its free amines and ferrocenylacetic acid.

The limiting currents obtained for the same enzyme batch, modified through the same experimental procedures, were found

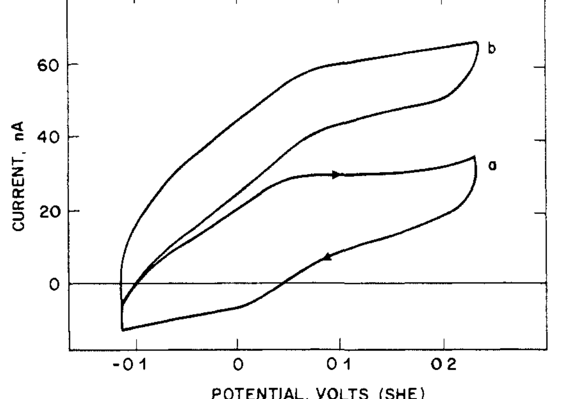


Figure 6. Cyclic voltammograms obtained with glucose oxidase modified with 14 ± 1 enzyme-bound ruthenium pentaammine functions without glucose (curve a) and at 20 mM glucose (curve b); 3-mm-diameter glassy carbon disk electrodes; scan rate, 1 mV/s.

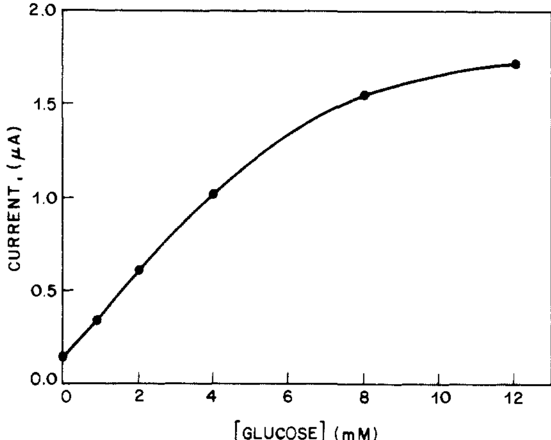
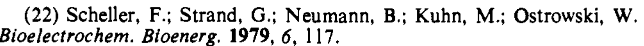


Figure 7. Glucose concentration dependence of the current at 0.5 V (vs SHE) for the ferrocylacetamide-modified electrode.



(22) Scheller, F.; Strand, G.; Neumann, B.; Kuhn, M.; Ostrowski, W. *Bioelectrochem. Bioenerg.* **1979**, *6*, 117.

Table III. Diffusion Coefficients of Modified Glucose Oxidase

MODIFICATION	$D \times 10^7$, $cm^2\ sec^{-1}$	COMMENTS
NONE	4.1 ± 0.4	NATIVE ENZYME
DEC	3.2 ± 0.3	TREATMENT OF THE ENZYME WITH THE COUPLING AGENT NO RELAYS
(ferrocenyl)–CO–NH–PROTEIN	2.9 ± 0.3	13 ± 1 RELAYS PER ENZYME MOLECULE
(ferrocenyl)–CH_2–CO–NH–PROTEIN	2.9 ± 0.3	13 ± 1 RELAYS PER ENZYME MOLECULE
$(NH_3)_5$Ru N(pyridyl)–C(=O)–NH–PROTEIN	4.9 ± 0.5	6 ± 1 RELAYS PER ENZYME MOLECULE
$(NH_3)_5$Ru N(pyridyl)–N=N–PROTEIN	4.3 ± 0.4	2 RELAYS PER ENZYME MOLECULE

to be reproducible within ±5%. The maximum variation for different batches of enzymes from the same supplier (Sigma) was ±10%. The enzyme with 13 ± 1 ferrocylacetamide relays was found to retain its high specificity for glucose. The addition of L-arabinose, lactose, or sucrose (to 30 mM concentration) did not produce a measurable current increment at 0.4 V (vs SHE). A measurable increment was observed for 30 mM D-sorbitol, but was quite small, only $^1/_{40}$th of the increment produced by the addition of glucose (30 mM).

The relay-modified enzyme electrodes vary in their chemical and electrochemical stability. In the group listed in Table II, the enzyme with 14 of its histidines bound to ruthenium pentaammine was both the most and the least stable. When the enzyme-bound ruthenium is predominantly in its trivalent state, the modified enzyme shows stable electrochemistry for over 1 week. If, however, the ruthenium is reduced to its divalent state, for example by adding glucose to the solution, some of the enzyme-bound ruthenium pentaammine complex dissociates and the glucose concentration dependent current drops rapidly: The modified enzyme solution loses 10% of its current in less than 5 min. Furthermore, assay of the number of bound ruthenium atoms after incubation of the modified enzyme (at 25 °C and at 30 mM glucose concentration) for 20 min shows a drop from 14 ruthenium atoms/enzyme molecule to 7 ruthenium atoms/enzyme molecule.

