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Direct Electrochemistry of Protein–Protein Complexes Involving Cytochrome *c*, Cytochrome b_5, and Plastocyanin†

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ABSTRACT: The direct electrochemistry of the cytochrome *c*/cytochrome b_5 and cytochrome *c*/plastocyanin complexes has been investigated at edge-plane graphite and modified gold electrode surfaces, which are selective for one of the two components of the complex. Electrochemical response of one protein at an otherwise electrostatically unfavorable electrode surface was achieved in the presence of the other protein, and the calculated heterogeneous electron-transfer rate constant and diffusion coefficient were found to be in good agreement with the values determined previously from the electrochemistry of the individual proteins [Armstrong, F. A., Hill, H. A. O., & Walton, N. J. (1988) *Acc. Chem. Res. 21*, 407 and references therein]. A dynamic model of the protein–protein–electrode ternary complex is proposed to explain the promotion effect, and this model is supported by a study comparing the electrochemical responses of covalent and electrostatic cytochrome *c*/plastocyanin complexes. It is also suggested that the behavior of protein–protein complexes at electrode surfaces could be related to that of the complexes associated with biological membranes.

Electron transfer between redox proteins is essential to many metabolic processes in biological organisms. In vitro inter-protein electron-transfer reactions are believed to involve formation of a kinetically detectable precursor complex which is stabilized and oriented by electrostatic and hydrophobic interactions (Poulos & Kraut, 1980). Among redox protein complexes, those between cytochrome *c* and cytochrome b_5 (cyt *c*/cyt b_5)[1] and between cytochrome *c* and plastocyanin (cyt *c*/Pc) have been extensively studied, partly due to the availability of the crystal structure of each protein (Dickerson et

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[1] Abbreviations: EPG, edge-plane graphite; FPLC, fast protein liquid chromatography; pySSpy, 4,4′-dipyridyl disulfide; $(Cys\text{-}Glu)_2$, L-cystinylbis(L-glutamic acid); $(Lys\text{-}Cys\text{-}OMe)_2$, bis(L-lysyl-L-cystine methyl ester); cyt *c*/cyt b_5, cytochrome *c*/cytochrome b_5; cyt *c*/Pc, cytochrome *c*/plastocyanin.

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Table I: Protein-Selective Electrode Surfaces[a]

	cyt *c*	cyt b_5	Pc
EPG	+	–	–
Au-(Cys-Glu)$_2$	+	–	–
Au-(Lys-Cys-OMe)$_2$	–	+	+
Au-pySSpy	+	(+)	–

[a] Symbols: +, good response; –, no response; (+), poor response.

al., 1971; Mathews et al., 1971, 1972; Guss & Freeman, 1983). Examples of the techniques that have been used to study the cyt *c*/cyt b_5 complex include ultraviolet/visible spectroscopy (Mauk et al., 1982), NMR (Eley & Moore, 1983; Hartshorn et al., 1987), Fourier transform infrared spectroscopy (Holloway & Mantsch, 1988), electrostatic analysis (Mauk et al., 1986), and molecular dynamics simulation (Wendoloski et al., 1987). The cyt *c*/Pc interaction has been studied by NMR (King et al., 1985; Bagby et al., 1990), chemical cross-linking (Geren et al., 1983; Peerey & Kostic, 1989), and kinetics (Augustin et al., 1983). A computer modeling study (Salemme, 1976) showed that proximity and orientation of the two heme groups in the cyt *c*/cyt b_5 complex is achieved through the selective formation of salt bridges between the acidic residues of the cytochrome b_5 heme face and the positively charged lysines around the heme edge of cytochrome *c*. Investigation of the cyt *c*/Pc complex by NMR spectroscopy indicated that complexation involves the acidic east site of plastocyanin and the lower heme site of cytochrome *c* and that the complex is nonstatic (Bagby et al., 1990).

The application of electrochemical techniques to the study of biological systems has furnished new insights into the electron-transfer processes and provided information on the thermodynamics, kinetics, and mechanisms of biological electron transfer (Dryhurst et al., 1982). In the last decade, direct electrochemistry of redox proteins such as cytochrome *c*, cytochrome b_5, and plastocyanin has been achieved at various functionalized solid electrodes (Frew & Hill, 1988; Armstrong et al., 1988; Hitchens, 1989). For example, cytochrome *c* electrochemistry was obtained at a 4,4′-bipyridyl-modified gold electrode (Eddowes & Hill, 1977), and detailed studies suggested that hydrogen bonding between the lysine residues surrounding the exposed heme edge of cytochrome *c* and pyridyl nitrogens at the electrode surface stabilizes a transient protein–electrode complex oriented so as to allow rapid electron transfer to and from the heme group. Thus the process appears to have some similarities to the manner in which cytochrome *c* interacts with its redox partners such as cytochrome b_5.

