VOLTAMMETRIC STUDIES OF INTERPROTEIN ELECTRON TRANSFER

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PREFACE

VOLTAMMETRIC STUDIES OF INTERPROTEIN ELECTRON TRANSFER

A number of chemical processes such as respiration, photosynthesis, nitrogen fixation, *etc.* occur in biological systems. Such reactions typically involve transfer of electrons between two redox centers. Therefore, there has been a widespread interest in developing methods to determine electron transfer rate constants between physiological partner proteins, in order to understand biological reaction rates in more detail. The electron transfer reaction for the transfer of an electron from ferrocytochrome b_5 (a heme-containing protein) to ferricytochrome c has been studied by earlier approaches such as flash photolysis in 1993, formation of a photoactive complex of cyt. b_5 in 1995.[1, 2] More recently, we proposed cyclic voltammetry as a viable approach. To this end, a mini gold electrode modified with β-mercaptopropionate, was used to determine protein-protein electron transfer rate constants.[3] When cyclic voltammetry was carried out on a solution containing a mixture of cytochrome b_5, cytochrome c, and the polycation, polylysine, it was found that cytochrome b_5 undergoes reversible electrochemistry at the modified electrode surface, while the electrode surface discriminates against cytochrome c. Modifying the electrode surface, therefore, provides a versatile method for selectively reducing a protein possessing a relatively low reduction potential (outer mitochondrial membrane cytochrome b_5, E° =-102 mV) in the presence of another protein possessing a

relatively high reduction potential (horse heart cytochrome c, E^{o} = 265 mV). This provides an alternative system for the transfer of an electron to a protein possessing a net negative charge in the presence of a protein possessing a net positive charge. An electrochemical-chemical reaction mechanism was proposed to account for the shape of the cyclic voltammogram obtained:

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

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

This reaction scheme was explicitly simulated, and a value of $k_f = 2.9 \times 10^8\ M^{-1}s^{-1}$ obtained, which was in good agreement with the value obtained previously by flash photolysis.[1] Further, cyclic voltammetric and spectroelectrochemical studies[4] of the wild type protein and its dimethyl ester have been carried out to investigate the factors that play a role in modulating the reduction potential of the wild type protein. The results of these studies may pave way for more investigative studies to explore the possibility that the reduction potential of cytochrome b_5 maybe modulated upon formation of a transient complex with cytochrome c.

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4) Rivera, M., Seetharaman, R., Girdhar, D., Wirtz, M., Zhang, X., Wang. X., & White, S (1998) *Biochemistry 37*, 1485.

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INTRODUCTION

Cytochrome b_5 is a heme-containing protein. The structure of heme is shown in Fig. 1.1. Cytochrome b_5 functions as an intermediate carrier in the electron transfer pathway from NADPH to fatty acyl CoA in the microsomal membrane [1] where it is anchored by means of a hydrophobic tail. Cytochromes b_5 maybe classified into:

(a) Microsomal cytochrome b_5, the genes coding for the microsomal rat liver and the microsomal bovine liver proteins have been cloned and expressed in *E. coli* [2, 3].

(b) Erythrocyte cytochrome b_5, a water-soluble protein in which the hydrophobic tail used to anchor the protein to the microsomal membrane is absent [4].

(c) Outer Mitochondrial Membrane (OM) cytochrome b_5 (cyt. b_5), isolated from the outer membranes of rat liver mitochondria by proteolytic cleavage [5] and by detergent solubilization [5 a]. The structure of OM cytochrome b_5 is shown in Fig. 1.2. The molecule is shown in the form of a space-filling diagram. The negatively charged amino acid residues (shown in red) present on the surface of the molecule imparts the molecule with a net negative charge. The heme is shown in blue. Fig. 1.2 a shows the views of the exposed and buried heme edge in OM cyt. b_5. OM cytochrome b_5 has not been as extensively studied as the microsomal protein. The proteolytically cleaved OM cytochrome b_5 was purified and proved to be distinct from the microsomal protein isolated from rat liver [6], and later shown to be 58% homologous to microsomal

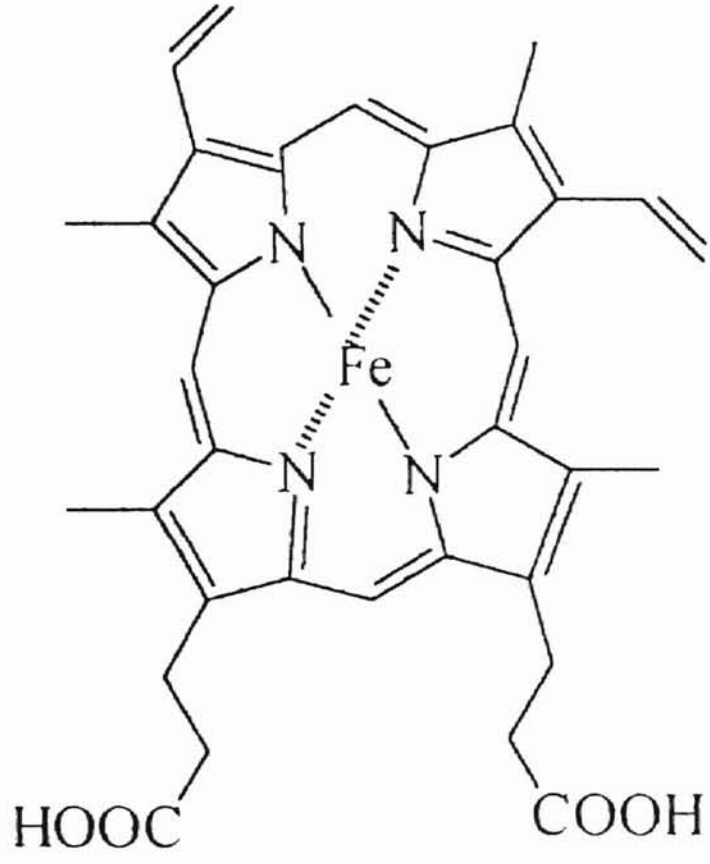


Fig. 1.1 Schematic representation of the heme in the outer mitochondrial membrane cytochrome b_5.

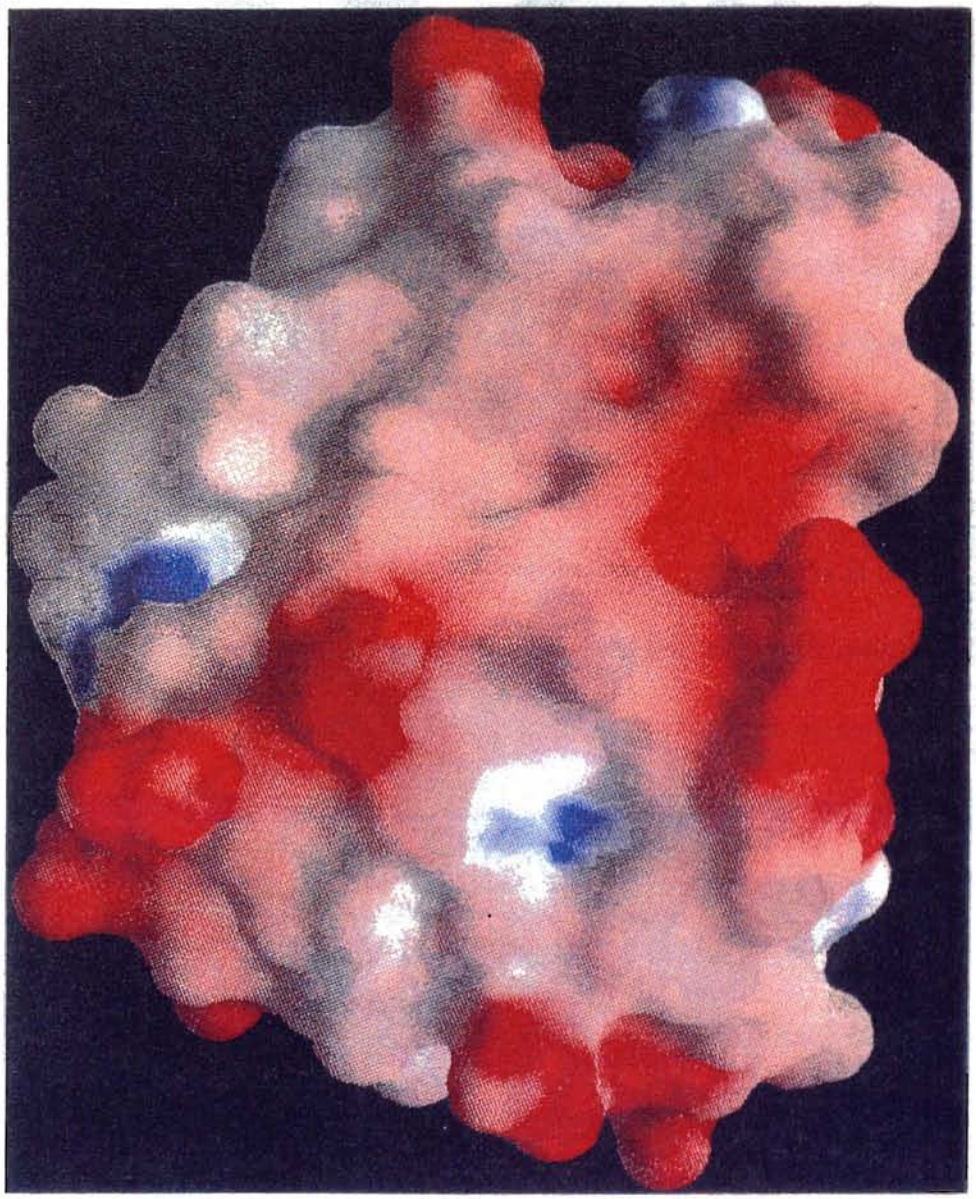


Fig. 1.2 Structure of OM cytochrome b_5.

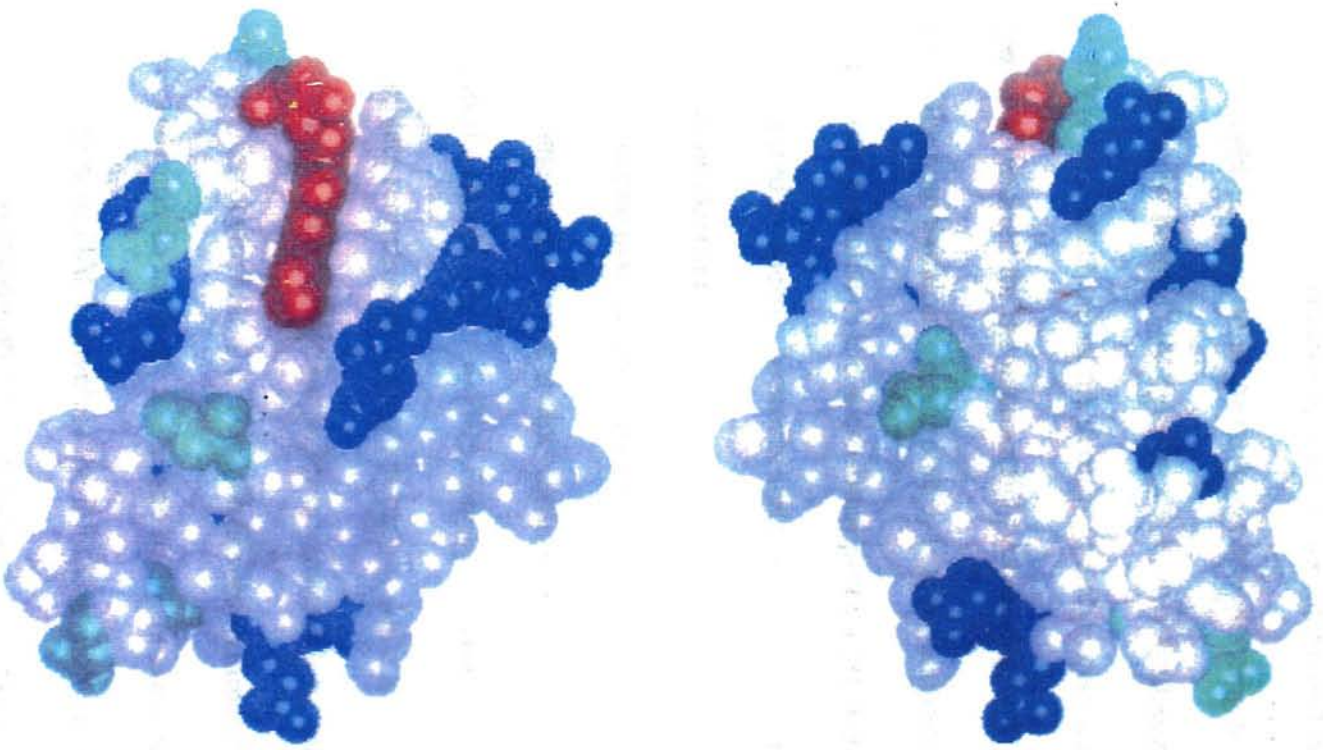


Fig. 1.2 a Views of the exposed and buried heme edge
in OM cytochrome b_5.

cytochrome b_5 [7]. It is important to note that most of the differences between OM cytochrome b_5 and microsomal 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 OM cytochrome b_5 was synthesized and expressed in *E. coli* [8]. The UV-visible spectrum of the overexpressed protein was shown to be identical to the spectrum reported for the microsomal protein (Fig. 1.3). However, the reduction potential of the mitochondrial cytochrome b_5 is approximately 100 mV more negative than the reduction potential of the microsomal protein [9]. The value of the reduction potential measured by spectroelectrochemistry is -102 mV for OM cytochrome b_5 *vs* the standard hydrogen electrode (SHE).

It is important to notice that rat OM cytochrome b_5 participates in electron transfer from NADH to cytochrome oxidase, with another small size and stable protein, viz., cytochrome c acting as the shuttle (Fig. 1.4) [10]. During oxidation of exogenous NADH there is a fast and complete reduction of ferricytochrome b_5 while endogenous ferricytochrome c is reduced. The reoxidation of ferrocytochrome b_5, after exhaustion of NADH, precedes that of ferrocytochrome c. These observations support the view of an electron transfer from the outer to the inner membrane of intact mitochondria. The aerobic oxidation of exogenous NADH involves the following pathway: NADH → NADH-cytochrome b_5 reductase → OM cytochrome b_5 → cytochrome c → cytochrome oxidase → oxygen. Thus, cytochrome c shuttles the electron across the intermembrane space in order to reduce ferricytochrome c oxidase in the inner mitochondrial membrane. This maybe further corroborated by the fact that a form of NADH dehydrogenase having the same

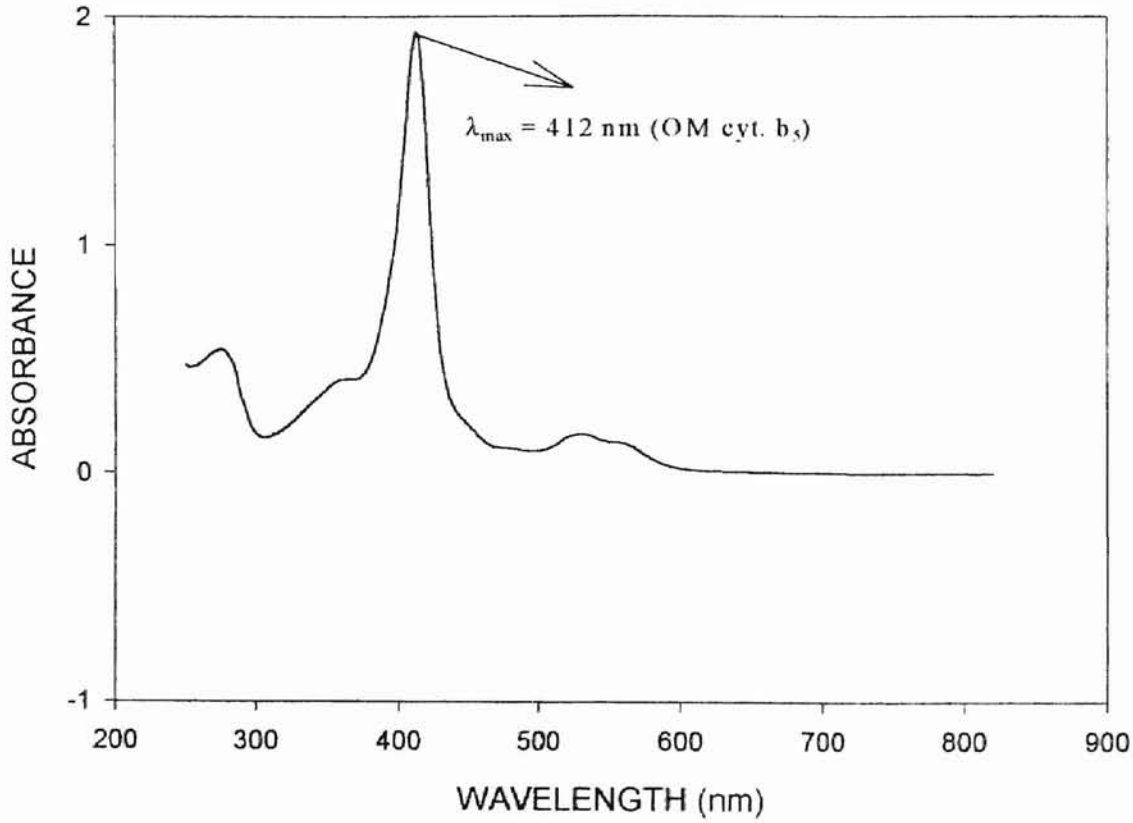


Fig. 1.3 UV-visible spectrum of OM cyt. b_5.

