Diffusionless Electrochemistry of Cytochrome b_5 Adsorbed on a Multilayer Film Electrode

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A multilayer film electrode has been developed that is well-suited for diffusionless electron transfer studies of acidic proteins. The multilayer electrode is comprised of trypsin-solubilized cytochrome b_5 adsorbed electrostatically to a self-assembled $HO_2C(CH_2)_7SH$/gold substrate via an intervening bridging layer of cationic poly-L-lysine.

Voltammetry has emerged over the past twenty years as an increasingly powerful tool for the characterization of redox proteins. By choosing to examine the electrochemistry of proteins in the adsorbed state,[1-3] one can realize certain advantages relative to ET kinetic studies performed in other formats. In particular, potential control affords the experimenter a continuously variable driving force, the electrode reagent exhibits no reorganization energy of its own, and the absence of diffusional limitations leads to a simplified kinetic analysis devoid of complications arising from adsorption/desorption processes.[4]

In previous work, our attention has been focused primarily upon a basic protein, horse cytochrome *c* (cyt *c*), immobilized by adsorption on carboxylic acid terminated self-assembled monolayers (SAMs) of the alkylthiol/gold type.[1,5] The cyt *c*/SAM/gold configuration has proven to be a useful model system for the investigation of cationic protein monolayer electrochemistry.

In the present contribution, we report a complementarily charged model system for the investigation of anionic protein monolayers. The acidic protein, recombinant trypsin-solubilized cytochrome b_5 (cyt b_5),[6,7] a 10062 dalton, hydrophilic, heme-containing fragment of cytochrome b_5, was chosen for this work. This fragment is negatively charged at pH 7 and exhibits several aspartate and glutamate residues clustered on the protein surface near the exposed heme edge. This cluster of residues, along with possibly the two heme propionates, comprise a negatively charged binding domain for interaction with oppositely charged redox partners or biocompatible surfaces.

Figure 1 is a proposed schematic representation of the cationic multilayer film electrode that has been constructed for adsorptive immobilization of cyt b_5. After first preparing a COOH-terminated alkylthiol SAM on gold, an overlayer of poly-L-lysine (PL) can be electrostatically adsorbed atop the SAM, onto which the cyt b_5 can subsequently be adsorbed. Previous characterization of the adsorption of PL onto alkanethiol modified gold surfaces by Corn and coworkers[8] suggests the roughly parallel orientation of PL shown in Figure 1. Some of the lysine residues form ammonium-carboxylate ion pairs with the terminal acids of the SAM, while other lysine residues remain free in the surrounding solution and presumably available for binding to cyt b_5.

To prepare this multilayer film, an evaporated gold mirror electrode (area = 0.32 cm^2) was first assembled into a cell[9] and contacted by a solution of 0.1 M H_2SO_4/0.01 M KCl. Electrochemical pretreatment was carried out in which anodic cycling to a final potential of 1.50 V vs Ag/AgCl resulted in a fresh gold/solution interface.[10] After rinsing, refilling with 0.5 M NaF and deaerating, background cyclic voltammograms were acquired. The SAM was then formed during a 3-day exposure to 5 mM 8-mercaptooctanoic acid [$HS(CH_2)_7COOH$] in absolute ethanol, after which additional cyclic voltammograms were acquired in deaerated 8.8 mM sodium phosphate buffer (pH 7.0, 20 mM ionic strength) (See Figure 2, "a"). All procedures were carried out at room temperature. The double-layer capacitance calculated from cyclic voltammograms was 5.3 ± 1.1 μF/cm^2. Next, 60 μL of ~1 mM poly-L-lysine (Sigma, average molecular weight of 3970 daltons) in 8.8 mM sodium phosphate buffer (pH 8.5) was introduced into the cell for 30 minutes. The cell was subsequently rinsed, refilled with buffer, deaerated, and cyclic voltammograms were again acquired. PL adsorption usually caused a slight decrease of C_{dl} (10% on average, Figure 2

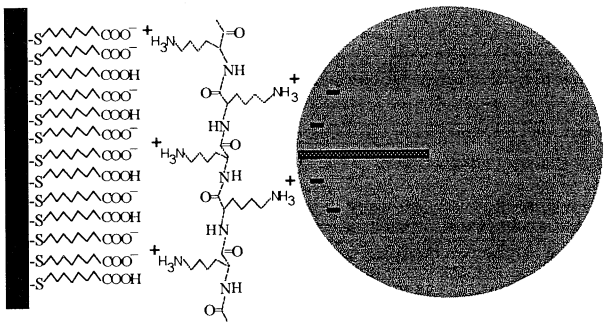

Figure 1. Schematic representation of the proposed cyt b_5/PL/SAM/gold structure. Horizontal bar represents heme in cyt b_5.

