Electrochemical Measurement of Second-Order Electron Transfer Rate Constants for the Reaction between Cytochrome b_5 and Cytochrome c[†]

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Received December 18, 1995; Revised Manuscript Received July 30, 1996[⊗]

ABSTRACT: The second-order electron transfer reaction between cytochrome b_5 and cytochrome c has been studied by cyclic voltammetry utilizing a gold electrode modified with β-mercaptopropionate. When cyclic voltammetry is performed on a solution containing a mixture of cytochrome b_5, cytochrome c and polylysine, cytochrome b_5 undergoes reversible electrochemistry at the electrode surface while cytochrome c discriminates against the electrode surface. The selectivity of the modified electrode for negatively charged proteins makes it possible to selectively reduce a protein possessing a net negative charge and a relatively low reduction potential (outer mitochondrial membrane cytochrome b_5, $E^\circ = -102$ mV; microsomal cytochrome b_5, $E^\circ = 3$ mV) in the presence of another protein possessing a net positive charge and a relatively high reduction potential (cytochrome c, $E^\circ = 265$ mV). The electrochemical reduction of ferricytochrome b_5 at the electrode surface is followed by a second-order electron transfer reaction between ferrocytochrome b_5 and ferricytochrome c that yields ferricytochrome b_5 and ferrocytochrome c. This fast homogeneous electron transfer reaction which is preceded by a heterogeneous electron transfer reaction results in a characteristic cyclic voltammogram containing a pre-peak to the reduction current. The second-order rate constant for the homogeneous reaction was obtained by invoking the above reaction scheme for digital simulation of a cyclic voltammogram which was subsequently fitted to the experimental data. Second-order rate constants obtained with this method are 2.9×10^8 and 8.9×10^8 for the homogeneous electron transfer reactions between rat liver outer mitochondrial membrane (OM) ferrocytochrome b_5 and beef liver microsomal ferrocytochrome b_5, with horse heart ferricytochrome c, respectively. These values are in good agreement with second-order rate constants obtained for the same protein systems by flash photolysis [Meyer, T. E., Rivera, M., Walker, F. A., Mauk, M. R., Mauk, A. G., Cusanovich, M. A., & Tollin, G. (1993) *Biochemistry 32,* 622−627].

Cytochrome b_5 is an intermediate carrier in the electron transfer pathway from NADPH to fatty acyl CoA in the microsomal membrane (Spatz & Strittmatter, 1971) where it is anchored by means of a hydrophobic tail. Proteolytic cleavage of cytochrome b_5 yields a fully active, water-soluble, heme-containing domain (Strittmatter & Ozols, 1996). Genes coding for the water-soluble domains of rat hepatic microsomal cytochrome b_5 (Bodman *et al.*, 1986) and bovine microsomal (Funk *et al.*, 1990) cytochromes b_5 have been synthesized and expressed in *Escherichia coli*. A different but closely related cytochrome b_5 is expressed in the erythrocyte (Hultquist *et al.*, 1984), except that this protein lacks the hydrophobic tail used to anchor the cytochrome to the microsomal membrane and is water-soluble. Erythrocyte cytochrome b_5 has been shown to reduce inactive methemoglobin to hemoglobin, thus playing an active role as part of the methemoglobin reductase system (Hultquist *et al.*, 1984). Recently, a gene coding for erythrocyte cytochrome b_5 has been synthesized and expressed in *E. coli* (Lloyd *et al.*, 1994). A third type of cytochrome b_5 was isolated from the outer membranes of rat liver mitochondria by proteolytic cleavage (Fukushima & Sato, 1972) and by detergent solubilization (Nisimoto *et al.*, 1977). The proteolytically cleaved rat liver outer mitochondrial membrane (OM) cytochrome b_5 was purified and proved to be distinct from the microsomal cytochrome b_5 isolated from rat liver (Ito, 1980), and later shown to be 58% homologous to microsomal cytochromes b_5 (Lederer *et al.*, 1983). It is noteworthy that most of the differences between mitochondrial cytochrome b_5 and microsomal beef liver cytochrome b_5 occur in the amino and carboxy termini, and that the polypeptide is highly conserved in the regions corresponding to the heme binding crevice. A gene coding for rat hepatic outer mitochondrial membrane (OM) cytochrome b_5 was synthesized and expressed in *E. coli* (Rivera *et al.*, 1992), and the UV−vis, EPR, and ^{1}H NMR spectra of the overexpressed protein were shown to be almost identical to the spectra reported for its microsomal counterparts. Subsequent studies of the mitochondrial protein showed that although the spectral signatures of microsomal and OM cytochromes b_5 were almost identical, the reduction potential of the mitochondrial cytochrome b_5 is approximately 100 mV more negative than the reduction potential of the microsomal proteins (Rivera *et al.*, 1994). The values of the reduction potential measured by spectroelectrochemical techniques under identical conditions of ionic strength and pH are −102 mV for outer mitochondrial membrane cyto-

[†] The financial support of NIH Grants GM 50503 and AHA 9507904S to M.R. is gratefully acknowledged.

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[⊗] Abstract published in *Advance ACS Abstracts,* September 1, 1996.

S0006-2960(95)02972-2 CCC: $12.00

chrome b_5 (Rivera *et al.*, 1994) and 5.1 mV (Reid *et al.*, 1982) or −1.9 mV (Walker *et al.*, 1988) for trypsin-cleaved bovine liver microsomal cytochrome b_5. All the above values are in reference to the standard hydrogen electrode.

It is important to notice that rat OM cytochrome b_5 has been shown to participate in the rotenone-insensitive transfer of electrons from NADH to cytochrome oxidase via cytochrome *c* (Bernardi & Azzone, 1981) and that the aerobic oxidation of exogenous NADH by mitochondria involves a pathway in which electrons flow from NADH → NADH cytochrome b_5 reductase → OM cytochrome b_5 → cytochrome *c* in the rat outer mitochondrial membrane. Cytochrome *c* in turn shuttles the electron across the intermembrane space in order to reduce cytochrome *c* oxidase in the inner mitochondrial membrane. It is also important to point out that a form of NADH dehydrogenase having the same stereochemical properties with respect to NADH and capable of reducing OM cytochrome b_5 has been found in the outer mitochondrial membrane. Whether this enzyme is identical to microsomal cytochrome b_5 reductase is not yet known (Raw *et al.*, 1960; Sottocasa *et al.*, 1967).

A hypothetical protein−protein electron transfer complex was proposed as a precursor for the reaction between cytochrome b_5 and cytochrome *c* (Salemme, 1976). This model was based on the known three-dimensional structures of both cytochromes, and it was postulated that the stabilization of the complex was provided by electrostatic recognition between the positively charged surface of cytochrome *c* and the negatively charged surface of cytochrome b_5. This model has triggered a large number of studies resulting in several lines of experimental information that corroborate the formation of a protein−protein complex between cytochrome b_5 and cytochrome *c*. Two recent reviews (Mauk *et al.*, 1995; Durham *et al.*, 1995) critically summarize the multidisciplinary approach taken by many investigators in an attempt to elucidate the more elusive subtleties of the cytochrome *c*-cytochrome b_5 complex, which has become a paradigm for the investigation of similar complexes formed by electron transfer proteins.