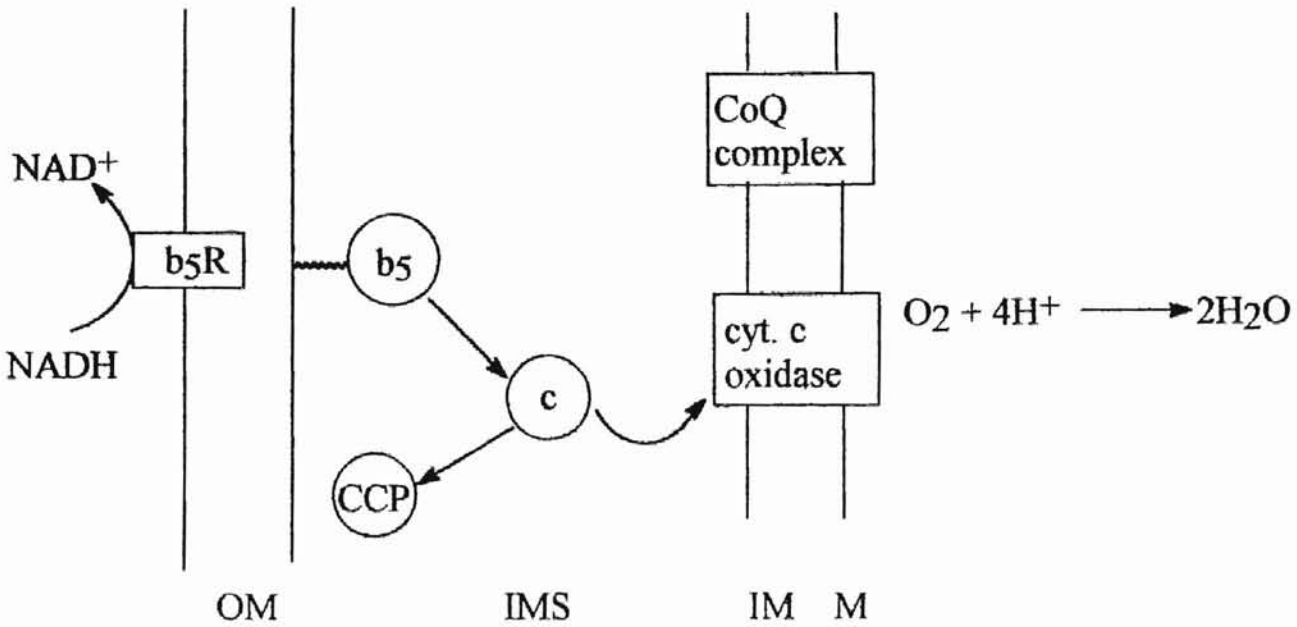


Fig. 1.4 The role of cyt. c in mitochondrial electron transport.
IM = Inner membrane. IMS = Intermembrane space.
OM = Outer membrane. M = Membrane.
b5R = b5 reductase. CCP = Cytochrome c peroxidase.
The arrows indicate the direction of electron flow to and from cyt. c.

stereochemical properties with respect to NADH and capable of reducing OM ferricytochrome b_5 has been found in the outer mitochondrial membrane. A hypothetical protein-protein electron transfer complex was proposed as a precursor for the reaction between cytochrome b_5 and cytochrome c [11]. According to this model, 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 that prove the formation of a protein-protein complex between cytochrome b_5 and cytochrome c [12 a, b].

Cyt. b_5 reduces cyt. c rapidly in vitro [1]. *The two proteins function as physiological redox proteins in vivo* [8]. Experimental and theoretical investigation of the interaction of cytochrome c and cytochrome b_5 has produced considerable insight into the manner in which these proteins recognize and bind to each other. The transfer of an electron from one protein to another is the simplest chemical reaction that can occur between two proteins. As a result, such reactions have attracted considerable experimental and theoretical attention.

Researchers interested in the studies of the rate of electron transfer between the proteins, ferrocytochrome b_5 and ferricytochrome c have traditionally approached the problem using several different techniques- (i) Pulse radiolysis [13];

(ii) Stopped-flow [14]; and

(iii) Flash photolysis [15, 16].

(iv) Computer Modeling studies [12 b].

The Problem:

In the approach of flash photolysis used by Meyer *et al.* [16], a laser flash at 400 nm was used to excite 5-deazariboflavin-EDTA (ethylene diamine tetraacetic acid) solution. This resulted in the formation of deazariboflavin semiquinone, the concentration of which was smaller than the cyt. b_5 or cyt. c concentrations. Thus, when both cytochromes were present simultaneously in a preformed complex at low ionic strength, free reduced cyt. b_5 reduces cyt. b_5 bound to oxidized cyt. c. To ensure that ferricyt. b_5 is initially reduced in a mixture of cyt. b_5-cyt. c, an 8:1 ratio of cyt. b_5 to cyt. c was prepared. The reaction between free cyt. b_5 and the stable complex of cyt. b_5-cyt. c proceeded faster under conditions of higher ionic strength, as complexed cyt. c reorients itself making it more accessible to free cyt. b_5.

This method provides a unique way to follow intracomplex electron transfer without having to chemically modify the protein. However, in order to selectively reduce cytochrome b_5 in the presence of cytochrome c, cytochrome b_5 had to be present in an eight-fold molar excess relative to cytochrome c, and the physiological molar ratio of b_5:c in the outer mitochondrial membrane of rat liver is 2:1.

In the approach involving ruthenium derivatives used by Durham *et al.* [12 b], cytochrome b_5 had to be converted to a tris(bipyridyl)rutheniumcysteine-65 derivative (a photoactive complex) for it to be reduced selectively in the presence of cytochrome c. This involved the complication of having to chemically modify the protein. Site-directed mutagenesis had to be performed on the protein to mutate tyrosine (Tyr) to a cysteine (Cys)-65, in order to obtain the ruthenium label of cytochrome b_5 as shown in Fig. 2.1.

Precaution had to be exercised when using the protein for electron transfer studies as Cys has a tendency to get oxidized resulting in the formation of a disulfide bridge between two Cys residues. The location of the ruthenium complex was critically important if the measured rate constants were to be representative of the rate constants that would be obtained if the reaction could be measured without the ruthenium present. The ruthenium complex had to be placed far from the surface domains involved in the interaction of the two proteins.

Placement of the ruthenium complex was also critical to efficient production of reduced cytochrome b_5. The ruthenium label had to be sufficiently close to the iron center to undergo electron transfer with a high yield. Otherwise, the concentration of reduced protein formed by laser excitation would be too small to detect.

Purpose and Significance of Study:

Purpose: To establish a relatively inexpensive, feasible, and reliable method of determining protein-protein electron transfer rate constants (between ferrocytochrome b_5 and ferricytochrome c). To these ends, cyclic voltammetric studies have been carried out using a gold electrode modified with β-mercaptopropionate, sodium salt. Chemical modification of metal surfaces provides a versatile method for the production of electrode interfaces which can be selective for the direct electrochemistry of one redox protein over another. In the presence of the polycation, polylysine, it has been possible to selectively reduce ferricytochrome b_5 possessing a net negative charge and a relatively low reduction potential, in the presence of cytochrome c possessing a net positive charge and a relatively high reduction potential. The reduced cytochrome b_5 is subsequently reoxidized by

ferricytochrome c in a homogeneous reaction. The second-order rate constant for this homogeneous electron transfer reaction was obtained by an explicit digital simulation of the experimental voltammograms by invoking an electrochemical-chemical (EC) reaction scheme. Furthermore, the experimental values obtained for the heterogeneous electron transfer rate constant, k_s, and the diffusion coefficient, D_o, for OM cytochrome b_5 have been found to be 1.05 X 10^{-2} cm/s and 4.34 X 10^{-6} cm^2/s, respectively, which are in good agreement with the values obtained by digital simulation of the cyclic voltammograms (Tables 3.1 and 3.2).

Significance: Such a study would give information on protein interfacial interactions and macromolecular recognition at various electrode surfaces and interfaces. This form of molecular recognition, *i. e.*, the ability to discriminate between the different sites on an electrode surface, mimics protein homogeneous electron transfer mechanisms wherein redox active proteins "recognize" their biological partners in a specific manner. It would tell whether the electron transfer rates occur at potentials near the standard redox potential.

Of all the chemical processes which occur in biological systems, the simplest, and thus inherently easier to understand is the transfer of a single electron between two redox centers. Such reactions form the basis for some of the most fundamental and important life processes (*e. g.*, photosynthesis, respiration, nitrogen fixation, etc.).

The electrochemical method developed has enabled the construction of an elegant system within which electron transfer has been made to occur in a useful manner (from cyt. b_5, a protein with a negative potential to cyt. c, a protein with a positive potential, as it

occurs in the mitochondrial electron transport chain. Usually it is difficult to get biological molecules to show a voltammetric response at ordinary electrodes using conventional electrochemical techniques. This method has helped overcome this problem, as it involves a modified electrode that selectively discriminates against cyt. c

Further, cyclic voltammetric and spectroelectrochemical studies of the wild type protein and its dimethyl ester (Fig. 1.5) have been carried out to investigate the factors that play a role in modulating the reduction potential of the wild type protein. The results of these studies may pave way for more investigative studies to explore the possibility that the reduction potential of cytochrome b_5 maybe modulated upon formation of a transient complex with cytochrome c.

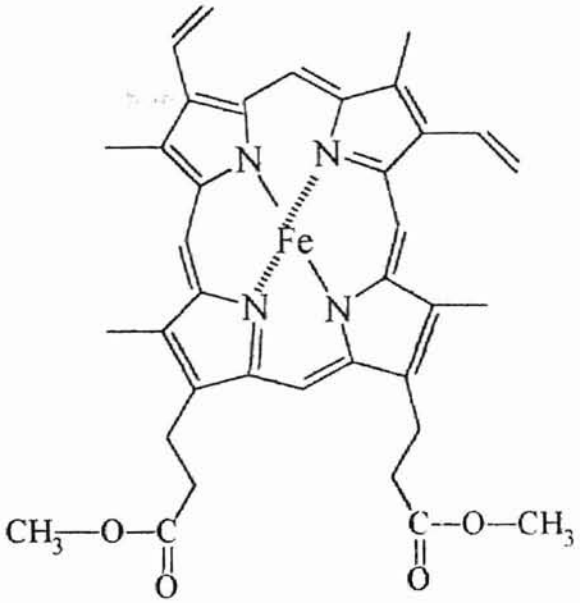


Fig. 1.5 Schematic representation of the dimethyl ester.

Chapter II

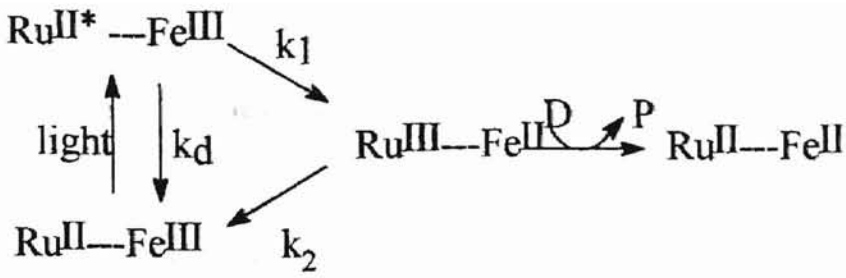
REVIEW OF THE PERTINENT LITERATURE

The measurement of second-order rate constants for the reaction between partner protein has been of tremendous interest to researchers. The traditional approach to the determination of such constants for the reaction between ferrocytochrome b_5 and ferricytochrome c has been using flash photolysis techniques.

Description of flash photolysis as carried out by Meyer *et al.* [15]:

The laser flash photolysis apparatus and methods of data collection and analysis were as previously described [16, 17]. A laser flash at 400 nm was used to excite 5-deazariboflavin. This in turn oxidizes EDTA, resulting in formation of 5-deazariboflavin semiquinone in less than 1 μs. The concentration of 5-deazariboflavin generated by the laser flash (< 1 μM) was smaller than the cytochrome b_5 or cytochrome c concentrations (> 1 μM), and it reacts with free cytochrome b_5 and with the bound cytochromes b_5 and c within a stabilized protein c complex as shown by arrows (Fig. 2.2).

In the study by **Durham and coworkers** (12 b), a ruthenium complex was covalently attached to cytochrome b_5 at locations which do not interfere with protein/protein binding. The photoinitiated reaction sequence is shown in Scheme I:



D = electron donor such as aniline or EDTA which can react with Ru(III) and essentially eliminate the back reaction, k_2.

Cyclic Voltammetric technique to determine protein-protein electron transfer rate constant (Hill and Walton):

Hill *et al.* [18] carried out an electrochemical investigation of the reaction of horse heart cytochrome c with the redox protein from *Pseudomonas aeruginosa*, cytochrome c_{551} and azurin, in the presence of *P. aeruginosa* nitrite reductase/cytochrome oxidase and dioxygen. Fig. 2.3 shows the respiratory chain of the bacterium *Pseudomonas aeruginosa*. For oxidation of horse heart cytochrome (II) c by azurin, the investigators obtained $k = 1 \times 10^4\ M^{-1}s^{-1}$, and, for oxidation by cytochrome c_{551}, $k = 2 \times 10^4\ M^{-1}s^{-1}$. Both rate constants were obtained at 293 K and an ionic strength of I = 0.135 M.

Formation of self-assembled monolayers of thiol compounds on the surface of gold:[19 a, b]

The self-assembly mechanism of alkanethiol monolayers on the (III) surface of gold was discovered with the use of an ultra-high vacuum scanning tunneling microscope. The use of gold substrates is important to the molecular self-assembly process. Gold is reasonably inert and, thus, allows substrates to be manipulated in air with lessened concern

for contamination. Substituted thiols of the form $HS(CH_2)_nCOOH$ bind strongly to gold. This is the most thoroughly characterized alkanethiol-type monolayer.