Table III summarizes the changes observed in the diffusion coefficients of the enzymes upon chemical modification.

Discussion

The results indicate the following: First, direct electrical communication can be established between redox enzymes (such as glucose oxidase or D-amino-acid oxidase) and metal electrodes, by chemically binding to the enzyme proteins' electron-transfer relays (Figures 2–6, Table II). Second, amperometric glucose and D-amino acid sensors can be made with the chemically modified enzymes and conventional metal or carbon electrodes. The electron-transfer sequence, on which these sensors are based, involves (a) selective reduction of the FAD centers of the enzyme by the substrate to $FADH_2$, (b) oxidation of the $FADH_2$ centers by the protein-bound electron-transfer relays, which are reduced, and (c) electron transfer from the reduced relays to the electrode, i.e., electrochemical reoxidation of the relays. In a sensor, the current associated with the latter process is measured. Over a range of glucose concentrations, there is a linear relationship between the concentration of the substrate and the current (figure 7). Third, the relays must have redox potentials that are oxidizing relative to the potential of the $FAD/FADH_2$ centers (Table II). Otherwise, step (b) cannot take place. Fourth, the most effective relays are fast redox couples. Fifth, electrodes made with enzymes having a greater density of the relays show greater current increment when the substrate concentration is increased; (Table II, Figures 2–6). Sixth, in relay-modified enzymes the structural and chemical changes affect the activity only slightly. The residual enzymatic activity after attachment of relays exceeds in glucose oxidase 60% of the activity of the native, unmodified enzyme (Table I). Upon attaching an average of two pyridyl azo groups complexed with ruthenium pentaammine, the enzymatic activity is actually tripled. Seventh, none of the enzyme modifications break the "dimeric" glucose oxidase, with four protein chains and two $FAD/FADH_2$ centers, into its "monomeric" halves. On the contrary, the carbodiimide-involving modifications cause some polymerization of the dimeric enzyme, as seen from the decrease in diffusion coefficients upon reaction with DEC. Bonding of relays by methods not involving carbodiimides does not substantially change the diffusion coefficients, i.e., the molecular weights, of the enzymes (Table III).

Our observations lead us to propose that in glucose oxidase and in D-amino-acid oxidase part of the protein or glycoprotein structure is not essential for enzymatic activity. Indeed, it had been reported that periodate oxidation of the polysaccharide of glucose oxidase decreases the thermal stabiity of the enzyme but does not reduce its activity.[23] It was also suggested that nature designed special molecules (such as cytochrome *c*) for specific and selective electron exchange with enzymes (such as cytochrome oxidase).[24] We now propose that, beyond providing structural stability, part of the protein or glycoprotein has the simple function of electrically insulating the $FAD/FADH_2$ centers. Such insulation is essential for the survival of any living system: Were different redox enzymes able to transfer electrons to each other in an uncontrolled way, the electrons would cascade thermodynamically downhill. Because of the cascade, enzymes could not be maintained at their proper oxidation potential (i.e., proper oxidation state) to fulfill their role. As a result, the rich inventory of biochemicals in nature would be reduced and synthetic routes, as well as metabolic paths, would close. In addition, the cascade of electrons would drastically reduce the amount of chemically stored free energy available to the organism. The biologically essential insulating shells also prevent, however, electrical communication between $FADH_2$ centers of native enzymes and metal electrodes. Incorporation of electron-transfer relays in the shells opens channels for electron transfer to the electrodes. As is evident from the fact that the modified enzymes retain most or all of their normal catalytic activity, the catalytic core of the enzyme is only mildly affected by the chemical changes in the insulating shell.

The structure of glucose oxidase has not yet been determined, and as far as we know, this large (150 000-Da) enzyme has not even been crystallized. The amino acid sequence in the four protein chains of the enzyme is also unknown. For these reasons we could not attempt to determine the spatial distribution of the relays in the enzymes, though we recognize the significant relationship between the spatial distribution and the electrocatalytic activity.

Considering the role of urea in the chemistry of attaching relays to the enzyme proteins, we note that interaction with urea at low concentration is known to open the structures.[20] Such opening exposes amino acid residues that are otherwise difficult to access and explains the superior electrochemical characteristics of enzymes modified in 2 M urea solutions.