We have developed a method for studying redox protein complexes that involves measuring the electrochemical response of one protein in the presence of a second protein. Selective electrode surfaces are required so that the electrode is interacting productively with only one component of the protein–protein complex. Such selectivity can be achieved as a result of the differing charges of the components of the respective complexes: at neutral pH, cytochrome *c* is highly positively charged whereas cytochrome b_5 and plastocyanin are highly negatively charged. The electrochemistry of these three proteins has been investigated previously (Frew & Hill, 1988; Armstrong et al., 1988; Bagby et al., 1988), and a summary of the results obtained at various electrode surfaces is presented in Table I.

For this electrochemical study of redox protein complexes, cyclic voltammetry (Bard & Faulkner, 1980) has been employed. In this technique, the potential applied to the electrode is swept linearly with time, forward and backward between two limits. The current output due to the oxidation and reduction of redox-active species is recorded as a function of the potential. From the resulting voltammograms the potential at the midpoint between the two peaks, $E_{1/2}$, is determined. Ideally, this is equal to or close to the thermodynamic redox potential. In cases where the cyclic voltammogram is peak-shaped (as opposed to sigmoidal), linear (rather than radial) diffusion is the dominant mode of mass transport (Armstrong et al., 1989). The methods of Nicholson and Shain (1964) can then be used to determine the heterogeneous electron-transfer rate constant, k_s, and diffusion coefficient, D_0, respectively. As shown in this paper, cyclic voltammetry offers a simple and yet powerful tool to investigate the effect of protein–protein complexation on the direct electrochemistry of either component of the complex.

Experimental Procedures

Horse heart cytochrome *c* (type VI) was purchased from Sigma and purified by fast protein liquid chromatography (FPLC) on a Mono-S cation-exchange column (HR10/10, Pharmacia) equilibrated with 30 mM phosphate buffer (pH 7.0). Potassium chloride gradients were used for elution from Mono-S. Spinach plastocyanin, prepared by the method of Borchert and Wessels (1970), was the kind gift of Dr. P. C. Driscoll and was further purified by FPLC using an anion-exchange column (Mono-Q HR5/5, Pharmacia) equilibrated with 20 mM Bis-Tris propane (Sigma), pH 7.4. The protein was eluted with potassium chloride gradients. Cytochrome b_5 was isolated as the soluble, trypsin-cleaved form from chicken livers essentially by the method of Reid and Mauk (1982). The final purity ratio of A_{413}/A_{280} was 5.8.

Zinc(II)-substituted cytochrome *c* [Zn(II) cytochrome *c*] was prepared from iron-free cytochrome *c* which was made in the laboratory of Dr. M. T. Wilson, University of Essex, according to the procedure of Vanderkooi et al. (1976). Metal incorporation was achieved by adding a concentrated solution of zinc acetate (1 M) to the protein solution (100 μM) to give a final zinc concentration of 1 mM. The reaction was carried out at 70 °C and was followed by monitoring the disappearance of the 404- and 506-nm absorbances of porphyrin cytochrome *c* and the appearance of the 424-nm absorbance of Zn(II) cytochrome *c*. To ensure complete reaction, a further 10 equiv of Zn(II) was added. The reaction was complete after 3 h. Excess zinc ions were removed by gel filtration over Sephadex G-25 (Pharmacia, 40 × 2.5 cm) equilibrated with 30 mM phosphate (pH 7.0). The protein was then purified by FPLC using a Mono-S cation-exchange column.

Mercury(II)-substituted plastocyanin [Hg(II) plastocyanin] was prepared according to the method of Tamilarasan and McMillin (1986) with minor modifications and was purified by using FPLC under the same conditions as were used for copper(II) plastocyanin. Fe(III) cytochrome *c* and Cu(II) plastocyanin were cross-linked in a 1:1 ratio by the method of Geren et al. (1983). The product was established as a dimer by using gel filtration and ultraviolet/visible difference spectroscopy. No effort was made to isolate individual species.

Published extinction coefficients were used to determine the concentrations of cytochrome *c* (Van Gelder & Slater, 1962), cytochrome b_5 (Ozols & Strittmatter, 1964), and plastocyanin (Davis & San Pietro, 1979).

Bovine pancreas ribonuclease, chicken egg white lysozyme, chicken egg albumin, calf thymus histone f_3, and soybean trypsin inhibitor samples were purchased from Sigma and used without further purification.

4,4′-Dipyridyl disulfide (pySSpy) was purchased from Aldrich and (Cys-Glu)$_2$ from Sigma. (Lys-Cys-OMe)$_2$ was synthesized in this laboratory by Drs. K. Di Gleria and V. J. Lowe. Sodium cacodylate and cacodylic acid were obtained

from BDH (U.K.) and HEPES from Sigma. All buffer solutions for electrochemical studies were prepared from sodium cacodylate (20 mM) or KCl (20 mM)/HEPES (1 mM). Water which had been purified by reverse osmosis (Millipore RO-6) and then by ion exchange (Millipore, Milli-Q) was used in all experiments. Buffer pH was adjusted to 7.0 by using Analar-grade KOH or HCl.