Voltammetric studies done by Rivera and Walker at the surface of modified gold electrodes:

Rivera and Walker [9] did reversible voltammetric studies at the surface of modified gold electrodes. Based on earlier studies [19], they modified the surface of the Au electrode with β-mercaptopropionate, a thiol compound. This method helped retain the biological activity of cytochrome b_5, and provided a unique way of obtaining voltammetric response at the gold surface. The addition of the polycation, polylysine facilitated electrostatic interaction between the modified gold surface and OM cytochrome b_5 that possessed like charge.

Voltammetric studies at modified gold surfaces were also carried out by other investigators [20, 21].

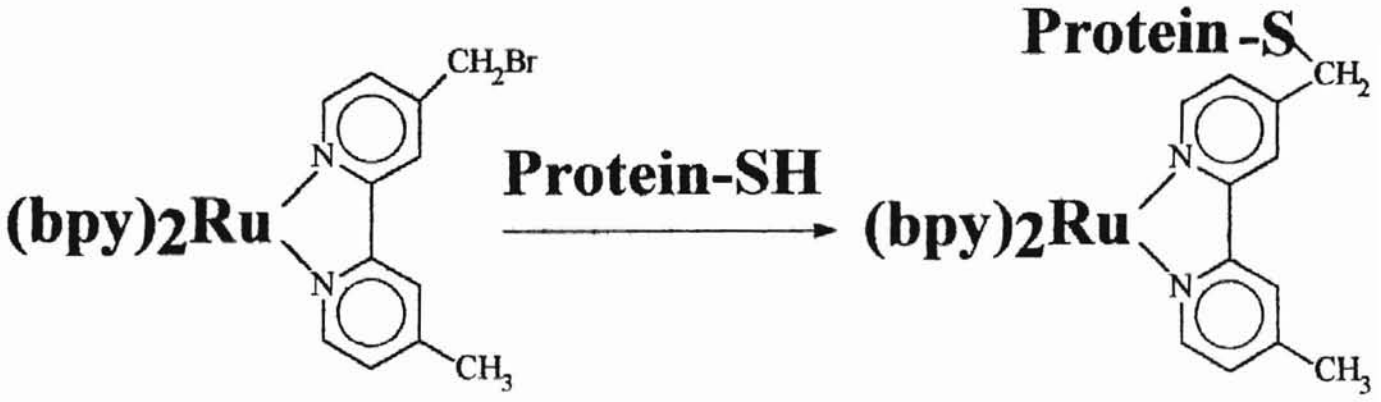


Fig. 2.1 Structure of Ru(II) bound to cyt. b5.

Adapted from Durham *et al.* (1997). *Journal of Chemical Education*, Vol. 74, No.6, pp. 636.

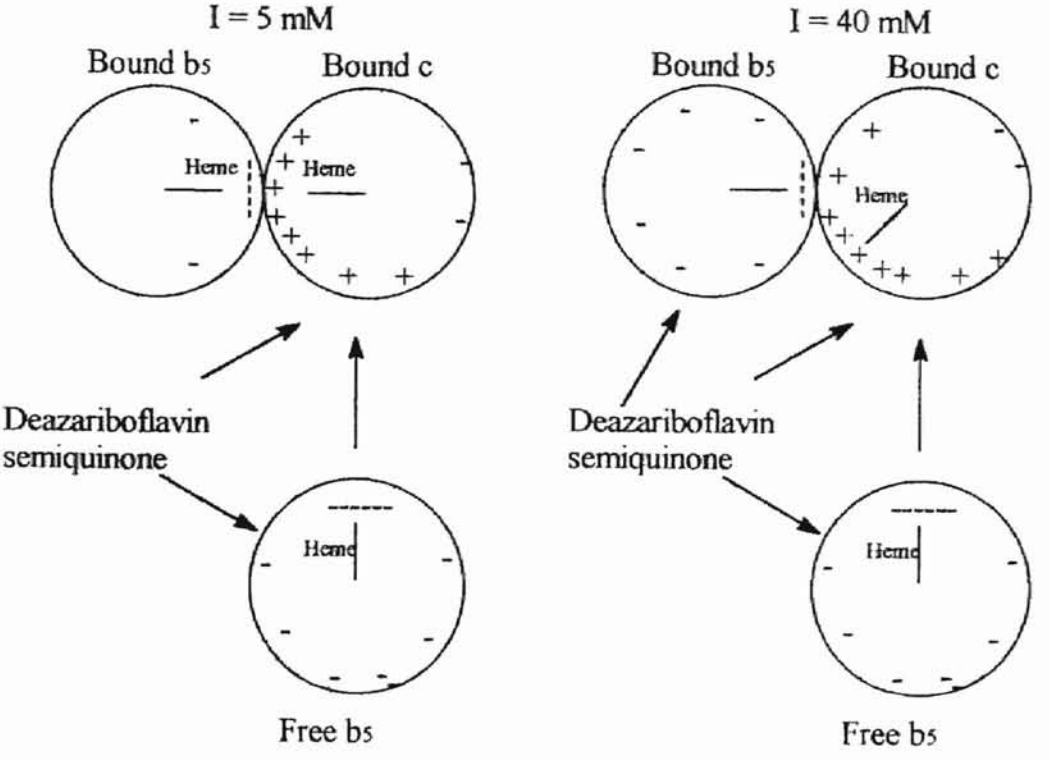


Fig. 2.2 Schematic representation of the cyt. b 5 - cyt. c system. Deazaribolavin semiquinone is rapidly generated by a flash of laser light at 400 nm, and subsequently reacts with free cyt. b 5 and with the bound cytochromes b 5 and c within a stabilized protein complex as shown by the arrows. Any cyt. b 5 which is reduced in this initial process is reoxidized by the bound cyt. c, also shown by arrows. (Adapted from Ref. 15).

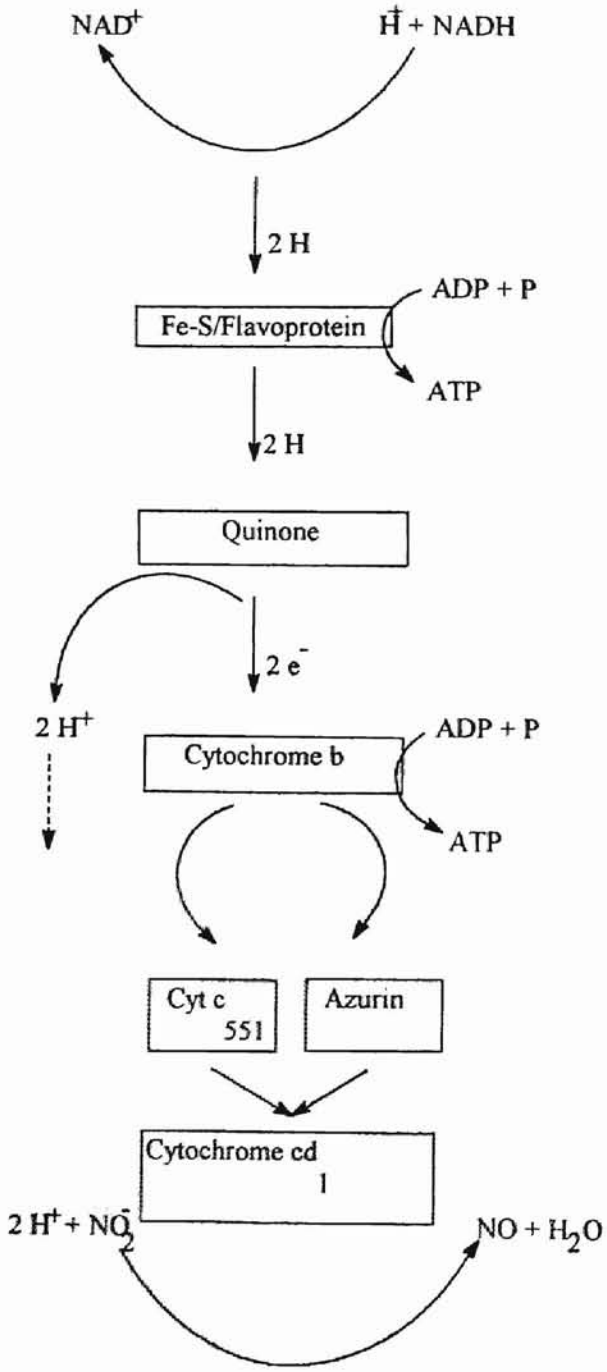


Fig. 2.3 Respiratory chain of the bacterium *Pseudomonas Aeruginosa*. (Adapted from Ref. 18).

Chapter III

METHODOLOGY

Experimental Procedures

Recombinant rat liver OM cytochrome b_5 was expressed in *E. coli* and purified as described below [8]:

Preparation of the overnight inoculum: 5 mL of Luria Bertani (LB) medium containing 10 μL of 1000X ampicillin sodium salt solution (stock 1000X ampicillin = 50 mg/mL) was placed in a culture tube. A single colony of the wild type OM cytochrome b_5. was introduced into the LB medium. The cultures were put in a shaker-incubator overnight for approximately 16 hours at 37 °C at 220 revolutions per minute (rpm). The overnight inoculum was used to innoculate 1 L of LB medium in a 2800 mL Fernbach flask. This was placed in a shaker-incubator at 37 °C at 220 rpm until the optical density (O. D.) at 600 nm ≈ 1 (approximately 3-5 hours). When O. D. = 1, protein synthesis was induced by the addition of 1 mL 1 M IPTG (isopropyl-β-D-thiogalactoside), 1 mL of 5-ALA (aminolevulinic acid), 17 mg/mL and 1 mL of $FeSO_4$, 100 mg/mL. This was placed in a shaker-incubator for 4.5 hours. The culture was subsequently cooled to 4 °C 4.5 hours after induction, and transferred to a pre-weighed centrifuge bottle and centrifuged at 3000 rpm for 10 minutes. The spent medium was decanted as much as possible. The pink pelleted cells in the bottle are weighed. The bottle containing *E. coli* was placed in -20 °C freezer overnight. *Note*: 4 L of cultures were prepared.

Purification of the Protein:

1. Three mL lysis buffer was added per gram of *E. coli*. The *E. coli* pellets containing the protein was resuspended. All cells were transferred to an Erlenmeyer flask of appropriate volume.

2. The pelleted cells were lysed with lysozyme (10 mg/mL): 160 µL of lysozyme was added per gram of *E. coli*. 32 µL of phenylmethylsulfonyl fluoride (PMSF) in ethanol was added per gram of *E. coli*, as a protease inhibitor. The cells were stirred every 5 minutes for 20 minutes.

3. The mixture was equilibrated at 37 °C for 20 minutes.

4. The mixture was stirred approximately 1 hour.

5. The resulting solution was sonicated for 20 minutes and the cell debris separated by ultracentrifugation at 45, 000 rpm for 1 hour using a Ti 50.2 Beckman rotor.

6. The supernatant was decanted and desalted using a dialysis membrane with a 6 kilo Dalton molecular weight cutoff. The solution was dialyzed against 10 mM EDTA, 50 mM Tris, pH 7.8 at 4 °C. A buffer change was made at least 3 times in a period of 16-24 hours.

7. The desalted proteinic solution was applied to a DE-52 (Whatman) anion exchange column equilibrated with 50 mM Tris, 100 mM EDTA, pH 7.8 at 4 °C.

8. The protein was eluted with a linear salt gradient , 0-500 mM in 50 mM tris, 10 mM EDTA, pH 7.8 at 4 °C.

9. The samples containing cytochrome b_5 were collected and concentrated by ultrafiltration using a membrane with a 3 kD molecular weight cutoff.

10. The concentrated protein solution was passed through a Sephadex G-50 column

equilibrated with 100 mM NaCl , 20 mM Tris, pH 7.8 at 4 °C.

11. The absorbance ratio, A_{280}/A_{412} of the fractions were read. The absorbance at 280 nm is due to the heme, and that at 412 nm is exhibited by the protein (the Soret band of OM cyt. b_5). All fractions that have nearly 4 times the absorbance shown by the heme were pooled.

12. The pooled fractions were dialyzed against deionized water at 4 °C.

13. The protein was then concentrated by ultrafiltration, and then lyophilized.

Solutions prepared for the above procedure:

LB Medium:

In a 1L Erlenmeyer flask containing 950 mL of deionized water, the following were added:

bacto-tryptone	10 g
Bacto-yeast extract	5 g
NaCl	10 g

The solutes were stirred until dissolved. The pH was adjusted to 7.0 with 5-N NaOH. The volume of the solution was adjusted to 1 L with deionized water. The solution was sterilized by autoclaving for 20 minutes at 15 pounds per square inch on liquid cycle.

Lysis buffer: 50 mM Tris. Cl, 1 mM EDTA, 100 mM NaCl (pH 8.0 at 4 °C).

Bovine erythrocyte cytochrome b_5 was a gift from Professor Ann Walker, and it was obtained as described previously [22]. 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 with a Bioanalytical Systems 50W computer-controlled potentiostat using a three-electrode configuration as shown in Figs. 3.1 a, b. 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 3M 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 minutes in deionized water. Surface modification of the electrode was achieved by dipping the polished gold working electrode into a 100 mM solution of β-mercaptopropionate for 20 minutes, followed by rinsing with deionized water. The modified electrode (shown schematically in Fig. 3.2) 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. Morpholino-propane-sulfonic acid, MOPS (100 mM, pH 7.0) was used to prepare all solutions. The protein concentrations were measured by UV-visible spectrophotometry and using the Soret band corresponding to the oxidized state in both proteins (Fig. 3.3). The extinction coefficients used were 130 $mM^{-1}cm^{-1}$

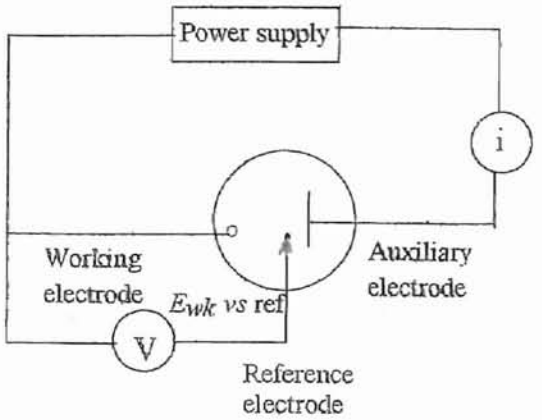


Fig. 3.1 a Three-electrode cell.

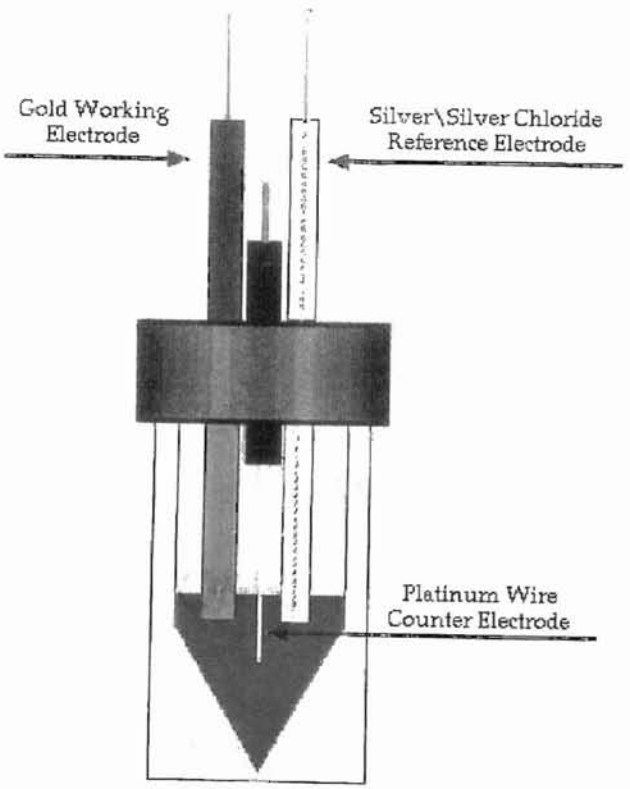


Fig. 3.1 b Electrochemical cell for voltammetry.