Our final comment relates to the mechanisms of electron transfer in the chemically modified enzymes. Work on electron tunneling in proteins[25–41] and on protein dynamics[42–44] shows that

(23) Nakamura, S.; Hayashi, S.; Koga, K. *Biochem. Biophys. Acta* **1986**, *445*, 294.
(24) Kraut, J. *Biochem. Soc. Trans.* **1981**, *9*, 197.
(25) Mayo, S. L.; Ellis, W. R., Jr.; Crutchley, R. J.; Gray, H. B. *Science (Washington, D.C.)* **1986**, *233*, 984.
(26) McLendon, G.; Miller, J. R.; Simolo, K.; Taylor, K.; Grant, A. G.; English, A. M. *ACS Symp. Ser.* **1986**, *n307*, 150.
(27) Miller, J. R. In *Antennas and Reaction Centers of Photosynthetic Bacteria*; Michel-Beyerle, M. E., Ed.; Springer-Verlag: Berlin, 1985; p 234.
(28) Bixon, M.; Jortner, J. *J. Phys. Chem.* **1986**, *90*, 3795.
(29) Isied, S. S. *Prog. Inorg. Chem.* **1984**, *32*, 443.
(30) McLendon, G.; Guarr, T.; McGuire, M.; Simolo, K.; Strauch, S.; Taylor, K. *Coord. Chem. Rev.* **1985**, *64*, 113.
(31) Peterson-Kennedy, S. E.; McGourty, J. L.; Ho, P. S.; Sutoris, C. J.; Liang, N.; Zemel, H.; Blough, N. V.; Margoliash, E.; Hoffman, B. M. *Coord. Chem. Rev.* **1984**, *64*, 125.
(32) Takaka, T.; Takenaka, K.; Kawamura, H.; Beppu, Y. *J. Biochem. (Tokyo)* **1986**, *99*, 833.
(33) Crutchley, R. J.; Ellis, W. R.; Gray, H. B. *J. Am. Chem. Soc.* **1985**, *107*, 5002.
(34) Larsson, S. *J. Chem. Soc., Faraday Trans. 2* **1983**, *79*, 1375.
(35) McGourty, J. L.; Blough, N. V.; Hoffman, B. M. *J. Am. Chem. Soc.* **1983**, *105*, 4470.
(36) Marcus, R. M.; Sutin, N. *Biochim. Biophys. Acta* **1985**, *81*, 265.

electrons can be relayed both by tunneling and by motion in and of protein chains. For distances of >8 Å tunneling rates (currents in our electrodes) decrease exponentially with the distance between electron donors ($FADH_2$ centers) and electron acceptors (oxidized relays) and also between relays and electrodes. The tunneling rates increase when the relays are fast redox couples, i.e., when there is little change in the structure of the relay and its solvent environment upon oxidation or reduction. Furthermore, directional or vectorial tunneling, i.e., flow of current, requires that the potentials of the donor and the acceptor differ. Our design of modified enzymes, involving shortened tunneling distances, fast redox couple relays, and potential gradients, is consistent with that required for effective electron-tunneling systems. At the same time, motion of the protein chains to which the relays are attached, i.e., protein dynamics, enhances electron transfer, through transiently reducing the tunneling distances between the $FADH_2$ centers and the relays and between the relays and the metal or carbon electrodes.

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(37) Beratan, D. N.; Hopfield, J. *J. Am. Chem. Soc.* **1984**, *106*, 1584.
(38) Isied, S. S.; Worosila, G.; Atherton, S. J. *J. Am. Chem. Soc.* **1982**, *104*, 7659.
(39) Hopfield, J. J. *Proc. Natl. Acad. Sci. U.S.A.* **1974**, *71*, 3640.
(40) Jortner, J. *Biochim. Biophys. Acta* **1980**, *594*, 193; *J. Am. Chem. Soc.* **1980**, *102*, 6676.
(41) Kuki, A.; Wolynes, P. G. *Science (Washington, D.C.)* **1987**, *236*, 1647.
(42) Churg, A. K.; Warshel, A. In *Structure and Motion: Membranes, Nucleic Acids and Proteins*; Clementi, E., Corongiu, G., Sarma, M. H., Sarma, R. H., Eds.; Adenine: Guilderland, NY, 1985; pp 361–374.
(43) Huber, R.; Bennett, W. S., Jr. *Biopolymers* **1983**, *22*, 261.
(44) Bennett, W. S.; Huber, R. *CRC Crit. Rev. Biochem.* **1984**, *15*, 291.