The rate of electron transfer between ferrocytochrome b_5 and ferricytochrome *c* has been studied using several different techniques, including pulse radiolysis (McLendon & Miller, 1985), stopped-flow (Eltis *et al.*, 1991), and flash photolysis (Qin *et al.*, 1991; Willie *et al.*, 1992; Meyer *et al.*, 1993). Early reports appeared to indicate that intramolecular electron transfer was relatively slow, 1600 s^{-1} (McLendon, 1985; Quin *et al.*, 1991). More recently, Willie *et al.* (1992) used a tris(bipyridyl)ruthenium-cysteine-65 derivative of cytochrome b_5 in a 1:1 complex with cytochrome *c* to show that the intracomplex electron transfer rate is actually 4×10^5 s^{-1}. Meyer *et al.* (1993) carried out experiments with an 8:1 cytochrome b_5: cytochrome *c* mole ratio and reported that the second-order electron transfer from free OM cytochrome b_5 to bound cytochrome *c* in a transient ternary complex was determined to be 6.7×10^8 M^{-1} s^{-1} (μ = 87 mM). The second-order rate constant for electron transfer between microsomal cytochrome b_5 and horse heart cytochrome *c* was also reported to be 3.8×10^8 M^{-1} s^{-1} (μ = 100 mM) by Willie *et al.* (1993). A tris(bipyridyl)ruthenium-cysteine-65 derivative of cytochrome b_5 was used in these studies, which allowed measurement of the second-order rate constants for a binary complex.

Cyclic voltammetry was established as a viable yet previously unexploited way of determining protein−protein electron transfer rate constants by Hill and Walton (1981, 1982), who used the voltammetric theory for homogeneous reactions coupled to a heterogeneous electrochemical reaction derived by Nicholson and Shain (1964). More recently, Barker *et al.* (1989) utilized the electrochemical response which results from selective interactions of proteins with surface-modified electrodes to study the homogeneous electron transfer between cytochrome (II) c and plastocyanin (II). In their studies, Barker *et al.* (1989) used gold electrodes modified with bis(4-pyridyl) bisulfide, or the dipeptide lysylcysteine dimethyl ester, to selectively promote the electrochemistry of cytochrome *c* in a solution containing a mixture of cytochrome *c* and plastocyanin. We describe an experimental approach similar to that reported by Barker *et al.* (1989), in order to carry out the electrochemical measurement of second-order electron transfer rate constants for the reaction between ferrocytochrome b_5 and ferricytochrome *c* using a gold electrode modified with β-mercaptopropionate. In the presence of polylysine, ferricytochrome b_5 is selectively reduced at the functionalized electrode surface and subsequently reoxidized by ferricytochrome *c* in a homogeneous reaction. The coupling of a homogeneous reaction to a heterogeneous electron transfer event has important implications in the shape of the observed cyclic voltammogram, which allow the measurement of second-order rate constants for the homogeneous reaction. The rate constants for the transfer of an electron from ferrocytochrome b_5 to ferricytochrome *c* were obtained by digitally simulating the experimental voltammograms by invoking an electrochemical−chemical (EC) reaction scheme.

EXPERIMENTAL PROCEDURES

Recombinant rat liver outer mitochondrial membrane cytochrome b_5 was expressed in *E. coli* and purified as described previously (Rivera *et al.*, 1992). Bovine erythrocyte cytochrome b_5 was a gift from Professor F. Ann Walker, and it was obtained as described previously (Walker *et al.*, 1988). Horse heart cytochrome *c* was purchased from Sigma and was used without further purification. Polylysine with MW = 3970, determined using viscosity measurements, was purchased from Sigma and was used without further purification. All other reagents were from Aldrich or Sigma and were used as received.

Cyclic voltammetry was carried out with a Bioanalytical Systems 50W computer-controlled potentiostat using a three-electrode configuration. A miniature 1 mm diameter gold disk working electrode, a platinum wire counter-electrode, and a silver/silver chloride miniature reference electrode with an internal filling solution consisting of 3 M KCl saturated with silver chloride and equipped with a fiber junction were purchased from Cypress Systems. All electrodes were placed in a single-compartment glass cell of approximately 700 μL. Before each experiment, the working electrode was successively polished using 15 μm, 6 μm, and 1 μm diamond polishing slurries on nylon followed by polishing with 0.05 μm silica polishing slurry on cotton wool, then thoroughly washed with deionized water, and sonicated for 3 min in deionized water. Surface modification of the electrode was achieved by dipping the polished gold working electrode into

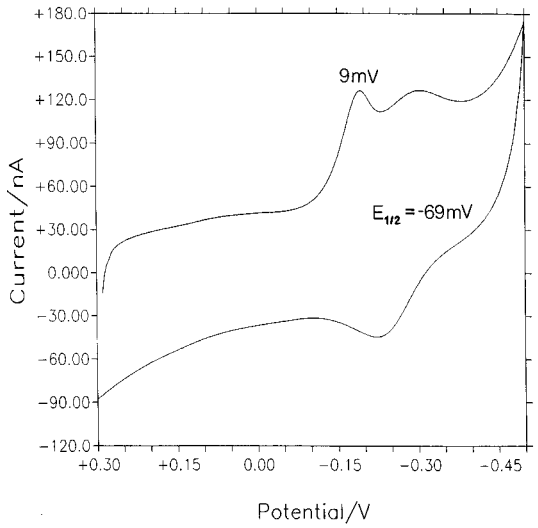

FIGURE 1: Cyclic voltammogram of a solution containing OM cytochrome b_5 (0.1 mM), horse heart cytochrome *c* (0.1 mM), and polylysine (0.025 mM) in 100 mM MOPS, pH 7.0. The scale of the voltammogram is shown *vs* the Ag/AgCl electrode, but the values 9 mV and −69 mV are with respect to the standard hydrogen electrode. Sweep rate = 50 mV/s. ΔE_p for the reversible wave in a typical experiment is between 56 and 63 mV.

a 100 mM solution of β-mercaptopropionate for 20 min, followed by rinsing with deionized water. The modified electrode was then immediately immersed in a deareated protein solution and a stream of nitrogen was blown gently across the surface of the protein solution in order to maintain the solution anaerobic.