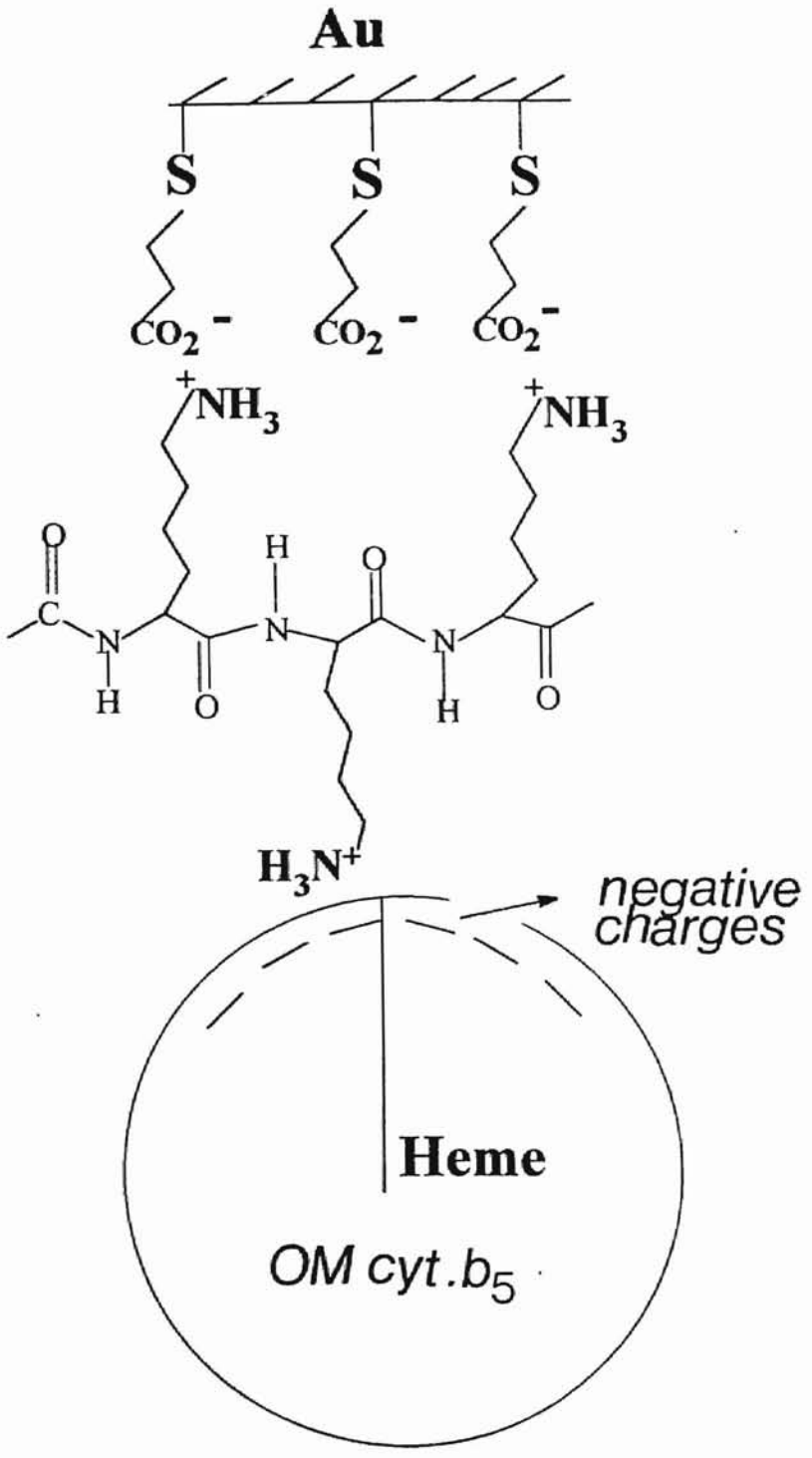


Fig. 3.2 Schematic representation of the gold electrode modified with β-mercaptopropionate. The polycation, poly-L-lysine, promotes the reversible electrochemistry of the negatively charged OM cytochrome b_5 at the negative surface of the modified electrode. (Adapted from Ref. 9).

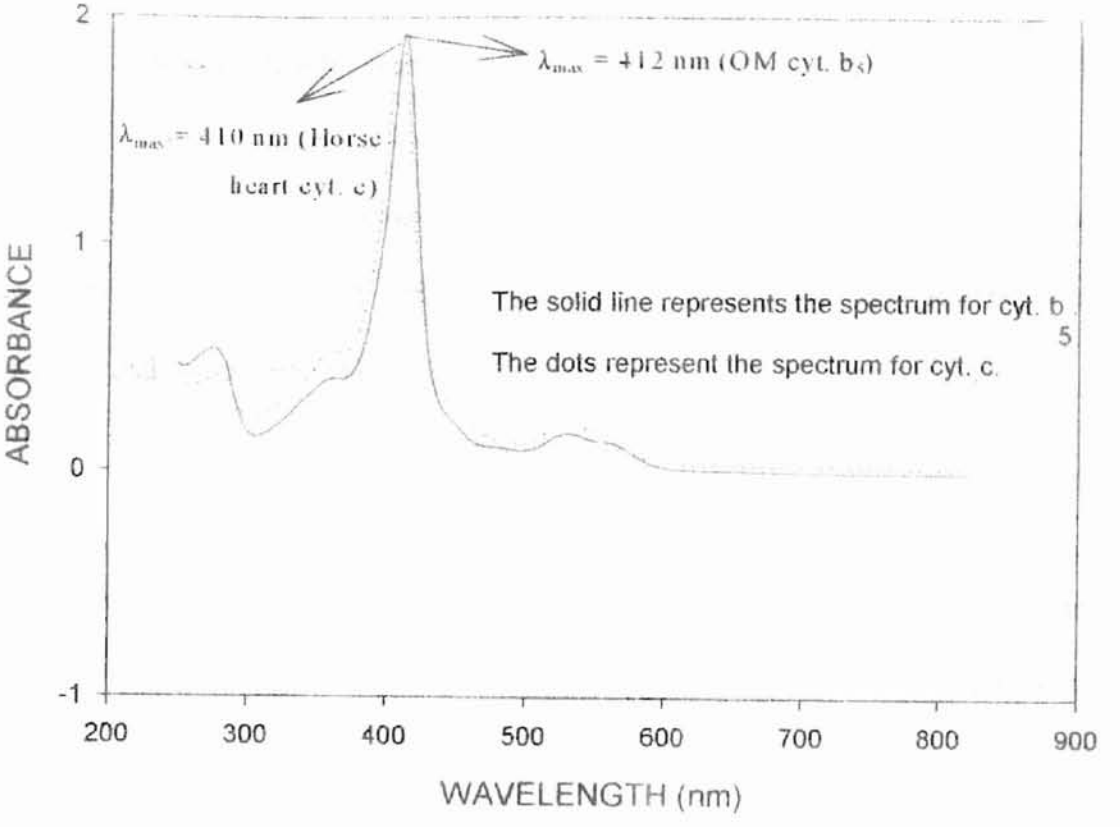


Fig. 3.3 UV-visible spectra of OM cyt. b_5 (the solid line) and horse heart cyt. c (dots).

for oxidized cytochrome b_5 [2] and 109.5 $mM^{-1}cm^{-1}$ for oxidized cytochrome c [23]. 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 the 100 mM solution of β-mercaptopropionate for 5 minutes 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 have been corrected so as to be referenced to the standard hydrogen electrode. Fig. 3.4 shows the cyclic voltammogram obtained from a solution containing equimolar amounts of the two proteins in the presence of polylysine in 100 mM MOPS, pH 7.0. Fig. 3.5 and Fig. 3.6 illustrate the significance of the shape of the voltammogram obtained in Fig. 3.4. This is discussed in detail in the following chapter. Fig. 3.7 shows the titration experiment carried out by adding incremental amounts of horse heart cyt. c to a solution of OM cyt. b_5 in the presence of polylysine in 100 mM MOPS, pH 7.0

Fig. 3.8 (A) was obtained by running a cyclic voltammogram of a solution containing horse heart cyt. c reduced with sodium dithionite to which a solution of an equimolar amount of OM cyt. b_5, in the presence of polylysine, in 100 mM MOPS, pH 7.0 was added. When this solution was allowed to stand in air for some time, cyclic voltammogram shown in Fig. 3.8 (B) was obtained. Fig. 3.9 was obtained by running a cyclic voltammogram of a solution containing equimolar amounts of microsomal cyt. b_5

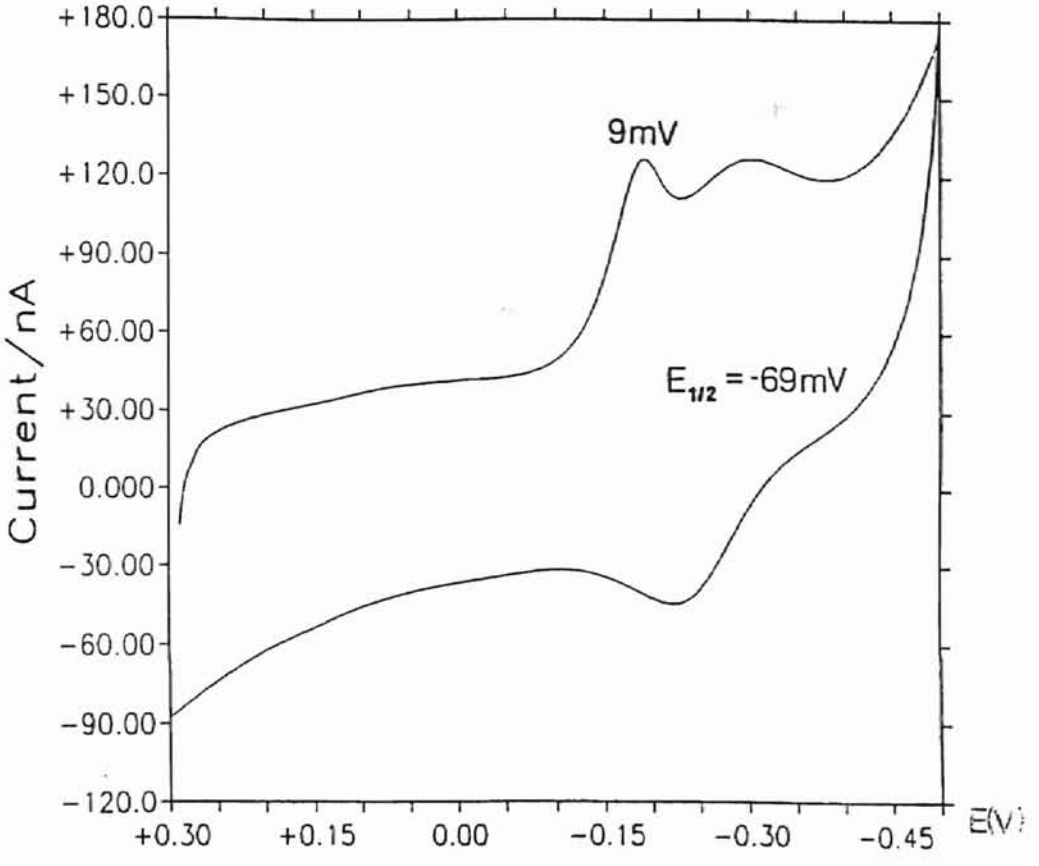


Fig. 3.4 Cyclic voltammogram of a solution containing OM cyt. b_5 (0.1 mM), horse heart cyt. c (0.1 mM), and polylysine (0.025 mM) in 100 mM MOPS, pH 7.0. The scale of the voltammogram is shown *vs* the Ag/AgCl electrode, but the values 9 mV and -69 mV are with respect to the standard hydrogen electrode. Sweep rate = 50 mV/s. ΔE_p for the reversible wave in a typical experiment is between 56 and 63 mV.

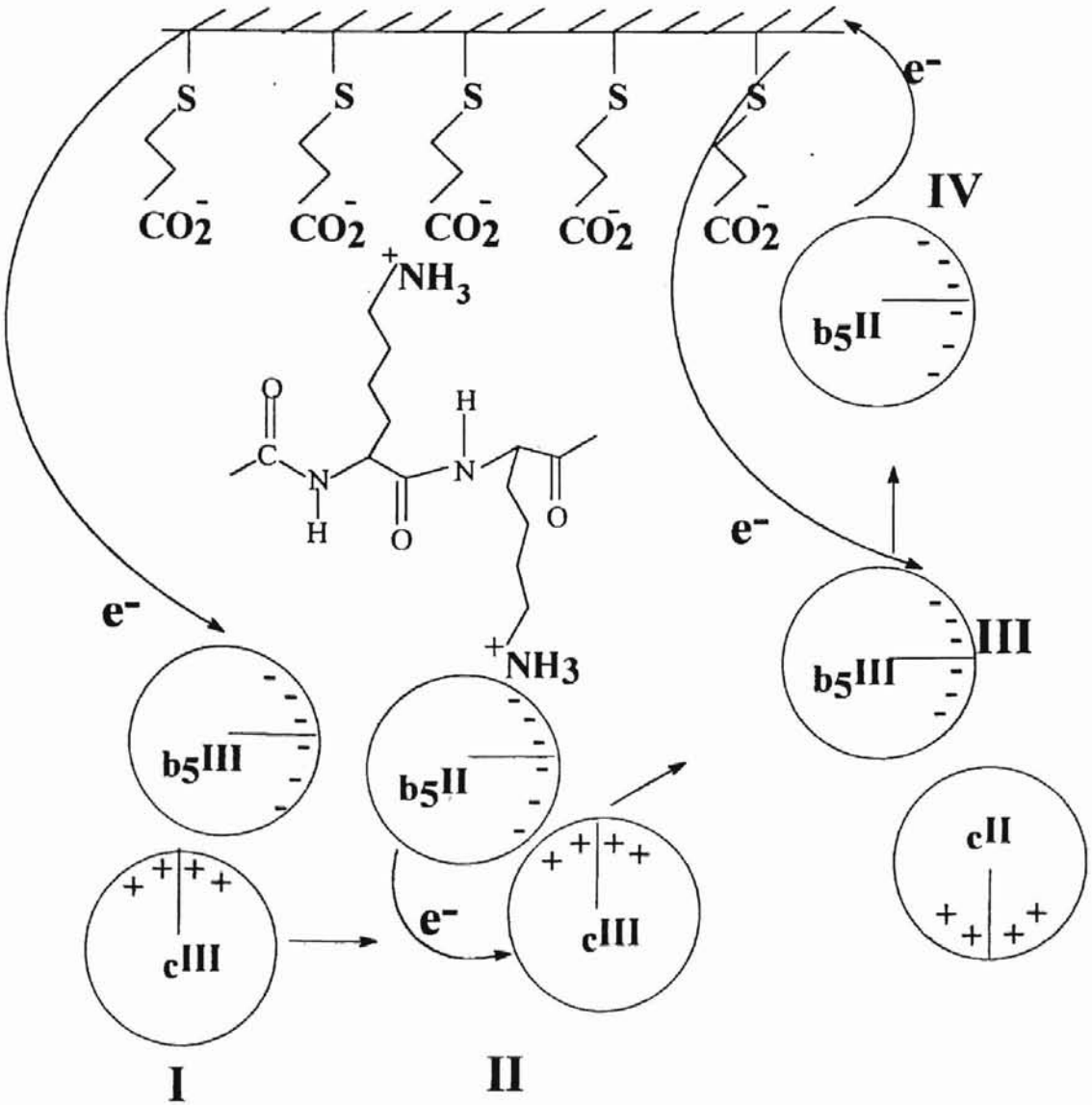


Fig. 3.5 Schematic representation of a gold electrode modified with β-mercaptopropionate in the presence of polylysine (shown as a polycation).