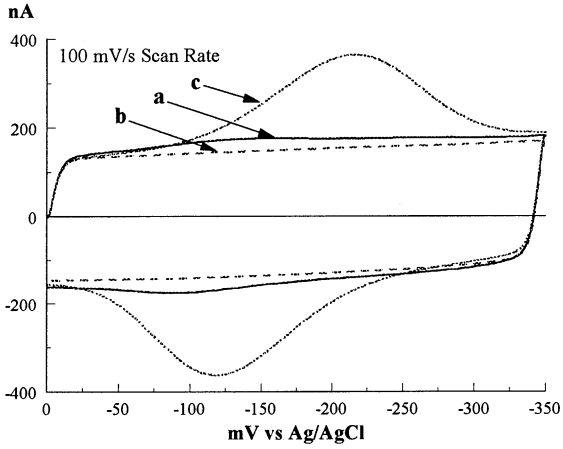

Figure 2. Cyclic voltammetric response at various steps in the multilayer film preparation. Solution: 8.8 mM sodium phosphate (pH 7.0, 20 mM ionic strength). (a) SAM/gold; (b) PL/SAM/gold; (c) cyt b_5/PL/SAM/gold.

"b"). 30 μL of 13.5 μM cyt b_5 (purity ratio $A_{412}/A_{280} > 5.7$) in sodium phosphate buffer (pH 7.0, 20 mM ionic strength) was then added directly atop the multilayer modified Au electrode for 30 min. Protein concentrations were determined spectrophotometrically ($\varepsilon_{412.5}$ = 117 mM^{-1}).[6] After rinsing the cell and refilling with buffer, cyclic voltammetry revealed a well-behaved and reproducible faradaic response (Figure 2, "c") that we attribute to electron transfer between the Au electrode and the adsorbed cyt b_5 through the PL monolayer and SAM. The electrochemical response reflects the diffusionless reduction and oxidation of an adsorbed (sub)monolayer of cyt b_5 (7-9 pmol/cm^2) and is stable for several hours at room temperature.

Figure 3 shows typical voltammetric responses of adsorbed cyt b_5 at three scan rates. For an ideal, reversible, one-electron

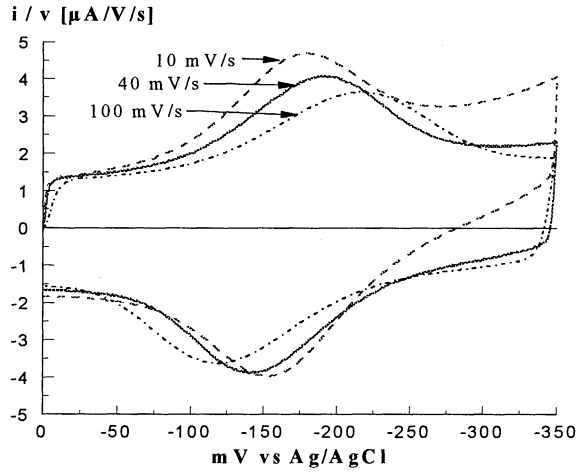

Figure 3. Cyclic voltammetry of cyt b_5/PL/SAM/gold. Current is normalized with respect to potential scan rate v (mV/s). 8.8 mM sodium phosphate (pH 7.0, 20 mM ionic strength).

redox adsorbate, the full-width-at-half-maximum (FWHM) value is 90 mV. Reversible FWHM values for the cyt b_5/PL/SAM/gold system were determined to be 90-100 mV, indicating only a slight E°′ dispersion. The formal potential value, 40 mV vs NHE, falls somewhat positive of the published value of ~ 9 mV vs NHE[11] for similar experimental conditions. This positive shift indicates some destabilization of ferricytochrome b_5 relative to ferrocytochrome b_5 as a consequence of adsorption. As scan rate was increased, the cyclic voltammetric response became quasi-reversible. Using Laviron's analysis,[12] an apparent ET rate constant at zero driving force of $k_{et}° = 1.2 \pm 0.3$ s^{-1} was determined.

Armstrong has previously shown that monolayers of acidic proteins, namely ferredoxins, can be prepared on edge pyrolytic graphite using co-adsorbing aminocyclitols as promoters.[3] In the present work, we have demonstrated an alternative interface that features a substantially lower interfacial capacitance, a poly-L-lysine-bridged self-assembled monolayer modified electrode. Currently, the role of alkane chainlength and the chemical nature of the electrostatic bridge on ET kinetics are under investigation. Newly synthesized aminothiols previously unavailable, are also being examined as cationic surface modifiers.

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