Cyclic voltammetry was carried out by using an Oxford Electrodes potentiostat with all voltammograms recorded on a x–y chart recorder (Gould 60000 series A3). The electrochemical cell employed a three-electrode system with two electrolyte compartments. The counter electrode was a semicylindrical piece of platinum gauze while the reference was a saturated calomel electrode linked to the main cell compartment through a Luggin capillary tip (~0.1-mm diameter). Oxygen was removed from the working compartment by passing humidified oxygen-free argon through the sealed cell. All the potentials reported in this paper are with respect to the normal hydrogen electrode (NHE).

The edge-plane graphite (EPG) electrode was constructed from a 5-mm disk of standard pyrolytic graphite (Le Carbonne, Portslade, Sussex) cut with the a–b plane perpendicular to the disk face and housed in a Teflon sheath. Prior to each experiment, the electrode was polished with an alumina/water slurry (high-purity Al_2O_3, particle size 0.3 μm, BDH) and sonicated briefly, followed by rinsing thoroughly with water.

The gold electrode was constructed in a manner similar to the EPG electrode and was cleaned initially by sanding with successively finer grades of sand paper, and then by wheel polishing and hand polishing with an alumina/water slurry. Subsequently, the electrode was cleaned electrochemically by cycling repeatedly in 1 M H_2SO_4 (Aristar grade) between −450 and +1250 mV. The freshly cleaned electrode was modified either by dipping into a 1 mM solution of pySSpy for 2–3 min or by cycling between −250 and −1100 mV for 10 successive scans in a saturated peptide solution.

Results

(1) Cytochrome c/Plastocyanin Complex. (i) Cytochrome c Selective Surfaces. From Table I, the simplest and most reproducible cytochrome c selective surface is polished edge-plane pyrolytic graphite, at which cytochrome c exhibits quasi-reversible electrochemistry but plastocyanin does not at low ionic strength and in the absence of multivalent cations such as $Cr(NH_3)_6^{3+}$ (Figure 1A,B). Figure 1C shows a cyclic voltammogram of a 1:1 mixture of fully oxidized cytochrome c and plastocyanin at low ionic strength. The response consists of two overlapping and symmetrical waves, and the midpoint potentials of each wave are estimated to be +270 and +340 mV, respectively. The lower potential is in good agreement with the cytochrome c redox potential value, but the second potential is somewhat lower than that of plastocyanin obtained by $Cr(NH_3)_6^{3+}$-promoted electrochemistry (+370 mV; see Figure 1B).

The cytochrome c/plastocyanin mixture was further investigated by using Zn(II) cytochrome c instead of Fe(III) cytochrome c. Zn(II) cytochrome c is redox-inactive between −550 and +850 mV and is structurally identical with the native protein (Moore et al., 1980). Figure 2 shows the cyclic voltammograms resulting from a 1:1 mixture of Zn(II) cytochrome c and plastocyanin at an EPG electrode. Only one wave is observed, with a midpoint potential of +345 mV. This is ascribed to plastocyanin. The peak currents are proportional to the plastocyanin concentration rather than the Zn(II) cytochrome c concentration. The shape of the voltammograms changes from sigmoidal to peak shaped (represented by the

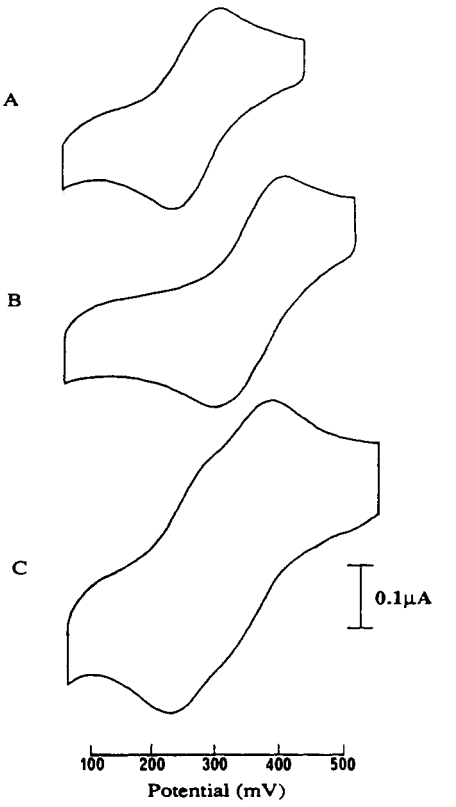


FIGURE 1: Cyclic voltammograms of (A) cytochrome c (50 μM), (B) plastocyanin (50 μM) and $Cr(NH_3)_6^{3+}$ (2 mM), and (C) cytochrome c and plastocyanin (50 μM each) at an edge-plane graphite electrode; 20 mM cacodylate, pH 7.0. Scan rate: 5 mV s^{-1}.