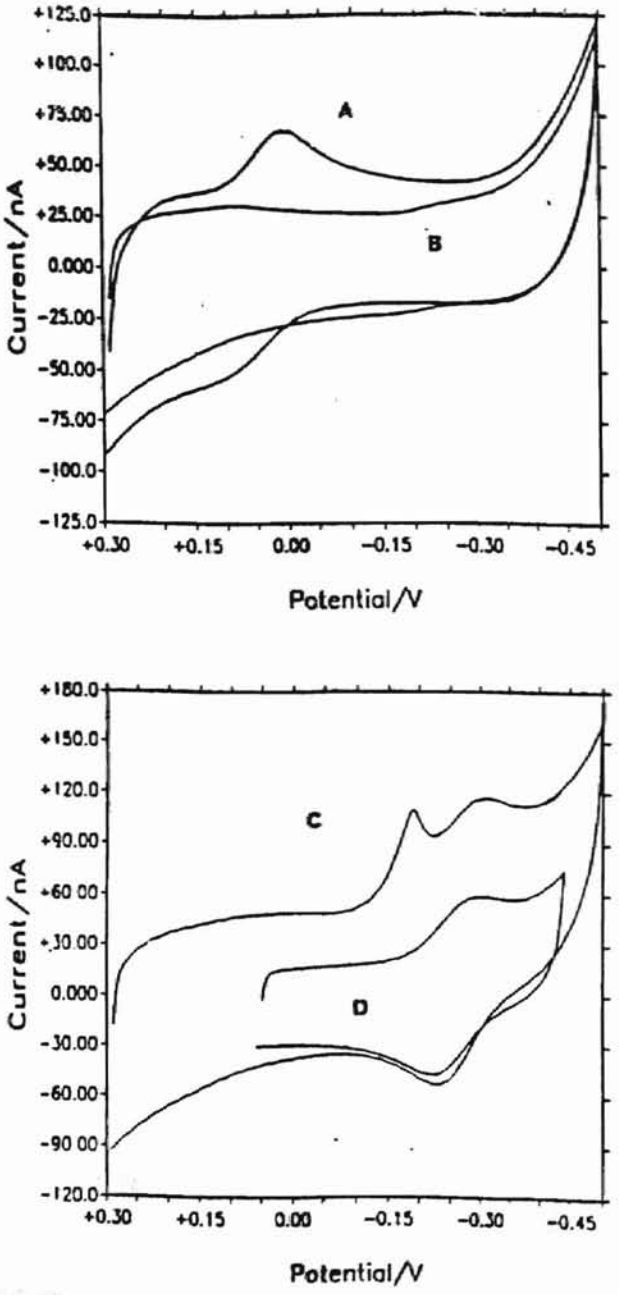


Fig. 3.6 (A) Cyclic voltammogram of cytochrome c (0.1 mM) in 100 mM MOPS, pH 7.0. (B) Faradaic response when polylysine is added to solution A to produce a final concentration of 0.025 mM. (C) Cyclic voltammogram when OM cytochrome b_5 is added to solution B to produce a final concentration of 0.1 mM. (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.

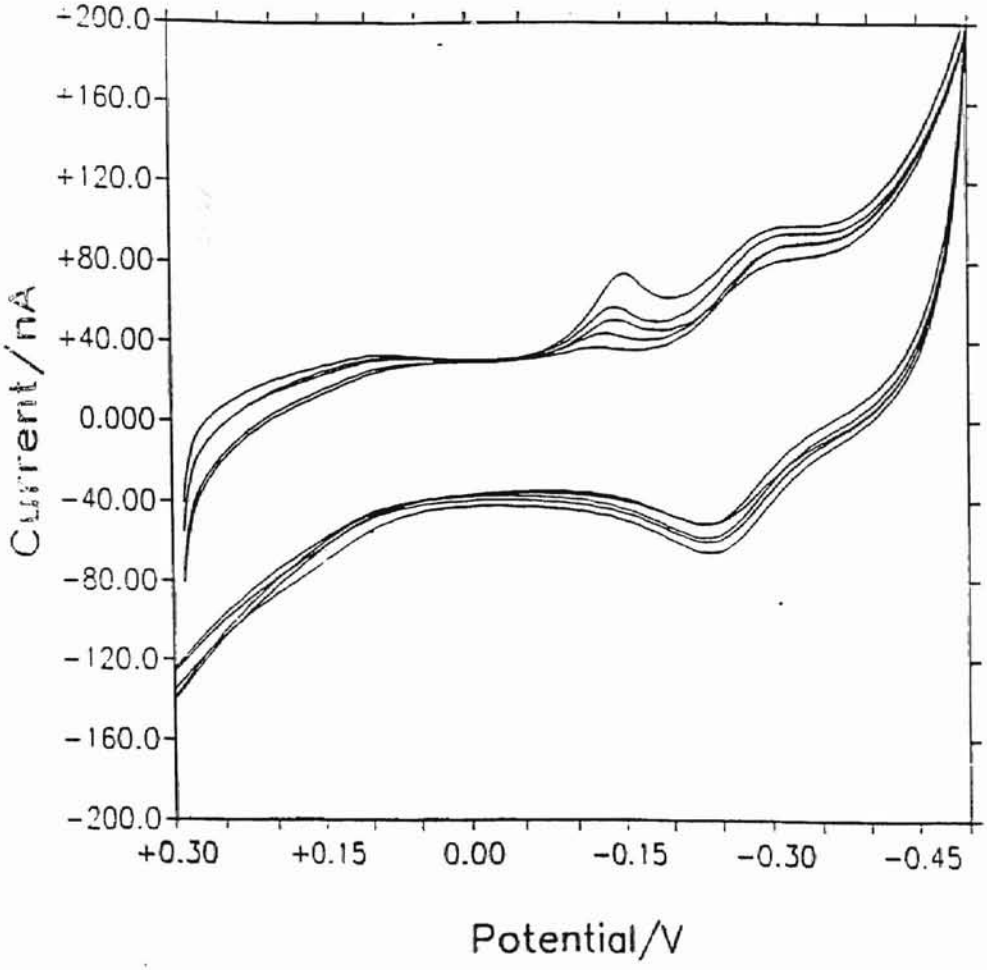


Fig. 3.7 Titration of a solution containing OM cytochrome b_5 (0.1 mM) and polylysine (0.025 mM) in 100 mM MOPS, pH 7.0, with 1 mM horse heart cytochrome c prepared in 100 mM MOPS, pH 7.0. The cyclic voltammograms were obtained with a gold electrode modified with β-mercaptopropionate and correspond to final cytochrome c concentrations of approximately 0.025, 0.05, 0.07, and 0.1 mM.

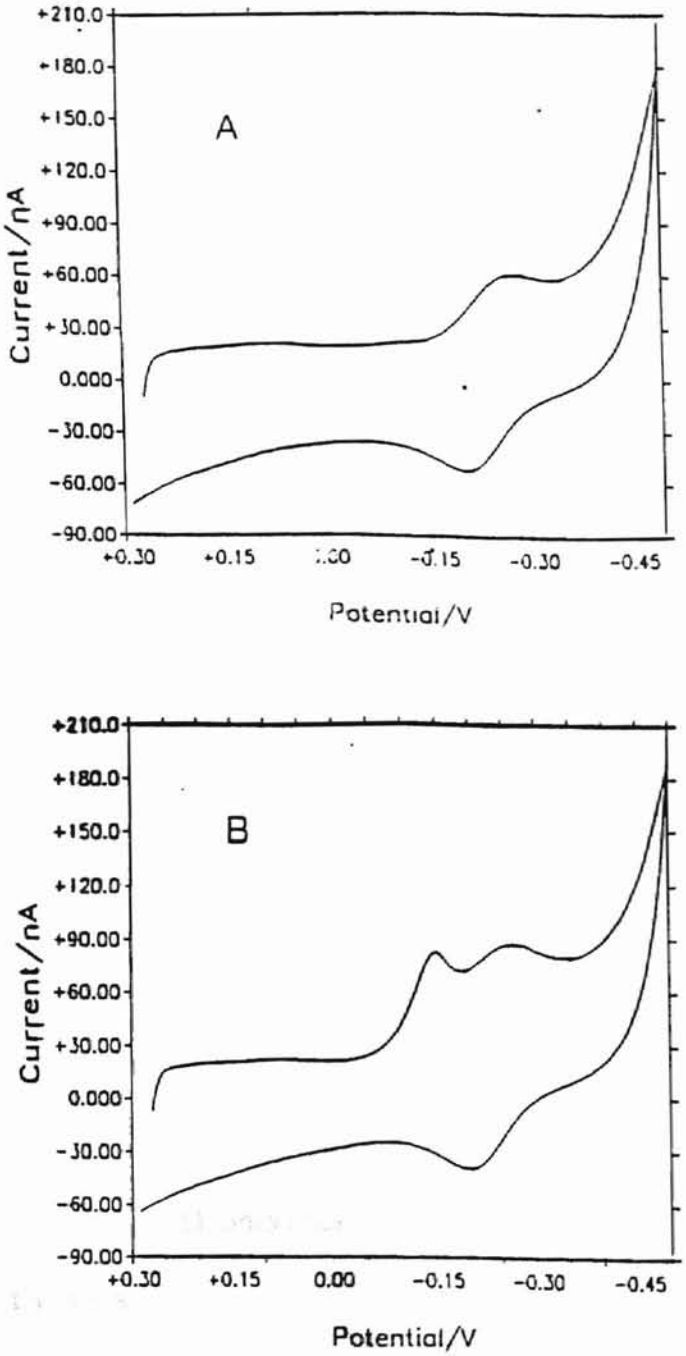


Fig. 3.8 (A) Cyclic voltammogram of a mixture containing rat liver OM ferricytochrome b_5 (0.1 mM), horse heart ferrocytochrome c (0.1 mM) and polylysine (0.025 mM) in 100 mM MOPS, pH 7.0. (B) Cyclic voltammogram obtained with a solution containing rat liver OM ferricytochrome b_5, horse heart ferricytochrome c, and polylysine. Concentrations as in (A). Sweep rate = 50 mV/s.

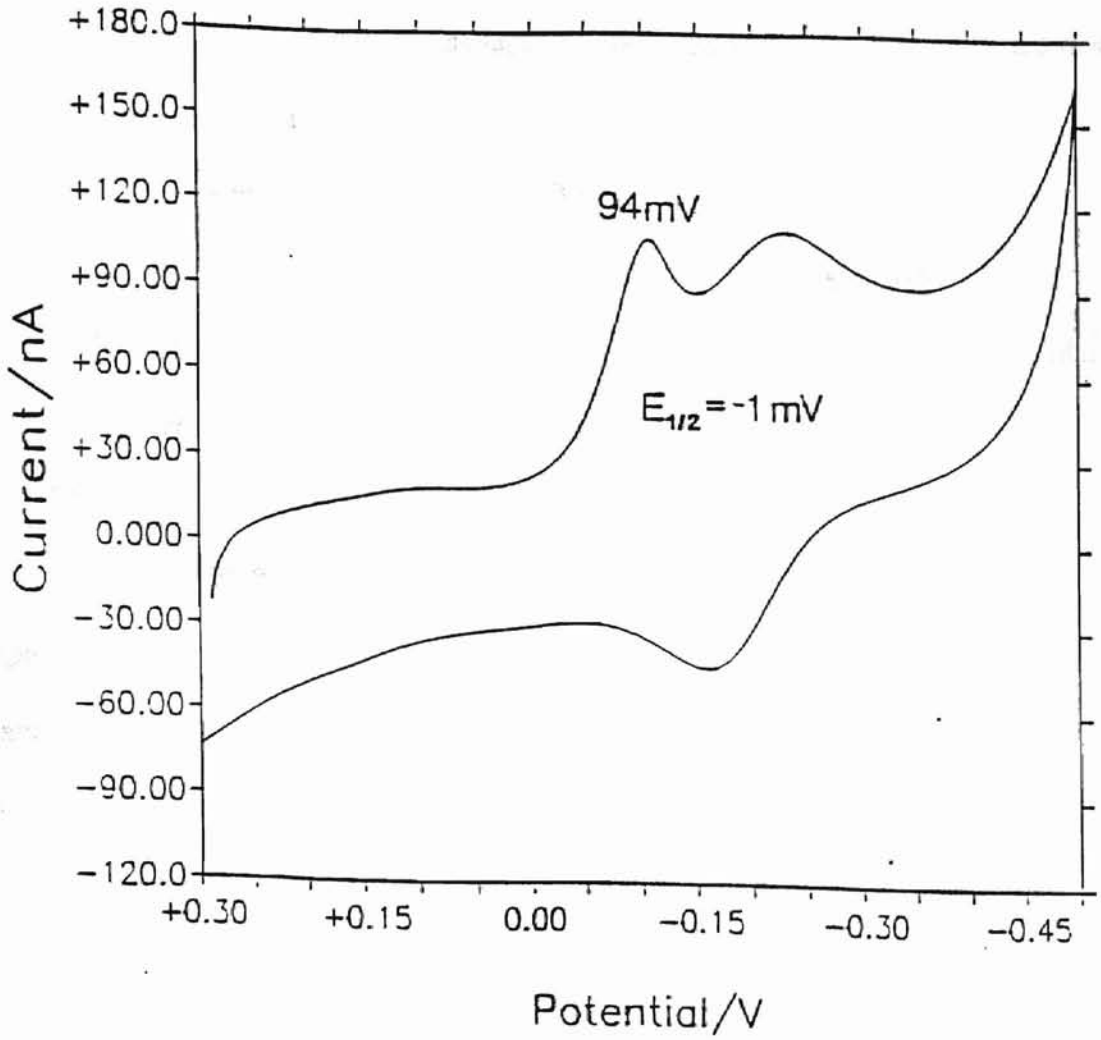


Fig. 3.9 Cyclic voltammogram of a mixture containing beef liver microsomal cytochrome b_5 (0.1 mM), horse heart cytochrome c (0.1 mM), and polylysine (0.025 mM) in 100 mM MOPS, pH 7.0. The scale of the voltammogram is shown *vs* the Ag/AgCl electrode, but the values 94 mV and -1 mV are with respect to 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.

and horse heart cytochrome c in the presence of polylysine in 0.10 M MOPS, pH 7.0

Digital simulation: Digital simulation of the cyclic voltammogram obtained from a mixture containing equimolar amounts of OM cytochrome b_5 and horse heart cytochrome c in the presence of polylysine in 0.10 M MOPS, pH 7.0 was performed using the program Digisim 2.0 (Bioanalytical Systems), a simulator for cyclic voltammetric responses [26]. 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} = -0.5 V, E_{end} = 0.30 V, scan rate = 0.05 V/s, electrode area = 0.00785 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 (the rate constant for the reaction involving transfer of an electron from OM ferrocytochrome b_5 to horse heart ferricytochrome c), k_s (the heterogeneous electron transfer rate constant for the electrochemical reduction of OM ferricytochrome b_5), K_{eq} (the equilibrium constant for the reaction involving electron transfer from OM ferrocytochrome b_5 to horse heart ferricytochrome c), discussed in the following chapter (Eqs. 4.3, 4.4) and D_o (diffusion coefficients of cyt. b_5 and cyt. c) were allowed to change throughout the fitting process. The simulated and experimental cyclic voltammograms are depicted in Fig. 3.10, and the parameters obtained from the simulations are listed in Tables 3.1 and 3.2, 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.* [17] using flash photolysis techniques were 2.6 X 10^8 $M^{-1}cm^{-1}$ (I = 5 mM), 1.3 X 10^9 $M^{-1}cm^{-1}$ (I = 35 mM), 6.7 X 10^8 $M^{-1}cm^{-1}$ (I = 87 mM) for rat liver OM cytochrome b_5 and 8 X 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 was 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 3.1 and 3.2). Furthermore, the experimental values obtained for the heterogeneous electron transfer rate constant, k_s, and diffusion coefficient, D_o, for OM cytochrome b_5 were 1.05 X 10^{-2} cm/s and 4.34 X 10^{-6} cm^2/s, respectively, which were in good agreement with the values obtained by digital simulation of the cyclic voltammogram (Tables 3.1 and 3.2) 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 voltammograms obtained with rat liver OM cytochrome b_5 (110 mV) as shown in Fig. 3.4 was smaller than the separation between the pre-peak and the main peak in the voltammogram obtained with microsomal cytochrome b_5 (125 mV) and cytochrome c as shown in Fig. 3.9.