Solutions used for cyclic voltammetry were typically 100 μM in OM cytochrome b_5 or microsomal cytochrome b_5, 100 μM cytochrome *c*, and 25 μM polylysine. MOPS (100 mM, pH 7.0) was used to prepare all solutions. The protein concentrations were measured by UV−vis spectrophotometry using the Soret band corresponding to the oxidized state in both proteins. The extinction coefficients used are 130 mM^{-1} cm^{-1} for oxidized cytochrome b_5 (Bodman *et al.*, 1986) and 109.5 mM^{-1} cm^{-1} for oxidized cytochrome *c* (Margoliash & Walaseck, 1967). The concentration of polylysine was obtained from the mass of substance weighed using the average molecular weight provided by Sigma. Titrations monitored by cyclic voltammetry were performed immediately after the electrode surface was modified. In between data points in any given titration, the working electrode was rinsed with deionized water and then immersed in a 100 mM solution of β-mercaptopropionate for 5 min in order to avoid deterioration of the modified surface throughout the experiment. Although the reduction potentials were obtained with respect to a silver−silver chloride electrode, the values of the reduction potential used under Results and Discussion have been corrected so as to be referenced with respect to the standard hydrogen electrode.

RESULTS AND DISCUSSION

The cyclic voltammogram of a solution containing equimolar concentrations of OM cytochrome b_5 and horse heart cytochrome *c* is shown in Figure 1. Noteworthy two reduction peaks are observed as the potential is scanned in the negative direction, and only one oxidation peak is observed when the scan direction is reversed. It should also be noted that the second reduction peak and the oxidation peak correspond to a reversible wave ($E_{1/2} = -69$ mV), and the peak at 9 mV is not accompanied by its oxidation counterpart. This peak is not due to heterogeneous electron transfer from the electrode to ferricytochrome *c* but from coupling of the heterogeneous electron transfer reaction to the homogeneous reaction between ferrocytochrome b_5 and ferricytochrome *c*. The peak at 9 mV therefore can be termed a pre-peak which corresponds to depletion of ferricytochrome *c* in the diffusion layer adjacent to the electrode surface which occurs due to fast homogeneous electron transfer between electrogenerated ferrocytochrome b_5 and ferricytochrome *c*, as shown by eqs 1 and 2, and schematically in Figure 2. In eq 1, k_s is the heterogeneous electron transfer rate constant, and in eq 2, k_f and k_b are the forward and backward second-order electron transfer rate constants.

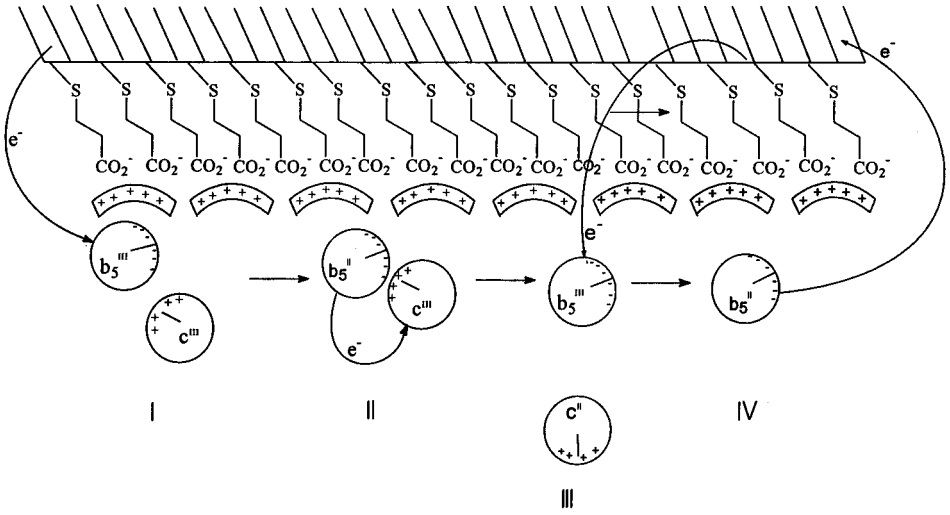

FIGURE 2: Schematic representation of a gold electrode modified with β-mercaptopropionate in the presence of polylysine (shown as a polycation). (I) Electrochemical reduction of ferricytochrome b_5 in the presence of ferricytochrome *c*. (II) Second-order electron transfer reaction between ferrocytochrome b_5 and ferricytochrome *c* which produces ferricytochrome b_5 and ferrocytochrome *c*. This reaction results in the depletion of ferricytochrome *c* in the diffusion layer, thus giving rise to the pre-peak observed in the voltammogram shown in Figure 1. (III) Electrochemical reduction of cytochrome b_5 that results in the depletion of ferricytochrome b_5 in the diffusion layer, thus giving rise to the cathodic peak observed in Figure 1. (IV) Electrochemical reoxidation of ferrocytochrome b_5 that takes place during the anodic scan, thus giving rise to the anodic peak observed in Figure 1.

$$\text{Fe(III) cyt } b_5 + e^- \overset{k_s}{\rightleftharpoons} \text{Fe(II) cyt } b_5 \quad (1)$$

$$\text{Fe(II) cyt } b_5 + \text{Fe(III) cyt } c \overset{k_f}{\underset{k_b}{\rightleftharpoons}} \text{Fe(III) cyt } b_5 + \text{Fe(II) cyt } c \quad (2)$$

The presence of polylysine in solution results in the selective reduction of ferricytochrome b_5, as shown schematically in Figure 2-I. The reduction of positively charged Fe(III) cytochrome *c*, which discriminates against the positively charged electrode surface, is possible if a second-order homogeneous electron transfer reaction takes place between electrogenerated ferrocytochrome b_5 and ferricytochrome *c* (Figure 2-II). If the ratio of k_f/k_b is large, a pre-peak separates from the main peak (Barker *et al.*, 1989). The larger the ratio of k_f/k_b, the larger the separation between the pre-peak and the main peak. The main peak is due to the depletion of ferricytochrome b_5 in the diffusion layer as shown schematically in Figure 2-III. In the return scan, the peak observed is due to the oxidation of ferrocytochrome b_5 at the electrode surface (Figure 2-IV).

These results are consistent with the reported cyclic voltammetric behavior of cytochrome *c* in the presence of plastocyanin at gold electrodes modified with bis (4-pyridyl) bisulfide, reported by Barker *et al.* (1989), who observed pre-shoulders to both reduction and reoxidation current peaks and interpreted their results in terms of a homogeneous electron transfer reaction between ferrocytochrome *c* and plastocyanin coupled to the selective heterogeneous reduction of ferricytochrome *c* at the electrode surface. Barker *et al.* (1989) substantiated their interpretation by carrying out digital simulations of the cyclic voltammograms using the electrochemical−chemical (EC) mechanism discussed above. In fact, using simulations, these authors predicted that the cyclic voltammogram of a mixture containing cytochrome b_5 and cytochrome *c* would have the characteristics seen in the voltammogram depicted in Figure 1, that is, a pre-peak preceding the reduction current peak if the electrode is selective to the negatively charged cytochrome b_5.