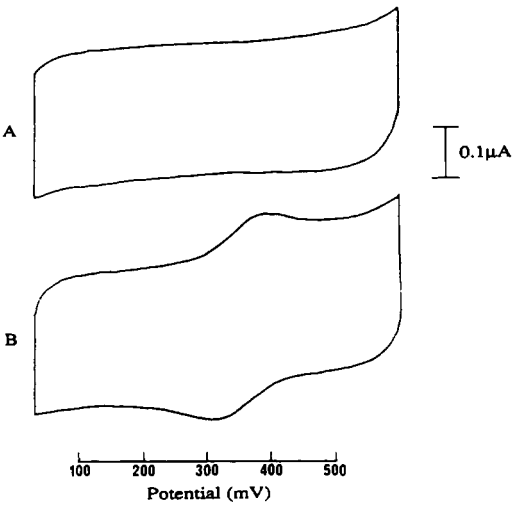


FIGURE 2: Promotion of plastocyanin electrochemistry by Zn(II) cytochrome c at an edge-plane graphite electrode; 20 mM cacodylate, pH 7.0. (A) Zn(II) cytochrome c alone (75 μM). (B) Zn(II) cytochrome c and plastocyanin (34 μM each). Scan rate: 10 mV s^{-1}.

decrease in "peak" separation shown in Figure 3A) as the Zn(II) cytochrome c to plastocyanin ratio increases. This change in shape is the result of a change from radial to linear diffusion as the number of surface-active sites [in this case, Zn(II) cytochrome c molecules] increases (Armstrong et al., 1989). No improvement in response is observed at ratios of Zn(II) cytochrome c to plastocyanin of greater than 1:1. The plot of peak current, i_p, against the square root of the scan rate, $\nu^{1/2}$ (Figure 3B), is linear up to ν = 100 mV s^{-1} only when the ratio of Zn(II) cytochrome c to plastocyanin is 1:1. Only in this case is the method of Nicholson and Shain (1964) used to determine the diffusion coefficient, D_0, and heterogeneous

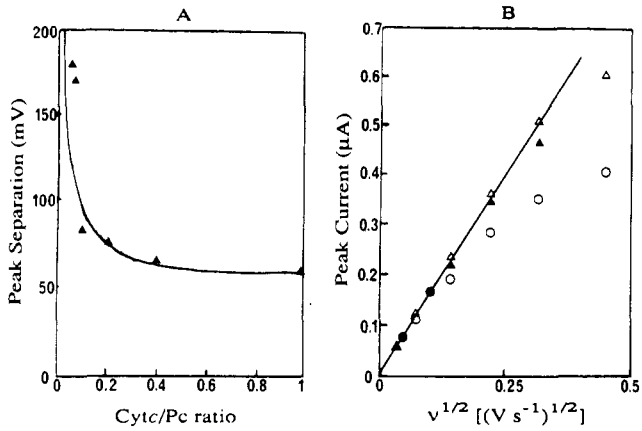


FIGURE 3: Zn(II) cytochrome *c* promoted plastocyanin electrochemistry; 20 mM cacodylate, pH 7.0. Ratios: open triangle = 1:1; filled triangle = 1:6; open circle = 1:12. (A) Variation of peak separation with Zn(II) cytochrome *c*/plastocyanin ratio. Scan rate: 20 mV s^{-1}. (B) Variation of peak current with the square root of scan rate, $\nu^{1/2}$, at different Zn(II) cytochrome *c*/plastocyanin ratios.

electron-transfer rate constant, k_s. [The criterion of linearity of the plot of i_p versus $\nu^{1/2}$ up to ν = 100 mV s^{-1} for the validity of the Nicholson and Shain (1964) theory is applied throughout this work.] The calculated values of D_o and k_s are 3.4×10^{-7} cm^2 s^{-1} and 6.3×10^{-3} cm s^{-1}, respectively, and are similar to those measured for plastocyanin in the presence of $Cr(NH_3)_6^{3+}$. There is no difference in the response for the 1:1 mixture at KCl concentrations of 1, 5, 10, 20, or 50 mM (constant 20 mM cacodylate), suggesting that the results obtained are independent of the extent of complexation between these two proteins.

It is thus concluded that the higher potential wave in the cyclic voltammograms of the Fe(III) cytochrome *c*/plastocyanin mixture is due to plastocyanin: the electrochemistry of plastocyanin is promoted by the presence of cytochrome *c* at a surface to which cytochrome *c* binds reversibly but plastocyanin does not.