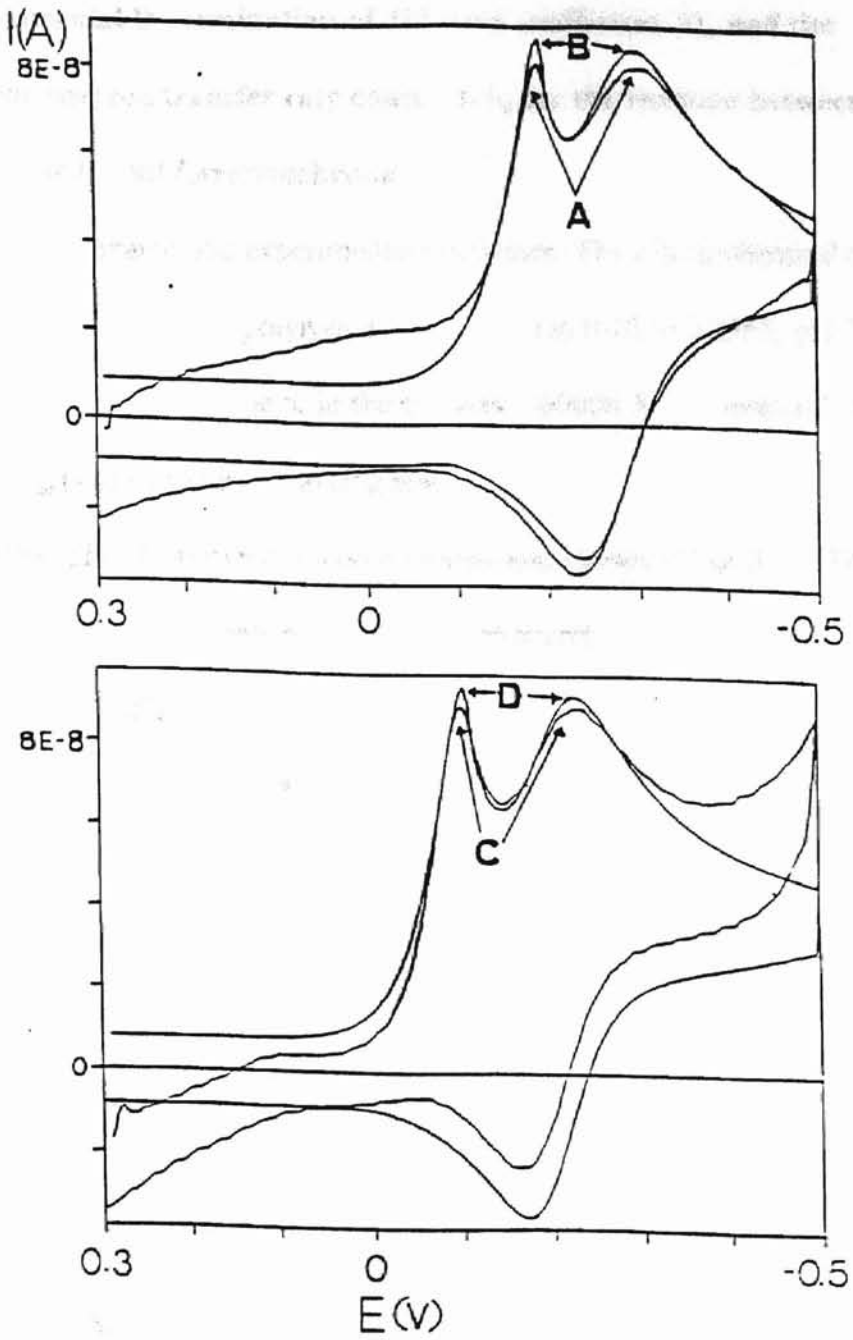


Fig. 3.10 (A) Experimental cyclic voltammogram obtained from a mixture containing rat liver OM cytochrome b_5 and horse heart cytochrome c. (B) Simulated cyclic voltammogram invoking the reaction scheme shown by eqs. 3.1 and 3.2. Parameters for simulation are shown in Table 3.1. (C) Experimental cyclic voltammogram of a mixture containing beef liver cytochrome b_5 and horse heart cytochrome c. (D) Simulated voltammogram invoking the reaction scheme shown by eqs. 3.1 and 3.2. Parameters for simulation are shown in Table 3.2.

Experimental Determination of diffusion coefficient, D_o, and the heterogeneous electron transfer rate constant, k_s for the reaction between ferrocytochrome b_5 and ferricytochrome c:

Solutions prepared and experimental procedure: The electrochemical cell contained the protein solution, polylysine (MW 3970) in 0.10 M MOPS, pH 7.0. The concentration of OM cytochrome b_5 in the cell was 1.00008 X 10^{-7} mol/cm^3. The cathodic peak current (i_{red} (A)) was read at varying scan rates, v, starting from 20 mV/s upto 150 mV/s. The graph of i_{red} (A) *vs* $(v)^{1/2}$ (V/s) is plotted and shown in Fig. 3.11. The values are tabulated in Table 3.3. The slope, m of the line was found to be 4.4 X 10^{-7}.

As per the Randles-Sevcik equation [25],

$$i = 2.69 \text{ X } 10^5 \, n^{3/2} \, A \, D_o^{1/2} \, c v^{1/2} \qquad (3.1)$$

where i = peak current, amperes; n = number of electrons involved in the reaction; A = electrode area, cm^2; c = concentration, mol/cm^3

Therefore,

$D_o^{1/2} = i / 2.69 \text{ X } 10^5 \, n^{3/2} \, A \, c \, v^{1/2}$

From the graph of i *vs* $v^{1/2}$, $y/x = i/v^{1/2} = m$, the slope.

Therefore, $D_o = (m / 2.69 \text{ X } 10^5 \, n^{3/2} \, A \, c)^2$ cm^2/s

Substituting for in the above equation, we have,

$D_o = 4.4 \text{ X } 10^{-7} / 2.69 \text{ X } 10^5 \text{ X } 1^{3/2} \text{ X } 0.00785 \text{ cm}^2 \text{ X } 1 \text{ X } 10^{-7}$ mol/cm^3

$D_o = 4.34 \text{ X } 10^{-6}$ cm^2/s

To determine k_s, a fresh solution of OM cytochrome b_5 and polylysine (MW 3970) in 0.10 M MOPS, pH 7.0 is prepared. For a totally reversible compound, the ΔE_p (mV),

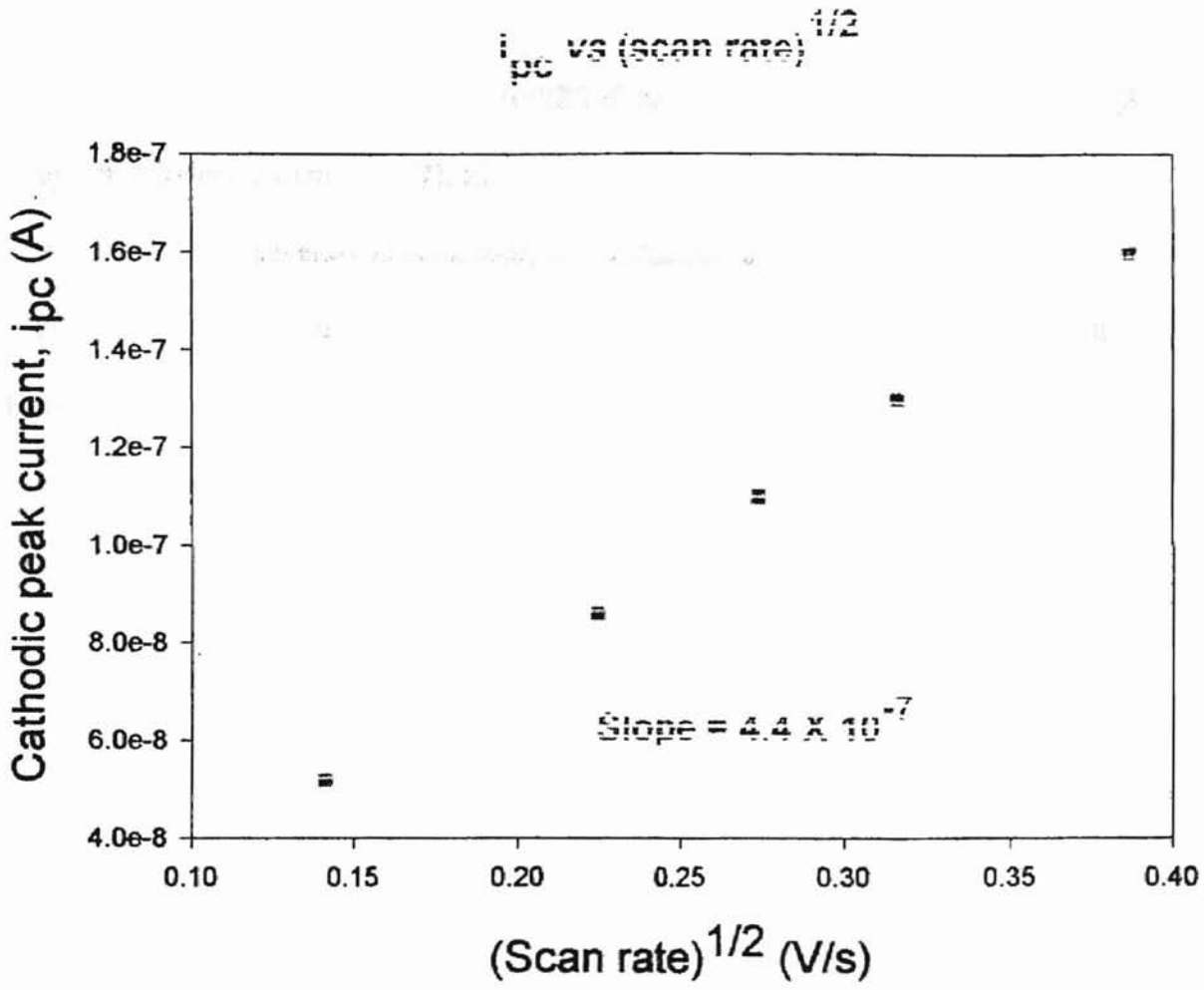


Fig. 3.11 Plot of cathodic peak current *vs* (scan rate)$^{1/2}$

peak-to-peak separation is 59/n mV. When the electron transfer reaction is not reversible the electron transfer rate, k_s, is slow. Thus, the measurements were made at a higher value of ν. The ΔE_p values were then read with a freshly prepared electrode each time. Corresponding to each value of ΔE_p (mV), the Ψ values were read from literature [27]. The values are tabulated in Table 3.4.

The Nicholson-Shain equation [28] was used to calculate the value of k_s.

$$k_s = \Psi[D_o \pi \nu (nF/RT)]^{1/2} \text{ cm/s} \qquad (3.2)$$

where Ψ = kinetic parameter [27]; nF/RT = 38.95, n = number of electrons involved; F = Faraday; R = universal gas constant; T = temperature, K.

Table 3.4 shows the values of ΔE_p (mV), the corresponding values of Ψ, and the values of k_s as calculated from the Nicholson-Shain equation.

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 3.5. Each of the rate constants listed in Table 3.5 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 OM cytochrome b_5 (0.050 mM) and horse heart cytochrome c (0.10 mM); 0.10 mM OM cytochrome b_5 and 0.050 mM horse heart cytochrome c, are shown in Figs. 3.12 a, 3.12 b. The rate constant for the transfer of an electron from ferrocytochrome b_5 to ferricytochrome c has been previously found to be affected by ionic strength [30]. Second-order rate constants for the reduction of horse heart ferricytochrome c by OM ferrocytochrome b_5 at three different values of ionic strength were measured with the aid of the electrochemical method described here (Table 3.5). 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

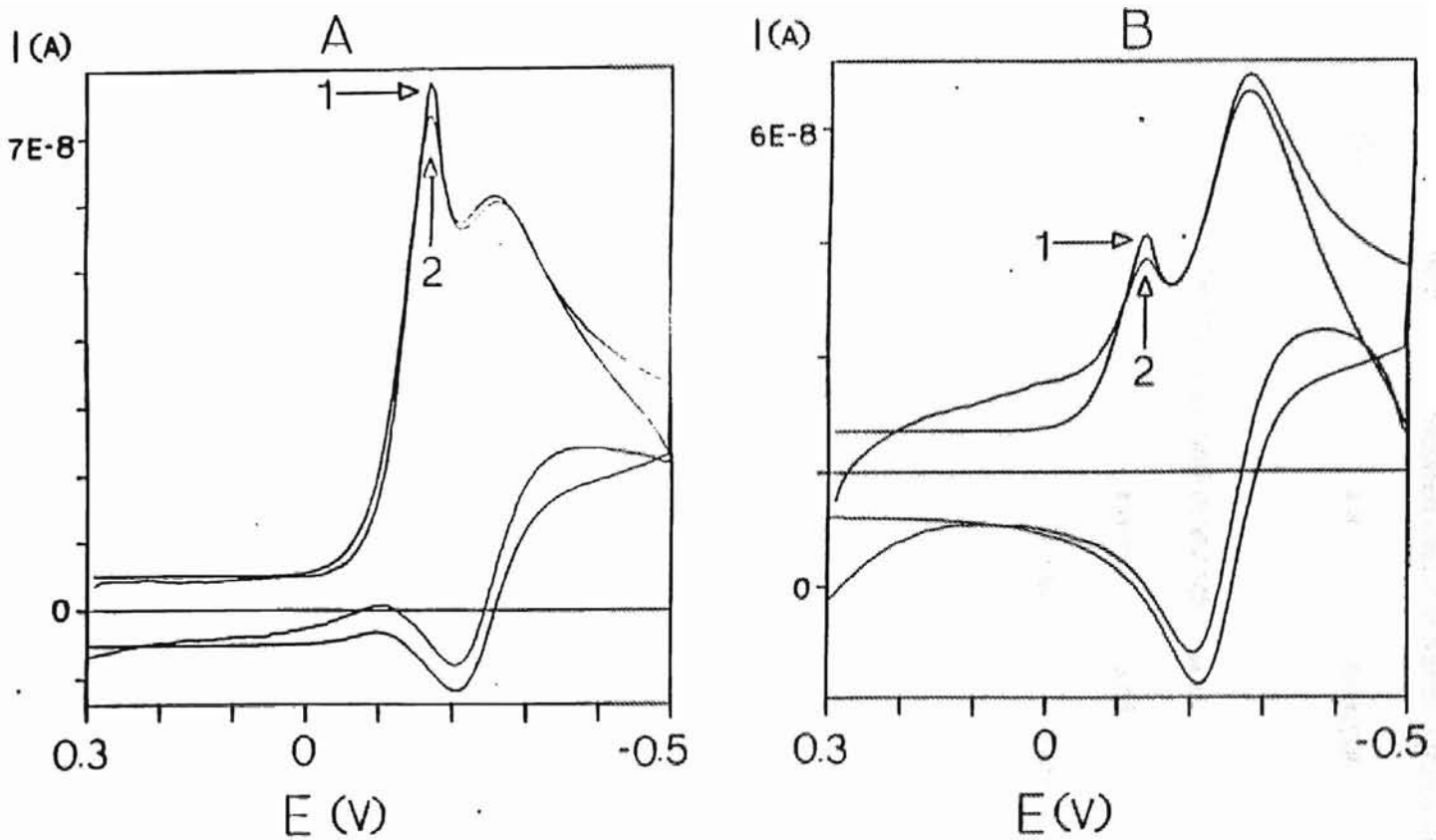


Fig. 3 12 (A) Simulated (1) Experimental (2) cyclic voltammogram from a solution containing OM cytochrome b_5 (0.050 mM) and horse heart cytochrome c (0.1 mM). (B) Simulated (1) and experimental (2) cyclic voltammogram from a solution containing 0.1 mM OM cytochrome b_5 and 0.050 mM horse heart cytochrome 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 3.3.

beef liver cytochrome b_5, using flash photolysis) at different ionic strengths.