What follows is a description of the experimental evidence supporting the above interpretation of the cyclic voltammogram shown in Figure 1. The cyclic voltammogram obtained from a solution containing only cytochrome *c* using a gold electrode modified with β-mercaptopropionate and in the absence of polylysine displays a reversible wave with a midpoint potential of 259 mV (Figure 3A). When polylysine is added to the solution containing cytochrome *c*, no Faradaic current is observed (Figure 3B) because polylysine binds to the negatively charged electrode and prevents diffusion of the positively charged cytochrome *c* toward the modified electrode. On the other hand, when an equimolar amount of OM cytochrome b_5 is added to the solution containing cytochrome *c* and polylysine, the cyclic voltammogram depicted in Figure 3C is obtained. This voltammogram shows the pre-peak, which results from the depletion of ferricytochrome *c* in the diffusion layer due to fast homogeneous second-order electron transfer between ferrocytochrome b_5 and ferricytochrome *c*, as indicated by eq 1 and 2 and schematically shown in Figure 2. The reversible wave with midpoint potential at −69 mV was attributed to the reduction of OM cytochrome b_5 in the presence of polylysine because a cyclic voltammogram obtained from a solution containing only cytochrome b_5 and polylysine shows a reversible wave with almost identical midpoint potential (Figure 3D). When ferricytochrome *c* was added stepwise to a solution of cytochrome b_5 and polylysine, while monitoring the titration by cyclic voltammetry (Figure 4), the pre-peak is absent when the solution contains only cytochrome b_5 and polylysine, but it appears upon addition of cytochrome *c* and its intensity increases gradually as a function of the concentration of cytochrome *c*. On the other hand, when cytochrome *c* previously reduced with sodium dithionite was added to a solution of OM cytochrome b_5 in order to produce an equimolar mixture of OM ferricytochrome b_5 and horse heart ferrocytochrome *c*, a voltammogram showing only the reversible wave at −69 mV was

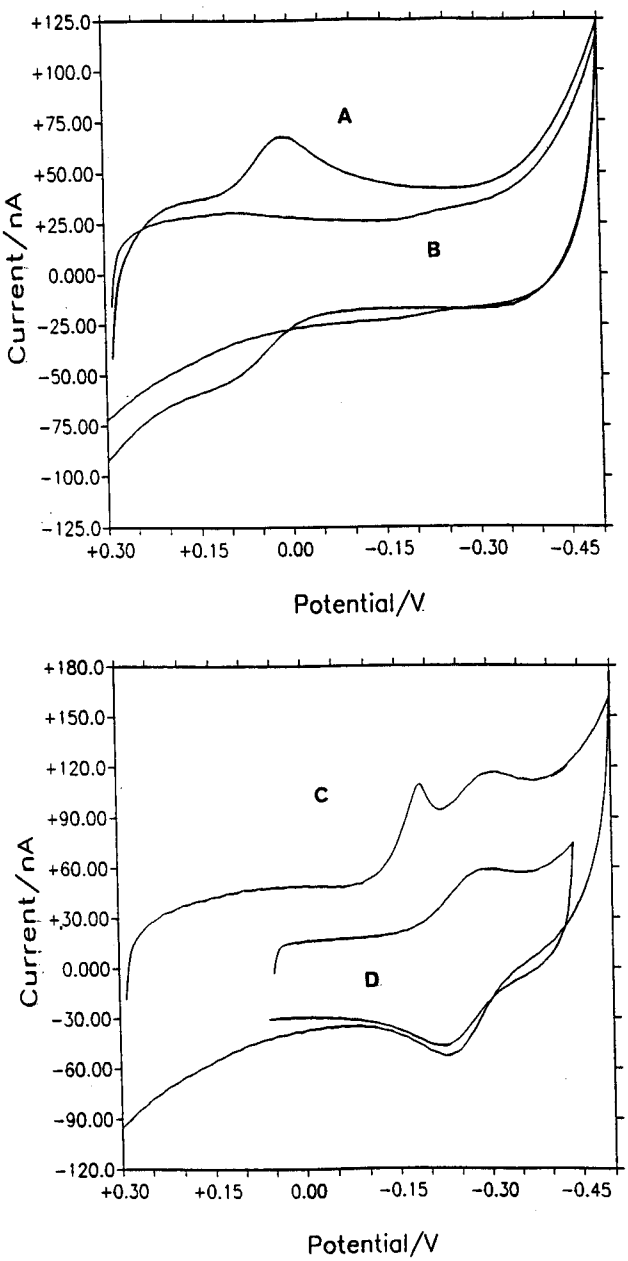

FIGURE 3: (A) Cyclic voltammogram of cytochrome *c* (0.1 mM) in 100 mM MOPS, pH 7.0. (B) Faradaic response when polylysine is added to solution A to produce a final concentration of 0.025 mM. (C) Cyclic voltammogram when OM cytochrome b_5 is added to solution B to produce a final concentration of 0.1 mM. (D) Cyclic voltammogram of a solution containing OM cytochrome b_5 (0.1 mM) and polylysine (0.025 mM) in 100 mM MOPS, pH 7.0. Sweep rate = 50 mV/s. Potential axis is with respect to the Ag/AgCl reference electrode.

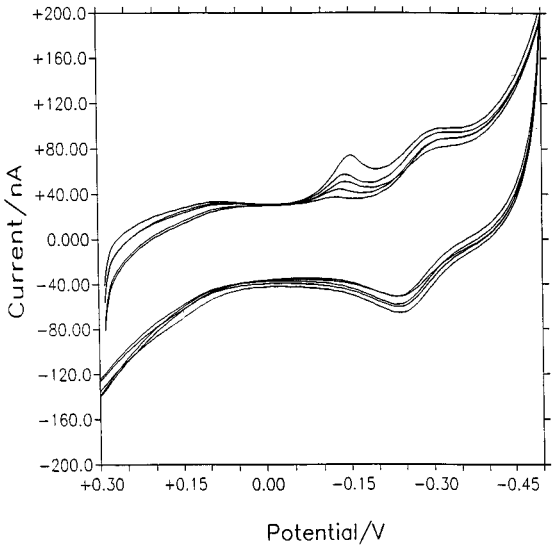

FIGURE 4: Titration of a solution containing OM cytochrome b_5 (0.1 mM) and polylysine (0.025 mM) in 100 mM MOPS, pH 7.0, with 1 mM horse heart cytochrome *c* prepared in 100 mM MOPS, pH 7.0. The cyclic voltammograms were obtained with a gold electrode modified with β-mercaptopropionate and correspond to final cytochrome *c* concentrations of approximately 0.025, 0.05, 0.07, and 0.1 mM.

obtained (Figure 5A). The absence of the pre-peak in Figure 5A is consistent with the fact that ferricytochrome *c* is absent in the bulk solution and in the diffusion layer. Consequently, ferrocytochrome b_5 cannot be oxidized by ferricytochrome *c*, and the pre-peak is abolished. When the same experiment was performed with the addition of oxidized cytochrome *c*, the voltammogram obtained shows the presence of the pre-peak due to homogeneous electron transfer between electrogenerated ferrocytochrome b_5 and ferricytochrome *c* in the diffusion layer (Figure 5B).