Two modified gold surfaces which are electrostatically compatible with cytochrome *c* (i.e., of opposite charge to cytochrome *c*) were also used to study the electrochemistry of the cytochrome *c*/plastocyanin mixture. At a $(Cys\text{-}Glu)_2$-modified electrode the promotion of plastocyanin electrochemistry by cytochrome *c* was again achieved and the diffusion coefficient and heterogeneous electron-transfer rate constant (2.4×10^{-7} cm^2 s^{-1} and 4.0×10^{-3} cm s^{-1} for a 1:1 solution of the two proteins) are quite close to those obtained with an EPG electrode. However, the responses with a pySSpy-modified gold electrode (Taniguchi et al., 1982) are rather different in that only a small cathodic wave at +200 mV is observed in a 1:1 Zn(II) cytochrome *c*/plastocyanin solution. This wave is not present when fully reduced plastocyanin is used and is thus attributed to the irreversible reduction of fully oxidized protein. It is clear that Zn(II) cytochrome *c* does not promote reversible electrochemistry of plastocyanin at a pySSpy-modified gold electrode, presumably because plastocyanin is binding to cytochrome *c* at the electrode surface but not in an orientation conducive to fast, reversible heterogeneous electron transfer.

The electrochemical behavior of the Fe cytochrome *c*/plastocyanin mixture at a pySSpy-modified gold electrode is complicated by the relatively fast homogeneous reaction between these two proteins and is discussed in detail elsewhere (Barker et al., 1989).

(*ii*) *Plastocyanin-Selective Surface.* A $(Lys\text{-}Cys\text{-}OMe)_2$-modified gold electrode was used as the plastocyanin-selective surface to investigate the promotion, by plastocyanin, of cytochrome *c* electrochemistry. Fully oxidized plastocyanin was added to Fe(III) cytochrome *c*, giving ratios ranging from 1:10 to 1:1. At a 1:10 ratio there is a poor cathodic wave accompanied by a better defined anodic wave on the first scan, but on following scans both waves increase in size until a steady state is reached. At this stage, the midpoint potential of this redox couple was measured to be +249 mV, suggesting that the response is due to cytochrome *c* electron transfer.

Table II: Midpoint Potentials (mV) of Complex Components at Various Electrodes

		midpoint potential		
	complexes	*a*	*b*	*c*
EPG	Fe^{III}cyt *c*/Pc	~+270	+340	
EPG	Zn^{II}cyt *c*/Pc		+345	
EPG	Fe^{III}cyt *c*/Pc[d]	~+270	~+340	
$Au\text{-}(Lys\text{-}Cys\text{-}OMe)_2$	Pc/cyt *c*	+250	~+370	
$Au\text{-}(Lys\text{-}Cys\text{-}OMe)_2$	Hg^{II}Pc/cyt *c*	+267		
EPG	Fe^{III}cyt *c*/cyt b_5	+270		-5
EPG	Zn^{II}cyt *c*/cyt b_5			-5
$Au\text{-}(Lys\text{-}Cys\text{-}OMe)_2$	cyt b_5/cyt *c*	+247		

[a] Midpoint potential associated with cytochrome *c*. [b] Midpoint potential associated with plastocyanin. [c] Midpoint potential associated with cytochrome b_5. [d] The cross-linked proteins.

As with cytochrome *c* selective surfaces, at high plastocyanin to cytochrome *c* ratios (for example, 1:1) two overlapping waves due to cytochrome *c* and plastocyanin are observed at a $(Lys\text{-}Cys\text{-}OMe)_2$-modified gold electrode. The midpoint potentials in the mixture were estimated to be lower than those of the individual proteins and are shown in Table II.

A single wave with a midpoint potential of +267 mV results from a mixture of Hg(II)-substituted plastocyanin and cytochrome *c* at a $(Lys\text{-}Cys\text{-}OMe)_2$-modified gold surface. As Hg(II)-substituted plastocyanin is electroinactive in the potential region of interest, the wave must be due to cytochrome *c*. This result confirms that the cytochrome *c* response at a $(Lys\text{-}Cys\text{-}OMe)_2$-modified gold surface is due to promotion rather than mediation by plastocyanin.

An alternative method of investigating the promotion of cytochrome *c* electrochemistry involved dipping the modified electrode in 100 μM plastocyanin for 5 min and then rinsing it with deionized water before placing it into the cytochrome *c* solution. A stable, well-defined redox wave appears at the first scan, in contrast to the response obtained from a 1:10 mixture of plastocyanin and cytochrome *c* in solution. (The latter is reported in the first paragraph of this section.) No response is observed when the modified electrode is dipped in plastocyanin solution, rinsed with water, and then transferred into buffer solution, indicating that only a very small amount of plastocyanin adsorbs at the electrode.

By comparison of the results obtained from the two different experiments mentioned above, it is proposed that, when the $(Lys\text{-}Cys\text{-}OMe)_2$-modified gold electrode is dipped into a solution of plastocyanin, the protein adsorbs weakly at the electrode surface. Promotion of cytochrome *c* electrochemistry then occurs by complexation with adsorbed plastocyanin. The improvement in the response obtained from a 1:10 solution mixture of the two proteins with time is tentatively ascribed to accumulation of plastocyanin at the electrode surface.