Experimental and simulated cyclic voltammograms obtained at μ=130 mM and 230 mM are depicted in Figs. 3.13 a, 3.13 b. 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 interpretation of these simulations is discussed in Chapter IV. 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 functions of ionic strength are discussed in Chapter IV.

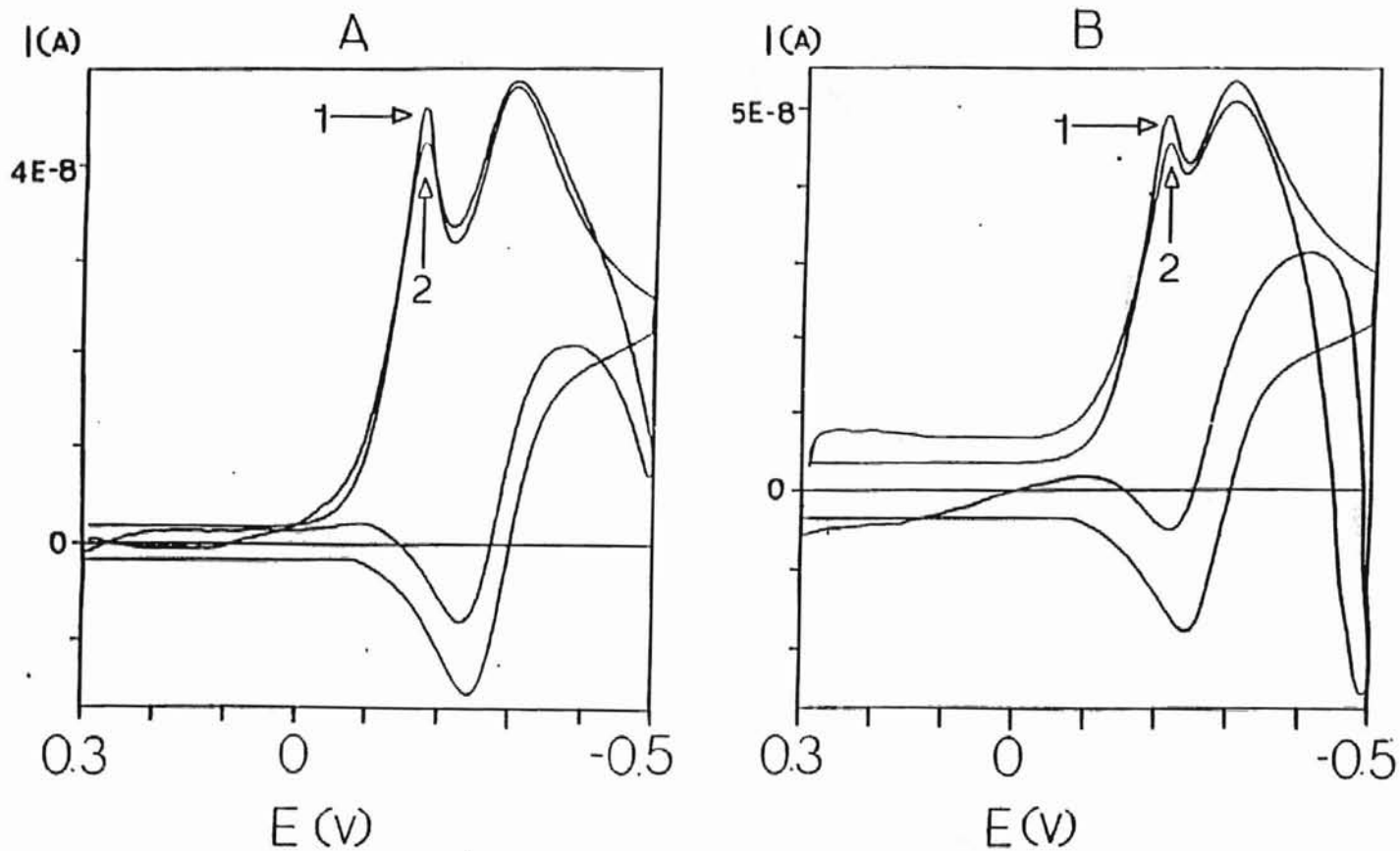


Fig. 3.13 Simulated (1) and experimental (2) cyclic voltammogram from a solution containing 0 10 mM OM cytochrome b_5 and 0.10 mM horse heart cytochrome c, scan rate = 25 mV/s. (A) μ = 130 mM and (B) μ = 230 mM. Solutions A and B also contained 0.025 mM polylysine. The second-order rate constants obtained from the digitally simulated voltammograms are listed in Table 3.3.

Preparation of the Dimethyl ester Heme Cytochrome b_5:

In order to further investigate the factors that play a role in modulating the reduction potential of OM cytochrome b_5, its dimethyl ester derivative was prepared [32], according to the following procedure:

Preparation of the apoprotein: Purified rat OM cytochrome b_5 [8] was placed in a 50 mL conical tube on ice. Its pH was lowered to 2.0 with ice-cold 1-N HCl. The heme was extracted by the addition of 2-butanone, previously chilled at -20 °C. The heme went into the ketone layer imparting it a dark brown color. The aqueous apoprotein layer was transparent. The apoprotein preparation was dialyzed against 4 L 0.6 mM $NaHCO_3$, 1 mM EDTA, pH 10.3 at 4 °C for about an hour. This was followed by dialysis against 4L 0.6 mM $NaHCO_3$ alone. Further dialysis was carried out against 20 mM NaH_2PO_4. H_2O, pH 7.2 overnight until the smell of 2-butanone disappeared (changing buffer a number of times).

Reconstitution with Dimethyl ester Heme: A solution of Fe(III) protoporphyrin (IX) dimethyl ester was prepared in dimethyl sulfoxide (DMSO). About 5.4 mg was weighed (for an initial mmol of OM cyt. b_5 = 4) and dissolved in 10 mL DMSO. The apoprotein was transferred from the dialysis bag into an Erlenmeyer flask at room temperature. About a 100 µL of the solution of Fe(III) protoporphyrin (IX) dimethyl ester was added to the apoprotein solution, allowed to react 2 minutes and a UV spectrum taken. The absorbance ratio, A_{280}/A_{412} was monitored for every 100 µL of the reagent added until the reaction mixture contained 5 mL of the reagent. The mixture was allowed to react 12 hours. The absorbance ratio was measured to check if it had remained constant. The dimethyl ester

cytochrome b_5 was concentrated by ultrafiltration to a volume of about 3 mL and loaded into a Sephadex G-50 column and eluted with 20 mM NaH_2PO_4. H_2O, 100 mM NaCl, pH 7.2 at 4 °C. The fractions bearing an A_{280}/A_{412} ratio in the range 0.2-0.25 were pooled. The fractions were concentrated by ultrafiltration and stored at -20 °C.

Spectroelectrochemical measurements- In spectroelectrochemistry, two quite different techniques, electrochemistry and spectroscopy, are combined [33]. It provides a useful tool for studying the redox chemistry of many biological molecules. Oxidation states are changed electrochemically by the addition of electrons at an electrode while spectral measurements on the solution adjacent to the electrode are made simultaneously. Such a technique is useful in obtaining spectra and redox potentials and for observing subsequent chemical reactions of electrogenerated species. The technique utilizes OTEs (Optically Transparent Electrodes), which enable light to be passed directly through the electrode and adjacent solution. The type of working electrode used was a minigrid electrode, consisting of a gold micromesh of about 200 wires per inch (Buckbee Mears Co., St. Paul, MN). The cell body consisted of two plates, with a quartz window, and a compartment for the reference electrode, which was a silver/silver chloride electrode. The counter electrode was a gold minigrid electrode (Fig. 3.14). The details of the spectroelectrochemical cell have been reported elsewhere [22]. Here the technique involved observation of a thin layer of solution ($\leq$ 0.2 mm) that was confined next to the OTE by placing the quartz window in the path of the optical beam of the spectrophotometer. The optical beam of the spectrophotometer is passed directly through the transparent electrode and the solution. This offered a simple way of controlling the

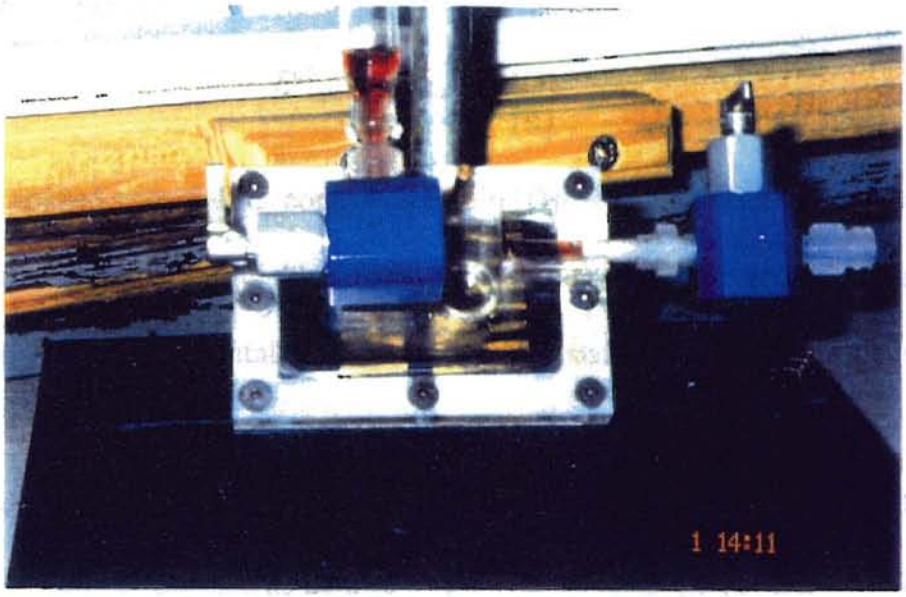


Fig. 3.14 Spectroelectrochemical cell.
The compartment to hold the reference electrode contains the protein solution. The gold minigrid on the left side serves as the counter electrode, and that on the right side, as the working electrode.

oxidation state of the species in a very small volume of solution for simultaneous spectral observation.

Consider the reduction of the species O

$$O + e^- \rightleftharpoons R \tag{3.3}$$

From the Nernst equation

$$E = E^{o'}_{O,R} + \frac{0.059}{n} \log \frac{[O]}{[R]} \tag{3.4}$$

Although the applied potential, E, controls the ratio [O]/[R] at the electrode surface, the ratio in the thin solution layer quickly adjusts to the same ratio by electrolysis. The redox couple was converted incrementally from one oxidation state to another by a series of applied potentials for which each corresponding value of [O]/[R] was determined spectrally. Each potential was maintained until electrolysis ceased so that the equilibrium value of [O]/[R] was established as defined by the Nernst equation. A nernstian plot was then made from the values of E and the corresponding values of [O]/[R] determined spectrally.

In the experiment carried out to determine the redox potential of OM cytochrome b_5, hexammineruthenium (III) chloride, and methyl viologen (Fig. 3.15), were added as the mediators. Ru(III) acted as a conveyor of electrons between the gold minigrid and Fe(III). Reduction/oxidation of the biological molecule was thus indirect through Ru(III). The nernstian plot is shown in Fig. 3.16. The experiment was repeated with OM cytochrome b_5 in the presence of: polylysine, MW 3970, polylysine, MW 9000. The same experiment was repeated with the dimethyl ester in the absence of polylysine, in the presence of polylysine (MW 3970), and in the presence of polylysine (MW 9000). The values of the redox

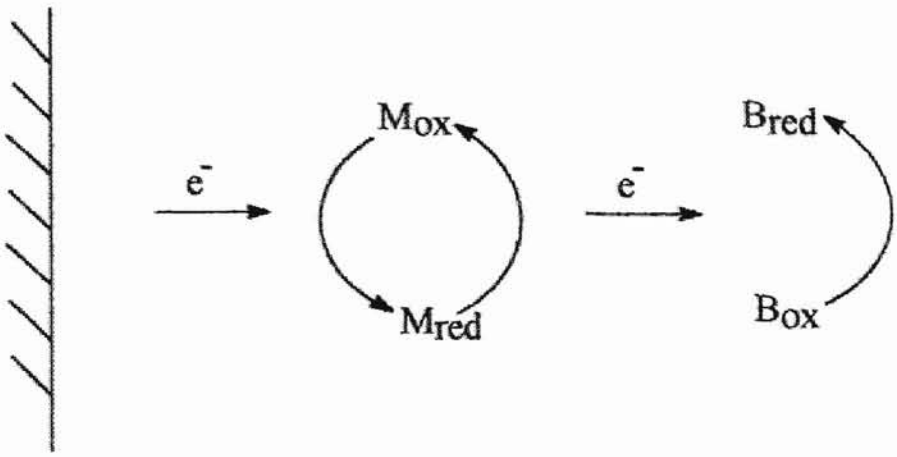


Fig. 3.15 M = Mediator-titrant, Ru(III).
B = protein, OM cyt. b5.
Ru(III) conveys electrons between the gold electrode and OM cyt. b 5.
Reduction/oxidation of OM cyt. b 5 is thus indirect through Ru(III).
(Adapted from Ref. 33).