Similar experiments were carried out with microsomal beef cytochrome b_5 and horse heart cytochrome *c*. The cyclic voltammogram of a solution containing equimolar concentrations of bovine liver microsomal cytochrome b_5 and horse heart cytochrome *c* was obtained with a gold electrode modified with β-mercaptopropionate under conditions identical to those used with the experiments involving mitochondrial cytochrome b_5. This voltammogram (Figure 6) is identical in shape to the one obtained with a solution of OM cytochrome b_5 under similar conditions, except that the pre-peak occurs at 94 mV and the midpoint potential at −1.0 mV.

Second-Order Rate Constants Obtained from Digital Simulation of Cyclic Voltammograms. If species O is reduced at an electrode surface to produce R (eq 3), which reacts with another species P in solution to regenerate O and produce Q (eq 4), the net effect is catalysis of the reduction of P by the O/R couple, thus giving rise to the EC mechanism:

$$O + e^- \overset{k_s}{\rightleftharpoons} R \tag{3}$$

$$R + P \underset{k_b}{\overset{k_f}{\rightleftharpoons}} O + Q \tag{4}$$

If the reduction of P by R is spontaneous, then P is thermodynamically more easily reducible than O. The EC mechanism requires that the reduction of P at the electrode

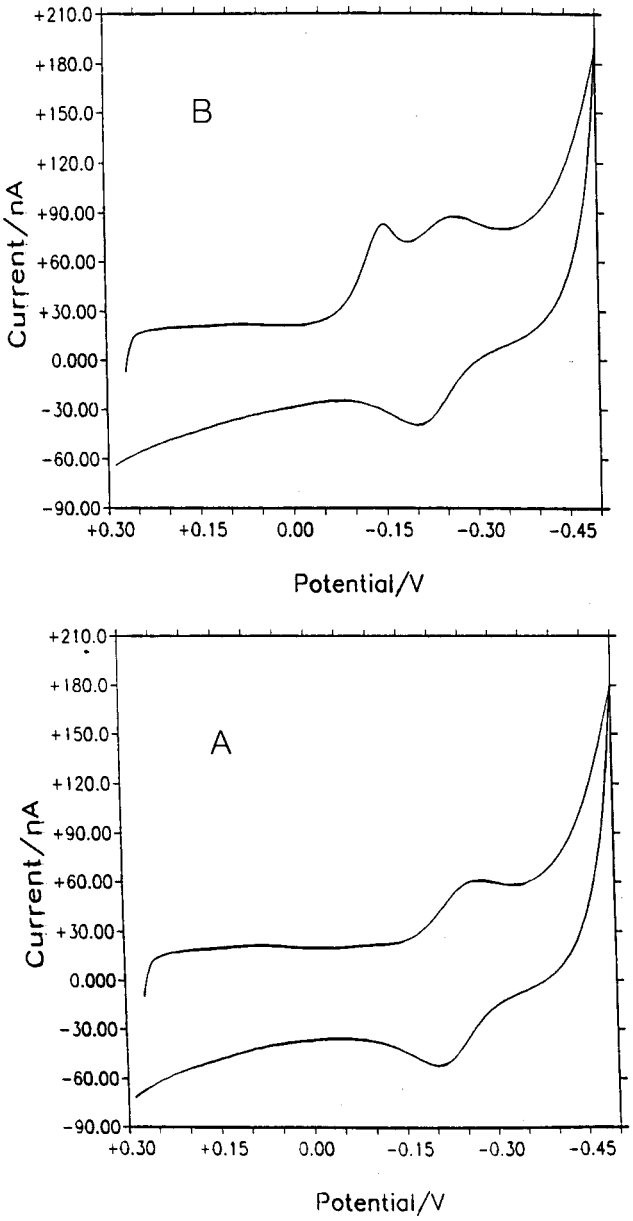

FIGURE 5: (A) Cyclic voltammogram of a mixture containing rat liver OM ferricytochrome b_5 (0.1 mM), horse heart ferrocytochrome *c* (0.1 mM) and polylysine (0.025 mM) in 100 mM MOPS, pH 7.0. (B) Cyclic voltammogram obtained with a solution containing rat liver OM ferricytochrome b_5, horse heart ferricytochrome *c*, and polylysine. Concentrations as in (A). Sweep rate = 50 mV/s.

is much slower than the homogeneous redox reaction between P and R so that the reduction of P at the electrode can be neglected (Bard & Faulkner, 1980; Reiger, 1994). For a mixture containing cytochrome b_5 and cytochrome *c* in solution, this condition is satisfied if the working electrode consists of a gold surface modified with β-mercaptopropionate and the solution contains polylysine. The working electrode then becomes selective for the negatively charged cytochrome b_5, as shown schematically in Figure 2. This implies that in eqs 3 and 4, O = ferricytochrome b_5, R = ferrocytochrome b_5, P = ferricytochrome *c*, and Q = ferrocytochrome *c*. Although ferricytochrome *c* is thermodynamically more easily reduced, it cannot interact with the electrode due to electrostatic repulsion between the positively charged protein and electrode surfaces. As a result, the ferricytochrome b_5/ferrocytochrome b_5 couple catalyzes the reduction of ferricytochrome *c* according to eqs 1 and 2.

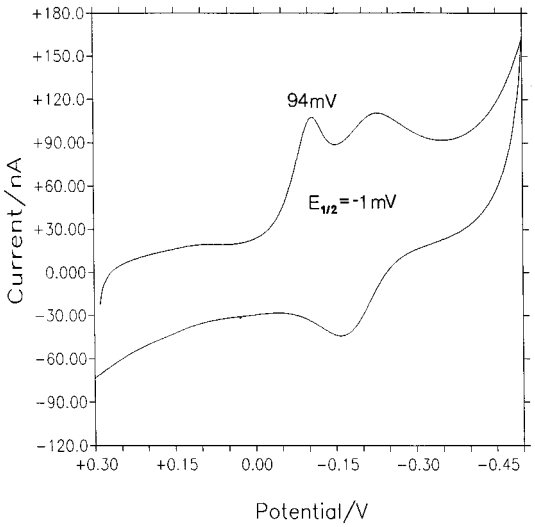

FIGURE 6: Cyclic voltammogram of a mixture containing beef liver microsomal cytochrome b_5 (0.1 mM), horse heart cytochrome *c* (0.1 mM), and polylysine (0.025 mM) in 100 mM MOPS, pH 7.0. The scale of the voltammogram is shown *vs* the Ag/AgCl electrode, but the values 94 mV and −1 mV are with respect to the standard hydrogen electrode. Sweep rate = 50 mV/s. ΔE_p for the reversible wave in a typical experiment is between 56 and 63 mV.