(*2*) *Cytochrome c/Cytochrome b_5 Complex.* (*i*) *Cytochrome c Selective Surfaces.* Titrations of cytochrome *c* with cytochrome b_5 at ratios from 1:10 to 2:1, similar to those with plastocyanin, were carried out at both edge-plane graphite and $(Cys\text{-}Glu)_2$-modified gold electrodes. Figure 4A–C shows the cyclic voltammograms of a 1:1 mixture of the two proteins as well as each protein alone at the graphite electrode. The two

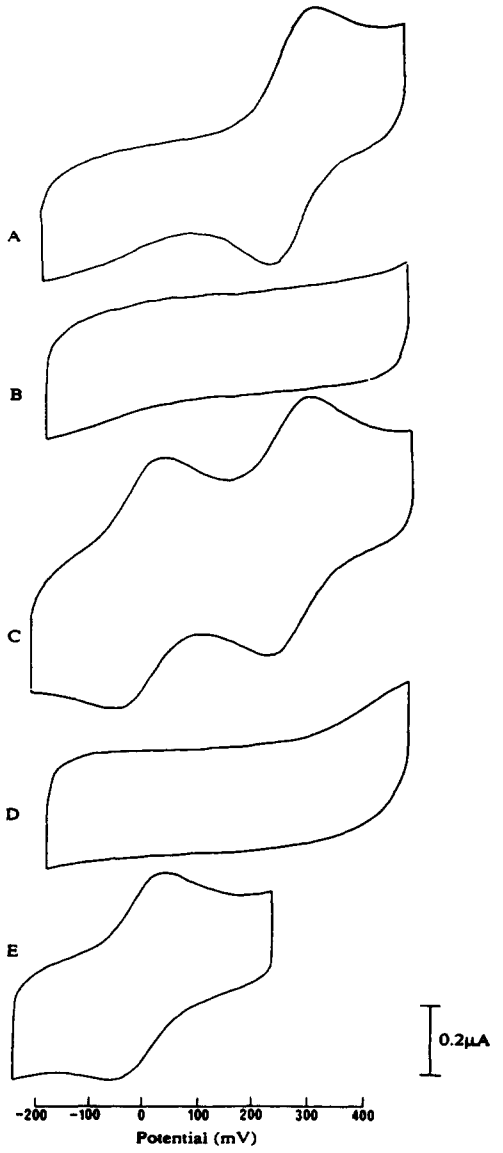


FIGURE 4: Cyclic voltammograms of (A) cytochrome *c* alone (95 μM), (B) cytochrome b_5 alone (95 μM), (C) cytochrome *c* with cytochrome b_5 (95 μM each), (D) Zn(II) cytochrome *c* alone (75 μM), and (E) cytochrome b_5 with Zn(II) cytochrome *c* (75 μM each) at an edge-plane graphite electrode; 1 mM HEPES/20 mM KCl, pH 7.0. Scan rate: 10 mV s^{-1}.

separate redox processes are well resolved because of the large midpoint potential difference between these two proteins (see below).

From the peak-shaped voltammograms, the measured midpoint potentials for cytochrome *c* and cytochrome b_5 are +270 and −5 mV, respectively. Titration experiments also suggest that the peak separations at a given scan rate decrease as the cytochrome *c* to cytochrome b_5 ratio increases until the value is 1:1, as observed for the cytochrome *c*/plastocyanin complex.

Figure 4D,E depicts the same experiments carried out with Zn(II) cytochrome *c*. It can be clearly seen that cytochrome *c* is promoting the electrochemistry of cytochrome b_5, rather than acting as a mediator of electrons to it.

(*ii*) *Cytochrome b_5 Selective Surface.* The experiments with the cytochrome *c*/plastocyanin mixture at a (Lys-Cys-OMe)$_2$-modified gold electrode were repeated by using a cytochrome *c*/cytochrome b_5 mixture. As with plastocyanin, promotion of cytochrome *c* electrochemistry is observed either by weakly adsorbed cytochrome b_5 or by "free" protein in solution.

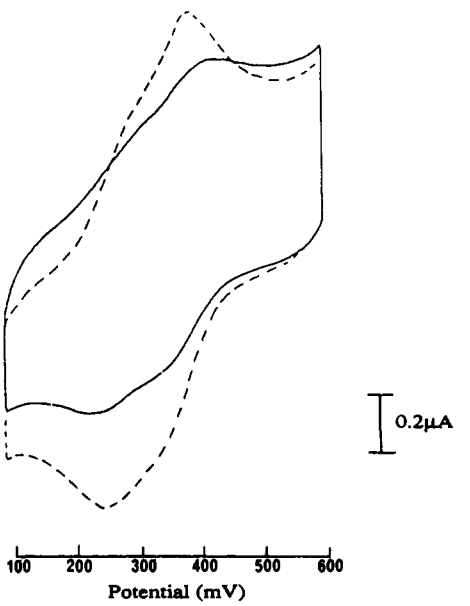


FIGURE 5: Cross-linked (1:1) cyt *c*/Pc (—) and a 1:1 mixture of "free" proteins (---) at an EPG electrode (50 μM); 20 mM cacodylate, pH 7.0. Scan rate: 10 mV s^{-1}.