Biosynthesis of Isoprenoid Membranes in the Methanogenic Archaebacterium *Methanospirillum hungatei*

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Abstract: The biosynthesis of 2,3-di-*O*-phytanyl-*sn*-glycerol (**1**) and 2,3-di-*O*-biphytanyl-*sn*-diglycerol (**2**), which are primary lipid components of the cell membrane of *Methanospirillum hungatei*, was studied by incorporation of ^{14}C-labeled diether **1** and its monophosphate **1-P**, ^{14}C-labeled tetraether **2** and its monophosphate **2-P**, [1-^3H]phytanol (**3**), [1-^3H]-(*E*)-phytol (**4**), and [1-^3H]-(*E,E,E*)-geranylgeraniol (**5**). No interconversion was found between the di- and tetraethers. Geranylgeraniol (**5**) was readily incorporated into ethers **1** and **2**; phytol (**4**) was incorporated poorly; and phytanol (**3**) was not incorporated. The results suggest that the ether linkages in archaebacterial lipids are established before the double bonds in the geranylgeranyl moiety are reduced and that diether **1** is not the immediate precursor of tetraether **2**.

Archaebacteria have recently been recognized as a distinct taxonomic unit that diverged from eubacteria and eukaryotes during the very early stages of evolution.[1] These unusual organisms inhabit hostile environments characterized by high salt (halophiles), high temperatures (thermophiles), low pH (acidophiles), or lack of oxygen (methanogens). Phenotypically, archaebacteria display distinct traits at the molecular level. Sequence analysis of 5S and 16S ribosomal RNA shows a distinct grouping within the archaebacterial kingdom that is far closer to other archaebacterial rRNAs than to eubacterial or eukaryotic rRNAs.[2–4] In addition, archaebacteria utilize metabolic cofactors not found in other organisms,[5–8] and the molecular architecture of lipids in their cell membranes is unique.[9]

In contrast to the fatty acid ester motif found in eubacteria and eukaryotes, archaebacterial membrane lipids are alkyl ether glycerolipids where the alkyl moieties are branched isoprenoid chains. The most commoly encountered structures are 2,3-di-*O*-[3*R*,7*R*,11*R*]-phytanyl-*sn*-glycerol (**1**)[10] and 2,3-di-*O*-[3*R*,7*R*,11*R*,15*S*,18*S*,22*R*,26*R*,30*R*]-biphytanyl-*sn*-diglycerol (**2**),[11] although several related variants have been reported, especially in the C_{40} units of tetraethers from thermophies.[12] Labeling patterns clearly indicate that the C_{20} and C_{40} isoprenoid chains are derived from acetate via mevalonate by the same general process utilized by eubacteria and eukaryotes.[12] Little is known, however, about the sequence of steps beyond the geranylgeranyl stage of chain construction, especially with respect to the timing of condensation and hydrogenation reactions. We now report incorporation studies with advanced precursors that help clarify the intermediate stages of ether lipid biosynthesis in the strict anaerobe *Methanospirillum hungatei*.

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(1) Woese, C. R.; Magrum, L. J.; Fox, G. E. *J. Mol. Evol.* **1978**, *11*, 245–252.
(2) Fox, G. E.; Luehrsen, K. R.; Woese, C. R. *Zentralbl. Bakteriol., Mikrobiol. Hyg., Abt. 1, Orig. C* **1982**, *3*, 330–345.
(3) Willekens, P.; Huysmans, E.; Vandenberghe, A.; DeWachter, R. *Syst. Appl. Microbiol.* **1986**, *7*, 151–159.
(4) Woese, C. R.; Olsen, G. S. *Syst. Appl. Microbiol.* **1986**, *7*, 161–177.
(5) Keltjens, J. T.; Caerteling, G. C.; Van der Drift, C.; Vogels, G. D. *Syst. Appl. Microbiol.* **1986**, *7*, 370–375.
(6) Ellefson, W. E.; Wolfe, R. S. *J. Biol. Chem.* **1981**, *256*, 4259–4262.
(7) Livingston, D. A.; Pfaltz, A.; Schreiber, J.; Eschenmoser, A.; Ankel-Fuchs, D.; Moll, J.; Jaenchen, R.; Thauer, R. K. *Helv. Chim. Acta.* **1984**, *67*, 334–351.
(8) Walsh, C. T. *Acc. Chem. Res.* **1986**, *19*, 216–221.
(9) Langworthy, T. A.; Pond, J. L. *Syst. Appl. Microbiol.* **1986**, *7*, 253–257.
(10) Kates, M. *Prog. Chem. Fats Other Lipids* **1978**, *15*, 301–342.
(11) Heathcock, C. H.; Finkelstein, B. L.; Aoki, T.; Poulter, C. D. *Science (Washington, D.C.)* **1985**, *229*, 862–864.
(12) DeRosa, M.; Gambacorta, A. *Syst. Appl. Microbiol.* **1986**, *7*, 278–285.

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