Digital simulation of the EC reaction scheme proposed above has been carried out in order to further substantiate the above interpretation of the cyclic voltammogram shown in Figure 1. If the potential of the P/Q couple is substantially more positive than the potential of the O/R couple, the ratio k_f/k_b is expected to be large, and consequently a pre-peak preceding the main peak is expected in the cyclic voltammogram of a solution containing O and P when the electrode is selective to O. Since the potential of cytochrome *c* is approximately 250 mV more positive than that of microsomal cytochrome b_5 and 350 mV more positive than that of OM cytochrome b_5, it is not likely that ferrocytochrome *c* can reduce ferricytochrome b_5, and consequently the cyclic voltammogram displays only one peak arising from the reoxidation of ferrocytochrome b_5 at the electrode surface.

The above mechanism was digitally simulated using the program Digisim 2.0 (Bioanalytical Systems), a simulator for cyclic voltammetric responses (Rudolph *et al.*, 1994). The simulation was carried out assuming semi-infinite diffusion and planar electrode geometry. The experimental parameters entered for digital simulation consisted of the following: $E_{start} = 0.29$ V, $E_{switch} = -0.50$ V, $E_{end} = 0.30$ V, scan rate = 0.05 V/s, electrode area 0.0078 cm^2, potential of the ferricytochrome b_5/ferrocytochrome b_5 couple, and the analytical concentrations of cytochrome b_5 and cytochrome *c*. All these parameters were kept constant throughout the fitting of the digitally simulated voltammogram to the experimental data. The parameters k_f, k_s, K_{eq}, and D_o (diffusion coefficients of cyt b_5 and cyt *c*) were allowed to change throughout the fitting process. Further refinement was accomplished by holding the diffusion coefficients constant and allowing k_f, k_s, and K_{eq} to vary throughout the fitting process. The simulated and experimental cyclic voltammograms are depicted in Figure 7, and the parameters obtained from the simulations are listed in Tables 1 and 2, which correspond to mixtures containing rat liver OM cytochrome b_5 and horse heart cytochrome *c*, and beef liver microsomal cytochrome b_5 and horse heart cytochrome *c*, respectively. The values of k_f obtained by Meyer *et al.* (1993) using flash photolysis techniques are 2.6×10^8 $M^{-1}cm^{-1}$ ($I = 5$ mM), 1.3×10^9 M^{-1} cm^{-1} ($I = 35$ mM), 6.7×10^8 M^{-1} cm^{-1} ($I = 87$ mM) for rat liver OM cytochrome b_5 and 8×10^8 M^{-1} cm^{-1} ($I = 5$ mM) for microsomal beef liver cytochrome b_5. These authors also noticed that the second-order reaction between beef microsomal cytochrome b_5 and horse heart cytochrome *c* is approximately 3 times faster than the reaction between rat liver OM cytochrome b_5 and horse heart cytochrome *c*, which is consistent with the values of k_f obtained by cyclic voltammetry (Tables 1 and 2). Furthermore, the experimental values obtained for the heterogeneous electron transfer rate constant, k_s, and diffusion coefficient, D_o, for OM cytochrome b_5 are 1.05×10^{-2} cm/s and 4.34×10^{-6} cm^2/s, respectively, which are in good agreement with the values

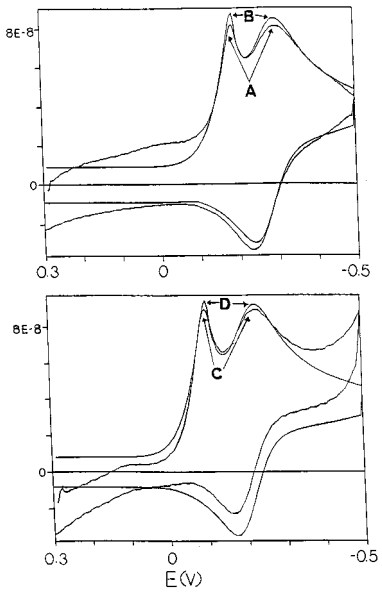

FIGURE 7: (A) Experimental cyclic voltammogram obtained from a mixture containing rat liver OM cytochrome b_5 and horse heart cytochrome *c*. (B) Simulated cyclic voltammogram invoking the reaction scheme shown by eqs 1 and 2. Parameters for simulation are shown in Table 1. (C) Experimental cyclic voltammogram of a mixture containing beef liver cytochrome b_5 and horse heart cytochrome *c*. (D) Simulated voltammogram invoking the reaction sheme shown by eqs 1 and 2. Parameters for simulation are shown in Table 2.

Table 1: Parameters for Digital Simulation of a Typical Cyclic Voltammogram Obtained from a Mixture Containing Rat OM Cytochrome b_5 and Horse Heart Cytochrome *c*[a]

Charge Transfer Parameters	
$E°$ (*vs* Ag/AgCl)	−0.266
transfer coefficient, α	0.5
k_s (cm/s)	0.0082
Chemical Reaction Parameters	
K_{eq}	2.5×10^5
k_f (M^{-1} s^{-1})	3.6×10^8
k_b (M^{-1} s^{-1})	1360
Species Parameters	
D_o(O)	1.0×10^{-6}
D_o(R)	1.0×10^{-6}
anal conc (O) (M)	0.00010
D_o(P)	1.1×10^{-6}
D_o(Q)	1.1×10^{-6}
anal concn (P) (M)	0.00010

[a] O = ferricytochrome b_5, R = ferrocytochrome b_5, P = ferricytochrome *c*, Q = ferrocytochrome *c*. Experimental voltammogram was background-subtracted. $I = 78$ mM.

Table 2: Parameters for Digital Simulation of a Typical Cyclic Voltammogram Obtained from a Mixture Containing Beef Liver Microsomal Cytochrome b_5 and Horse Heart Cytochrome c[a]

Charge Transfer Parameters	
$E°$ (vs Ag/AgCl)	−0.201
transfer coefficient, α	0.5
k_s (cm/s)	0.013
Chemical Reaction Parameters	
K_{eq}	2.0×10^5
k_f (M^{-1} s^{-1})	8.9×10^8
k_b (M^{-1} s^{-1})	4450
Species Parameters	
D_o (O)	1.4×10^{-6}
D_o (R)	1.4×10^{-6}
anal conc (O) (M)	0.00010
D_o(P)	1.6×10^{-6}
D_o(Q)	1.6×10^{-6}
anal concn (P) (M)	0.00010

[a] O = ferricytochrome b_5, R = ferrocytochrome b_5, P = ferricytochrome c, Q = ferrocytochrome c. Experimental voltammogram was background-subtracted. I = 78 mM.