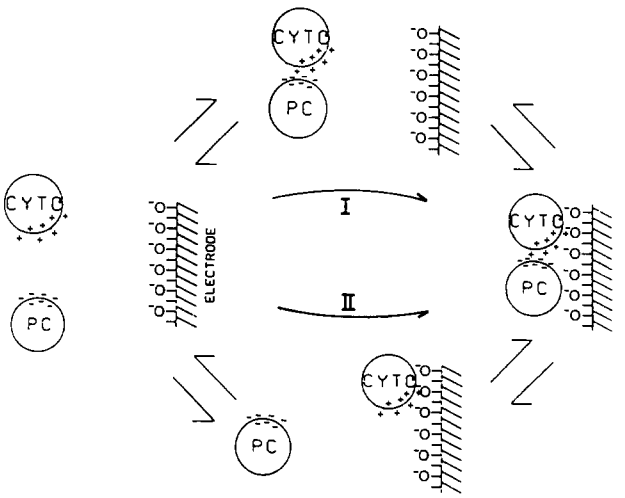
(*3*) *Electrochemistry of Cross-Linked Cyt c/Pc Complexes as a Probe of Dynamics at Electrode Surfaces.* It has been recently proposed [e.g., Mauk et al. (1986)] that protein–protein interactions are highly dynamic and that complexes of different orientations may exist between two interacting proteins. To see if cross-linking the two proteins inhibits electrochemical response, covalently linked cyt *c*/Pc complexes were prepared and studied. The voltammetric responses of the covalent complex at a graphite electrode are shown in Figure 5. Also shown is the response of the "free" mixture of 1:1 cyt *c*/Pc. The currents are significantly smaller in the case of the covalent complex. It is difficult to quantify the response, but the linked proteins still give two overlapping waves, and the more positive wave has a higher midpoint potential than that in the "free" mixture.

(*4*) *Promotion by Other Proteins.* The observation that the electrochemical promotion of one of the complex components by the other is not ionic strength dependent and therefore is not influenced by the extent of complexation between the proteins in solution suggests that the complex formation at the electrode could be nonspecific. To investigate this possibility, proteins without redox centers were tested as possible promoters of plastocyanin electrochemistry. These include lysozyme (M_r 14000, p*I* 11.2), ribonuclease (M_r 13000, pI 8.0), histone f_3 (M_r 15000, p*I* 11.0), albumin (M_r 43000, p*K* 6.5), and trypsin inhibitor (M_r 23000, p*I* 4.5).

Of these proteins, only the basic ones act as promoters of plastocyanin electrochemistry at an EPG electrode. The midpoint potentials are closer to 370 mV [the value obtained from plastocyanin electrochemistry with $Cr(NH_3)_6^{3+}$ as promoter] than to 340 mV (the value obtained with cytochrome *c* as promoter). In the case of histone promotion, the plastocyanin response reaches its maximum at 1:5 histone to plastocyanin and then declines at higher ratios, presumably due to the blockage of the electrode surface by strongly adsorbed histone molecules. It is therefore reasonable to assume that, for plastocyanin electrochemistry to occur, plastocyanin should bind to the basic proteins at the electrode in a side-by-side fashion.

The fact that no plastocyanin response is observed in the presence of neutral and acidic proteins confirms that the promotion of plastocyanin electrochemistry by basic proteins

Scheme I



is not simply due to the denaturation of the polypeptides at the electrode surface. It is not known whether these basic proteins form stable electrostatic complexes with plastocyanin, but they do not have any specific binding sites for other proteins, and any complexes that do form will probably not have very high association constants. Indeed, lysozyme has a uniform distribution of basic groups on its surface (Delepierre et al., 1987).

Discussion

Selective electrode surfaces have been used to probe the electrochemical behavior of one of the two components in a protein–protein complex, and two effects have been observed. First, the protein that cannot bind to the electrode in a manner favorable for heterogeneous electron transfer binds to the other protein at the electrode surface in such a way that its redox center is still available for heterogeneous electron transfer. Thus the electrochemistry of the initially unresponsive protein is promoted by a second protein that binds productively to the electrode. Alternatively, as with the cyt *c*/Pc mixture at a pySSpy-modified gold electrode, the unresponsive protein (plastocyanin) may not bind to the other at the electrode, or if it does, it is unfavorably disposed to the electrode for direct electron transfer. A pySSpy-modified gold electrode differs from the EPG and peptide-modified gold electrodes in that it presents a weakly basic hydrogen-bonding surface while the others have ionized negative surfaces, so it is perhaps not surprising that different electrochemical characteristics are exhibited.