Table 3: Second-Order Rate Constants for the Reduction of Horse Heart Ferricytochrome c by OM Ferrocytochrome b_5

protein concn (mM)	μ (M)	scan rate (mV/s)	k_f (M^{-1} s^{-1})[a]
0.10 cyt b_5; 0.10 cyt c	0.078	50	$2.9 \times 10^8 \pm 0.82$
0.10 cyt b_5; 0.10 cyt c	0.078	25	$2.2 \times 10^8 \pm 1.0$
0.10 cyt b_5; 0.10 cyt c	0.13	25	$1.3 \times 10^8 \pm 0.25$
0.10 cyt b_5; 0.10 cyt c	0.23	25	$1.6 \times 10^7 \pm 0.67$
0.050 cyt b_5; 0.10 cyt c	0.078	50	$2.7 \times 10^8 \pm 0.70$
0.10 cyt b_5; 0.050 cyt c	0.078	50	$2.2 \times 10^8 \pm 0.95$

[a] Each value of k_f is an average of four measurements.

obtained by digital simulation of the cyclic voltammogram (Tables 1 and 2) and hence provide additional evidence that the model simulated digitally resembles the experimental events. The separation between the main peak and the pre-peak in the voltammogram obtained with rat liver OM cytochrome b_5 (110 mV) is smaller than the separation between the pre-peak and the main peak in the voltammogram obtained with beef liver microsomal cytochrome b_5 (125 mV). This is consistent with the faster electron transfer reaction observed between microsomal cytochrome b_5 and cytochrome c (Tables 1 and 2), despite the larger thermodynamic driving force for the reaction between rat liver OM cytochrome b_5 and cytochrome c.

Electron Transfer Rate Constants Measured Electrochemically under Different Experimental Conditions. In order to assess the reliability and reproducibility of the electrochemical method described here, rate constants for the reduction of horse heart ferricytochrome c by OM ferrocytochrome b_5 were measured under different conditions, including protein ratios, scan rates, and ionic strengths, and the results of these experiments are summarized in Table 3. Each of the rate constants listed in Table 3 is the result of four measurements. Every measurement was carried out with a freshly derivatized electrode, and a freshly prepared protein-containing solution was used every two measurements in order to determine reproducibility from sample to sample. Experimental and simulated cyclic voltammograms obtained with solutions containing ratios of cytochrome b_5/cytochrome c different from 1 are shown in Figure 8. The cyclic voltammogram depicted in Figure 8A was obtained with a cytochrome b_5/cytochrome c ratio = 0.5. Consistent with the model and

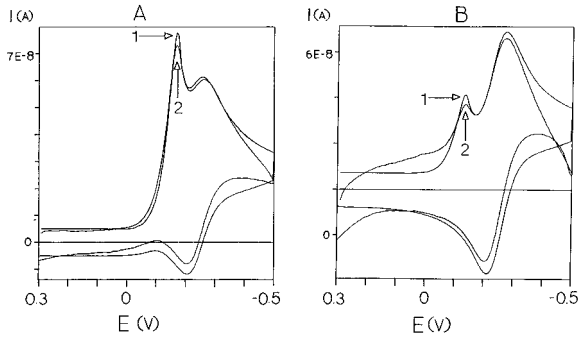

FIGURE 8: (A) Simulated (1) and experimental (2) cyclic voltammogram from a solution containing OM cytochrome b_5 (0.050 mM) and horse heart cytochrome c (0.10 mM). (B) Simulated (1) and experimental (2) cyclic voltammogram from a solution containing 0.10 mM OM cytochrome b_5 and 0.050 mM horse heart cytochrome c. For solutions A and B, polylysine was added to a final concentration of 0.025 mM, μ = 78 mM, scan rate = 50 mV/s. The second-order rate constants obtained from the digitally simulated voltammograms are listed in Table 3.

with the relative analytical concentrations of both cytochromes, the pre-peak which corresponds to the reduction of ferricytochrome c by electrogenerated ferrocytochrome b_5 is more intense than the main peak which corresponds to the electrochemical reduction of ferricytochrome b_5. The opposite is observed when the cytochrome b_5/cytochrome c ratio is 2. The resultant cyclic voltammogram (Figure 8B) consists of a pre-peak that is less intense than the main peak, as expected from the analytical concentrations of both cytochromes.

The rate constant for the transfer of an electron from ferrocytochrome b_5 to ferricytochrome c has been found to be affected by ionic strength (Willie *et al.*, 1992). At low ionic strengths, 2 mM, the kinetic behavior was reported to be consistent with electron transfer within a 1:1 complex formed between microsomal cytochrome b_5 and horse heart cytochrome c (Willie *et al.*, 1992). Increasing the ionic strength to approximately 20 mM decreases the stability of the complex and results in second-order kinetics whose rate constants decrease rapidly as the ionic strength is increased (Willie *et al.*,1992). Second-order rate constants for the reduction of horse heart ferricytochrome c by OM ferrocytochrome b_5 were measured with the aid of the electrochemical method described here, at three different values of ionic strength (Table 3). It was found that the second-order rate constants become progressively lower as the ionic strength of the solution is increased. Furthermore, the values of the rate constants obtained for this pair of proteins as a function of ionic strength are in good agreement with the previously reported second-order rate constants obtained for the reduction of horse heart cytochrome c by microsomal beef liver cytochrome b_5, using flash photolysis (Willie *et al.*, 1992) and stopped flow methods (Eltis *et al.*, 1991) at different ionic strengths. Experimental and simulated cyclic voltammograms obtained at μ = 130 mM and 230 mM are depicted in Figure 9 A and B, respectively. The separation between the pre-peak and the main peak is larger at lower ionic strengths than at higher ionic strengths, which is consistent with the lower second-order rate constants obtained at higher ionic strengths.

The observation of second-order kinetics at ionic strengths higher than 20 mM is also consistent with the large effect of ionic strength on the stability of the cytochrome b_5/

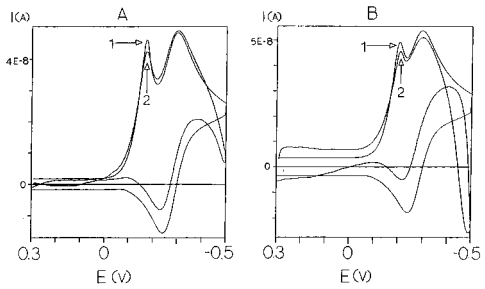

FIGURE 9: Simulated (1) and experimental (2) cyclic voltammogram from a solution containing 0.10 mM OM cytochrome b_5 and 0.10 mM horse heart cytochrome *c*, scan rate = 25 mV/s. (A) μ = 130 mM and (B) μ = 230 mM. Solutions A and B also contained 0.025 mM polylysine. The second-order rate constants obtained from the digitally simulated voltammograms are listed in Table 3.