Obviously, for a certain diprotein complex, isomers of different conformations may exist at an electrode. Some are favorable for homogeneous and some for heterogeneous electron-transfer reactions. Which of these two processes dominates depends on the two proteins involved as well as the electrode surface used. The possibility cannot be ruled out that the isomers favorable for direct electron transfer between either protein and the electrode are quite different from those favorable for fast intracomplex electron transfer.

Consider the mixture of cytochrome *c* and plastocyanin at an EPG electrode. Cytochrome *c* binds reversibly to the electrode surface, but plastocyanin does not. Therefore, for plastocyanin to be favorably disposed at the electrode, it must bind to cytochrome *c* at that surface in a side-by-side fashion. There are two possible pathways by which the complex could form at the electrode, as depicted in Scheme I. The complex could form in solution and then diffuse to the electrode surface where it binds reversibly. Alternatively, cytochrome *c* could bind first to the electrode followed by plastocyanin binding to cytochrome *c*. The fact that the promotion effect is independent of the extent of complexation between the proteins and that the promoted plastocyanin responses depend on the concentration of plastocyanin alone suggests that diffusion of the complex to the electrode is not responsible for the promotion effect, and therefore, the second pathway looks more reasonable. This suggestion is supported by the observation [see Results, section 1(ii)] that cytochrome *c* electrochemistry promoted by plastocyanin depends on the amount of plastocyanin at the electrode surface.

For electrochemical promotion of one protein by another, the formation of a ternary complex of protein–protein–electrode must be considered. The question arises as to how one protein can bind to *both* the electrode and the other protein in such a way as to present its redox center for heterogeneous electron transfer. In a static, computer-generated model of the cyt *c*/cyt b_5 complex (Salemme, 1976), the two heme surfaces are masked, so it would be impossible for one protein to bind simultaneously to the other protein and the electrode in an orientation conducive to heterogeneous electron trnsfer. However, there has been an increasing body of evidence [e.g., Mauk et al. (1986)] that protein–protein complexes are highly dynamic and that Salemme's model represents one of the most stable of a range of orientations of the complex of similar energy. Two limiting cases of the cyt *c*/cyt b_5 interaction (Eltis et al., 1988) show that, in the complex, both heme groups are exposed to the solvent. This fact, in addition to the flexibility of the complementary electrostatic interactions in the complex (Wendoloski et al., 1987) and the relatively low recognition selectivity of the functionalized electrode surfaces, makes it highly possible that either cytochrome *c* or cytochrome b_5 can bind productively to the other protein and the electrode simultaneously. A similar argument can be applied to the cyt *c*/Pc system, although less information on this complex is available at present.

The results of experiments on the covalently linked cyt *c*/Pc complexes can also be interpreted in terms of the dynamic nature of protein–protein interactions. The decrease in electrochemical response of the covalently linked complex compared to that of the electrostatic complex cannot be solely ascribed to the heterogeneity of the covalent complex, nor to the increase in molecular mass of the diffusing species in solution (the covalent complex is about 2 times larger than a single protein). Rather, the reduction in electrochemical activity is probably largely due to the limitation of relative movement of the two proteins within the covalent complex. It has been shown (Geren et al., 1983) that, when cross-linked to plastocyanin, cytochrome *c* can still interact with small reductants but not with physiological partners such as succinate–cytochrome *c* reductase and cytochrome *c* oxidase, indicting that the heme crevice of cytochrome *c* is inaccessible to large enzymes but not to small molecules. In this case, the requirements of interaction specificity between physiological redox partners cannot be met. Since a nonzero electrochemical response from a similarly linked complex has been observed at a cytochrome *c* selective electrode, it appears that the redox centers of the linked cyt *c*/Pc complex are not completely masked but are significantly less accessible than those of the electrostatic complex. The lower interaction specificity of the edge-plane graphite electrode relative to that of an enzyme means that the partly exposed lysine residues around the heme crevice of the covalently linked cytochrome *c* are sufficient to allow effective binding of the protein to the electrode.

The basic proteins lysozyme, ribonuclease, and histone f_3 have been shown to promote the electrochemistry of plastocyanin. The uniform surface distribution of basic residues on lysozyme (Delepierre et al., 1987) indicates that interaction between regions consisting of high concentrations of charged residues is not essential to the promotion effect.

Electrode interfaces are similar to biological membrane interfaces in that both are electically charged and have layers of polarized water molecules. Therefore, the electron-transfer processes of proteins at modified electrode surfaces could be compared to the events associated with biological membranes. Similar comparison could be made between the protein–protein complexes at electrode surfaces and those associated with biological membranes. In making these comparisons, however, it should be remembered that we are probably some way from reproducing biological specificity at an electrode surface.

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