cytochrome *c* complex (Mauk *et al.*, 1982). These investigators found that the binding constant for the formation of the cytochrome b_5/cytochrome *c* complex is 4×10^6 M^{-1} at μ = 1 mM and 8×10^4 M^{-1} at μ = 10 mM. Subsequently, Eley and Moore (1983) measured the binding constant at μ = 40 mM and reported a value of 9.6×10^2 M^{-1}, which corroborated the strong dependency of the stability of the cytochrome b_5/cytochrome *c* complex on ionic strength. The similar trends followed by the second-order rate constants obtained for the systems OM cytochrome b_5/horse heart cytochrome *c* and microsomal cytochrome b_5/horse heart cytochrome *c* as a function of ionic strength are consistent with the large degree of homology in the sequences of these proteins. The majority of the differences in amino acid sequence between the microsomal and mitochondrial cytochromes b_5 occur in the amino- and carboxy-terminal domains which are removed from the heme active site and have not been implicated in binding to cytochrome *c*. On the other hand, the polypeptides are highly conserved in the region corresponding to the heme binding crevice (Lederer *et al.*, 1983; Rivera *et al.*, 1992). In addition, all of the acidic residues on the surface of cytochrome b_5 which have been implicated in electrostatic binding to cytochrome *c*, namely, Glu 48, Glu 44, Glu 56, Asp 60, and heme propionate (Mauk *et al.*, 1995), are also conserved in the mitochondrial protein, thus indicating that both proteins may form complexes with cytochrome *c* that exhibit similar second-order rate constants as a function of ionic strength, consistent with the experimental data presented in this communication.

Importance of the Electrode Surface. The results of the digital simulation described in the previous section corroborate the idea pioneered by Hill and Walton (1981, 1982) that cyclic voltammetry is a viable method for the determination of homogeneous electron transfer reactions between proteins. It should be emphasized, however, that the voltammetric measurement of homogeneous electron transfer reactions between proteins using surface-modified electrodes is possible because the structure of most electron transfer proteins allows them to discriminate surfaces based on electrostatic interactions, therefore pointing out the importance of the electrode surface. The extensive research that has been carried out on the topic of "direct" electrochemistry of redox proteins, which followed the work of Eddowes and Hill (1977, 1979), who demonstrated that reversible voltammetry of redox proteins can be obtained at the surface of modified electrodes, has been summarized by Armstrong *et al.* (1990) and Bond (1994). The picture that emerged from these studies is one of molecular recognition between the redox proteins and the functionalized electrodes which results in modified electrodes discriminating against certain proteins on the basis of mainly electrostatic interactions. In this context, it is interesting to compare the results described here with the elegant cyclic voltammetric studies of the complexes formed by microsomal beef cytochrome b_5 with several mutants of yeast cytochrome *c* (Burrows *et al.*, 1991) and with horse heart cytochrome *c* (Bagby *et al.*, 1990) using pyrolytic graphite electrodes. These authors observed two independent electrochemical responses, one for each protein, with some differences in the shapes of the peaks, which were correlated to the type of cytochrome *c* mutant involved in each experiment (Burrows *et al.*, 1991). It is important to notice that when cyclic voltammetric experiments were carried out using pyrolytic graphite electrodes to study the cytochrome b_5/cytochrome *c* complex, two independent electrochemical signals were observed, one for cytochrome *c* and the other for cytochrome b_5, whereas when gold electrodes modified with β-mercaptopropionate are used in the presence of polylysine in order to study the same complex, the electrochemical signal depicted in Figure 1 is obtained.

Edge-plane pyrolytic graphite electrodes are obtained by cutting a pyrolytic graphite crystal perpendicular to its basal plane in order to expose a relatively reactive edge face (Armstrong *et al.*, 1987), which in the presence of oxygen forms various types of carbon−oxygen-containing functional groups. It is noteworthy that these surfaces are heterogeneous in that they contain hydrophobic areas with low density of oxygen containing functional groups, areas with a high density of these groups, and areas with intermediate density of C−O-containing functional groups (Panzer & Elving, 1975). Furthermore, it has been shown by X-ray photoelectron spectroscopy (Armstrong *et al.*, 1987) that when an edge-plane pyrolytic graphite electrode is polished with 0.3 μM alumina, a typical procedure when using these electrodes, C−O-containing functional groups amount to only 33% of surface coverage. In contrast, self-assembled monolayers of mercapto compounds have been shown to provide close to monolayer coverage of the electrode surface (Creager & Olsen, 1995; Everett & Fritsch-Faules, 1995), thus resulting in a high density of carboxylate groups on the electrode surface when β-mercaptopropionate is used to derivatize a gold surface. As a result, when a gold surface is modified with β-mercaptopropionate and the solution contains polylysine, the electrode surface is expected to have a large density of positive charge (Figure 2), thus resulting in selective interactions between the modified electrode and the negatively charged cytochrome b_5. The selectivity of the β-mercaptopropionate electrode against cytochrome *c* in the presence of polylysine is clearly demonstrated by the cyclic voltammogram shown in Figure 3B. If polylysine is not present in solution, on the other hand, the electrode is selective to cytochrome *c* and discriminates against cytochrome b_5.

CONCLUDING REMARKS

Researchers interested in the measurement of second-order rate constants for the reaction between ferrocytochrome b_5 and ferricytochrome *c* have traditionally approached the problem using flash photolysis techniques. In order to carry out the measurements, it was necessary to resort to different

schemes to selectively reduce cytochrome b_5 in the presence of cytochrome *c*. Meyer *et al.* (1993) used a mixture containing an eight-fold molar excess of cytochrome b_5 to measure the second-order rate constant for the oxidation of ferrocytochrome b_5 by ferricytochrome *c* in a transient ternary complex consisting of two molecules of cytochrome b_5 and one molecule of cytochrome *c*. Willie *et al.* (1993) covalently attached the $[Ru(bypiridine)_3]^{2+}$ complex to the surface of cytochrome b_5. The photochemical properties of ruthenium complexes of this type provide a means of generating ferrocytochrome b_5 in the presence of ferricytochrome *c*. The electrochemical method described in this report provides an alternative experimental scheme to selectively reduce a protein possessing a relatively low potential (*e.g.*, cytochrome b_5) in the presence of equimolar concentrations of a protein possessing a relatively high potential (*e.g.*, cytochrome *c*). The resulting electrochemical signal consists of a pre-peak that corresponds to the depletion of ferricytochrome *c* in the diffusion layer due to fast homogeneous electron transfer between electrogenerated ferrocytochrome b_5 and ferricytochrome *c* and a reversible wave corresponding to the reduction and oxidation of cytochrome b_5 in the diffusion layer (Figure 2). Digital simulation of these electrochemical signals obtained under a variety of different experimental conditions, such as different protein ratios, ionic strengths, and scan rates, was used to calculate second-order rate constants for the reduction of ferricytochrome *c* by ferrocytochrome b_5. The results from these experiments conclusively demonstrate that the electrochemical method described here can be reliably used to determine second-order rate constants for interprotein electron transfer systems under a variety of experimental conditions.

ACKNOWLEDGMENT

We express our gratitude to the reviewers for their insightful comments which played an important role in the interpretation of the electrochemical response. We also express our gratitude to Prof. F. Ann Walker for a sample of bovine liver microsomal cytochrome b_5.

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BI9529723