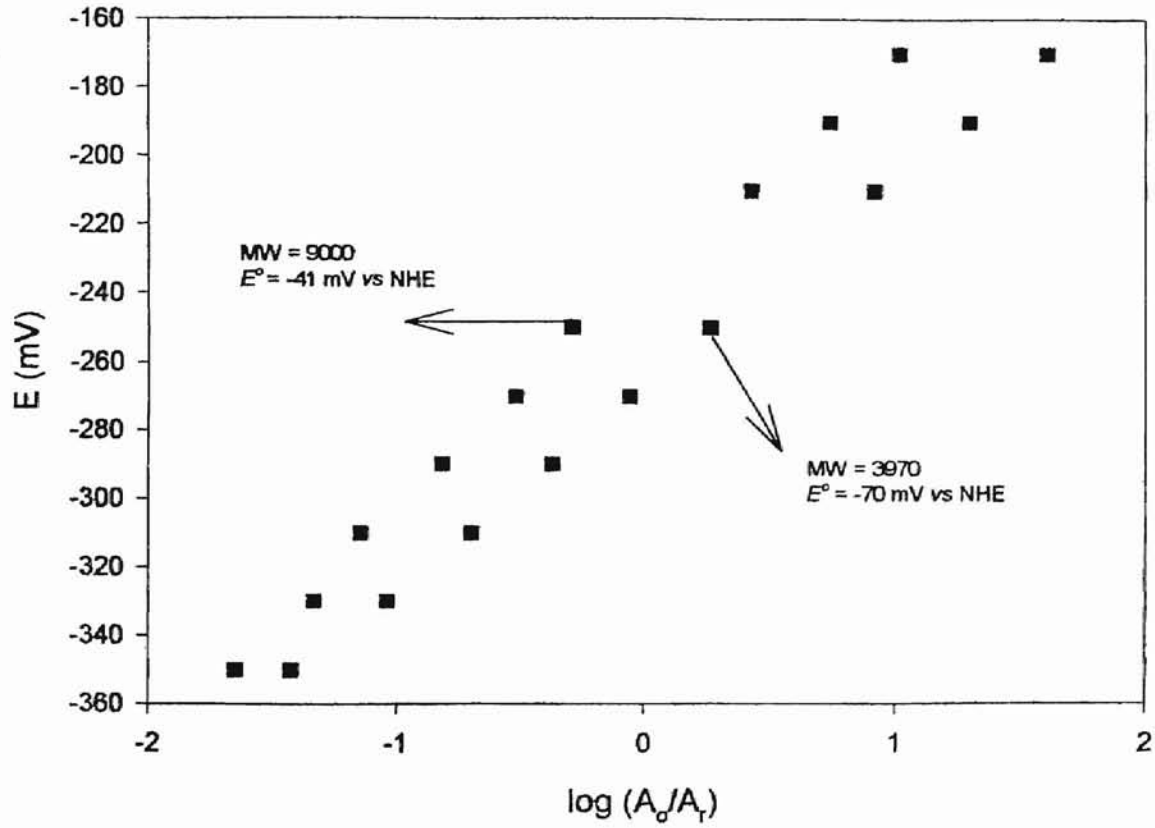


Fig. 3.16 Nernstian plots obtained for a solution of OM cyt. b_5 (52 micromolar) in 100 mM MOPS, pH 7.0 in the presence of polylysine (103 micromolar), MWs = 3970, 9000

potential are tabulated in Table 3.6.

Fig. 3.17 was obtained by running a cyclic voltammogram of OM cyt. b_5 (0.1 mM) in 100 mM MOPS, pH 7.0, in the presence of polylysine (0.2 mM), MW 3970. The working electrode was a gold disk electrode modified with β-mercaptopropionate. The scan rate was set at 50 mV/s. *Inset*: A plot of the square root of scan rate *vs* cathodic peak current, made for the solution of OM cyt. b_5, in the presence of polylysine, MW 3970, in 100 mM MOPS, pH 7.0. The voltammogram is discussed in the following chapter.

Fig. 3.18 was obtained by carrying out a titration of a solution of OM cyt. b_5 in 100 mM MOPS, pH 7.0, by adding increments of polylysine (MW 3970) to the solution. The same was repeated with polylysine (MW 9000).

Fig. 3.19 was obtained by carrying out an NMR titration of a C-13 labeled solution of 2 mM OM cyt. b_5 in 5 mM perdeuterated phosphate buffer, pH 7.0, to which increments of polylysine, MW 3970 were added, and the corresponding chemical shifts read. This was done in order to gain insight into the factors modulating the reduction potential of cyt. b_5.

NMR Spectroscopy Experimental details: δ-ALA that was C-13 labeled was used to prepare the heme that was incorporated into cyt. b_5 [23 a]. NMR titrations of the protein by adding increments of polylysine (MW 3970) were carried out using a Varian Unitiy Inova 400 spectrometer (C-13 operating frequency = 100. 6 MHZ). OM cyt. b_5 was dialyzed against water, exchanged with D_2O , and freeze-dried. Acqusition of the resonances due to the carbonyl carbons of the heme propionate at room temperature was carried out. The spectral width was set at 9.5 kHz, the acquisition time at 1.1 s, and the

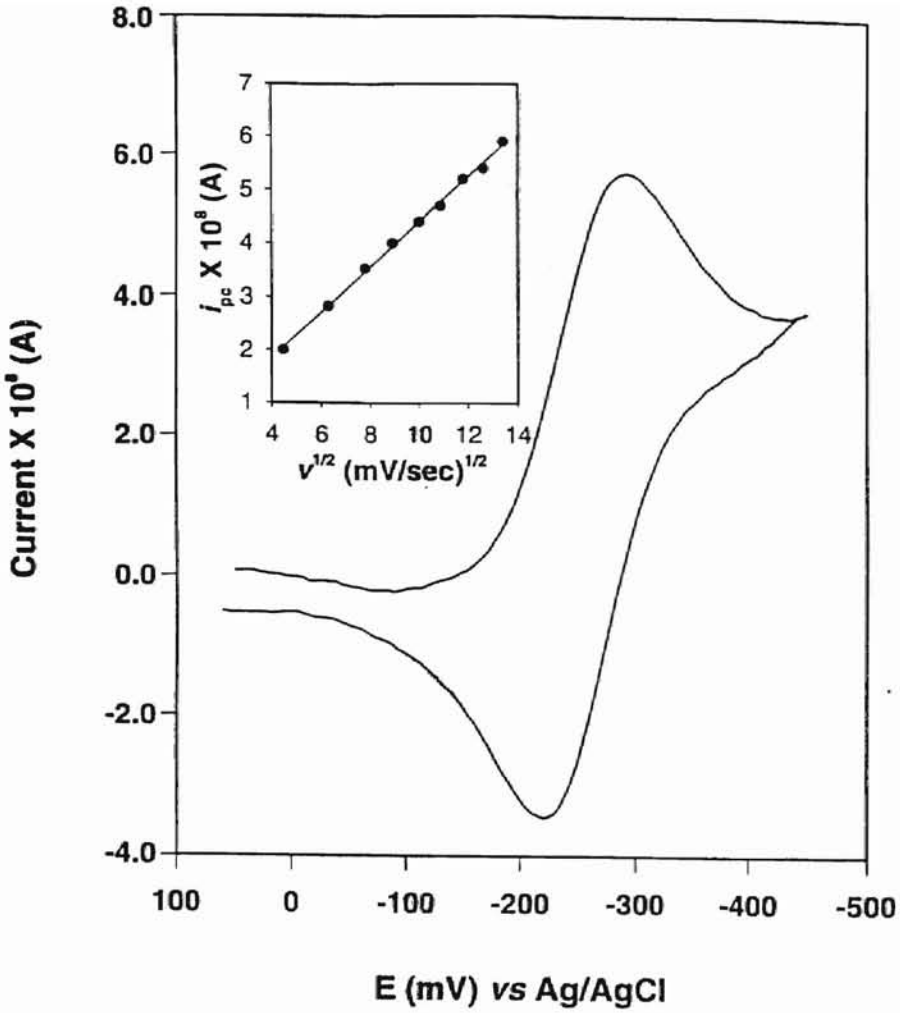


Fig. 3.17 Background-subtracted cyclic voltammogram of the rat OM cyt. b_5 (0.1 mM) in 100 mM MOPS at pH 7.0. The gold disk electrode was modified with β-mercaptopropionate, and the voltammogram was obtained in the presence of 0.2 mM polylysine (MW = 3970), with a scan rate of 50 mV/s. The reduction potential of OM cyt. b_5 depends on the concentration and molecular weight of polylysine (see Fig. 3.19), but the attributes for reversibility are independent of the concentration of polylysine when the polylysine/cyt. b_5 ratio is larger than 0.2.

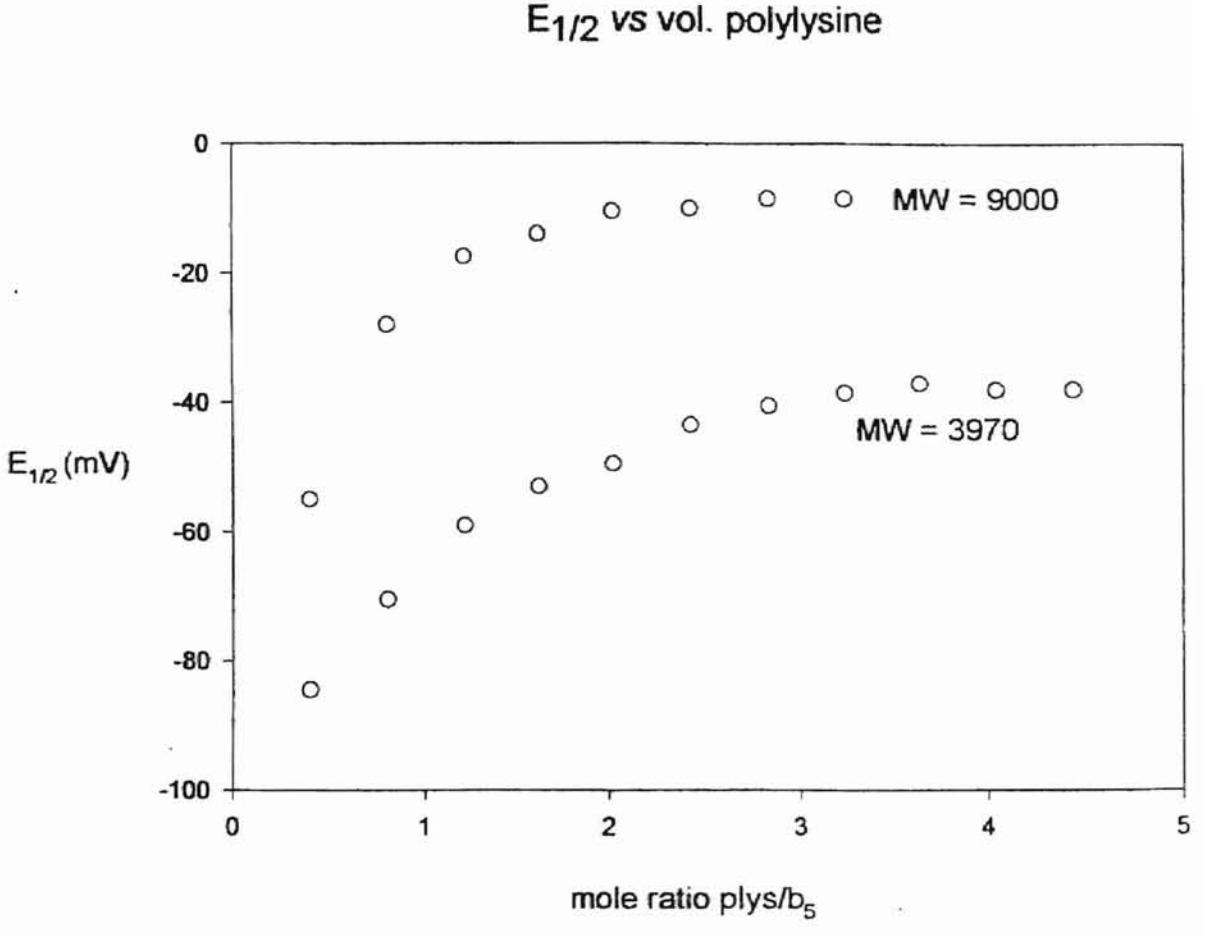


Fig. 3.18 Graph of $E_{1/2}$ *vs* molar ratio plys/b_5.

The protein solution was titrated separately with two different MWs of polylysine. One titration was carried out with MW = 3970. Another titration was carried out on a fresh protein solution with MW = 9000.

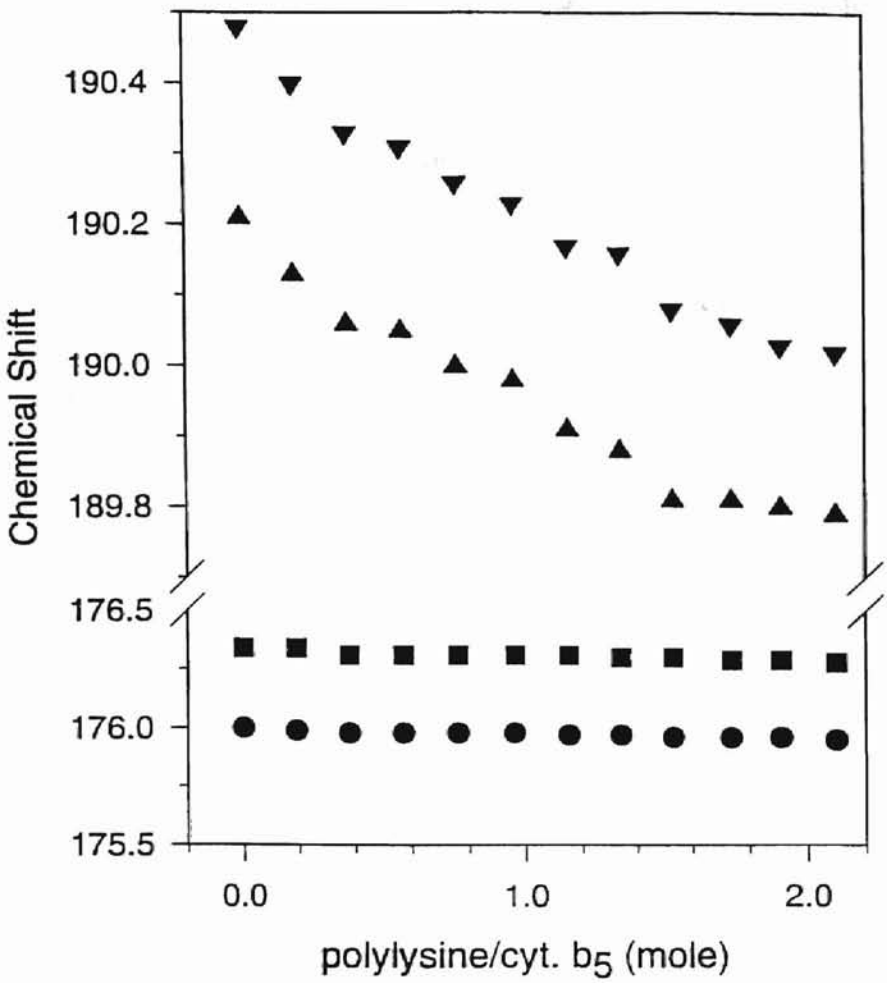


Fig. 3.19 NMR titration curves of OM cyt. b_5
with polylysine (MW = 3970).
(▲ and ▼) indicate the protein resonances due to
the heme propionate located on the exposed heme edge.
(• and ■) indicate the protein resonances due to
the heme propionate located on the buried heme edge.

relaxation delay at 200 ms. Two thousand scans were acquired for each data point.

Fig. 3.20 was obtained by carrying out titrations of OM cyt. b_5 with polylysine (MWs 3970, 9000). The same was repeated with the dimethyl ester. The * in the plot corresponds to the value of E° obtained for OM cyt. b_5 by spectroelectrochemistry. The trends noticed in the plot are discussed in the following chapter.

Fig. 3.21 shows the titration of the polylysine-cyt. b_5 complex (polylysine/cyt. b_5 ratio = 2.0) with $MgCl_2$. First, a solution of OM cyt. b_5 in 100 mM MOPS, pH 7.0 was titrated with increments of polylysine, MW 3970. When a polylysine/cyt. b_5 ratio of 2.0 was reached, the titration was performed by adding increments of Mg^{2+}.

Chapter IV

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 Fig. 3.4. The striking feature is two reduction peaks 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 corresponding 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 the 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. 4.1 and 4.2, and schematically in Fig. 3.5-I, II, III, IV.

$$\text{Fe(III) cyt. } b_5 + e^- \overset{k_s}{\rightleftharpoons} \text{Fe(II) cyt. } b_5 \qquad (4.1)$$

$$\text{Fe(II) cyt. } b_5 + \text{Fe(II) cyt. c} \underset{k_b}{\overset{k_f}{\rightleftharpoons}} \text{Fe(III) cyt. } b_5 + \text{Fe(II) cyt. c} \qquad (4.2)$$

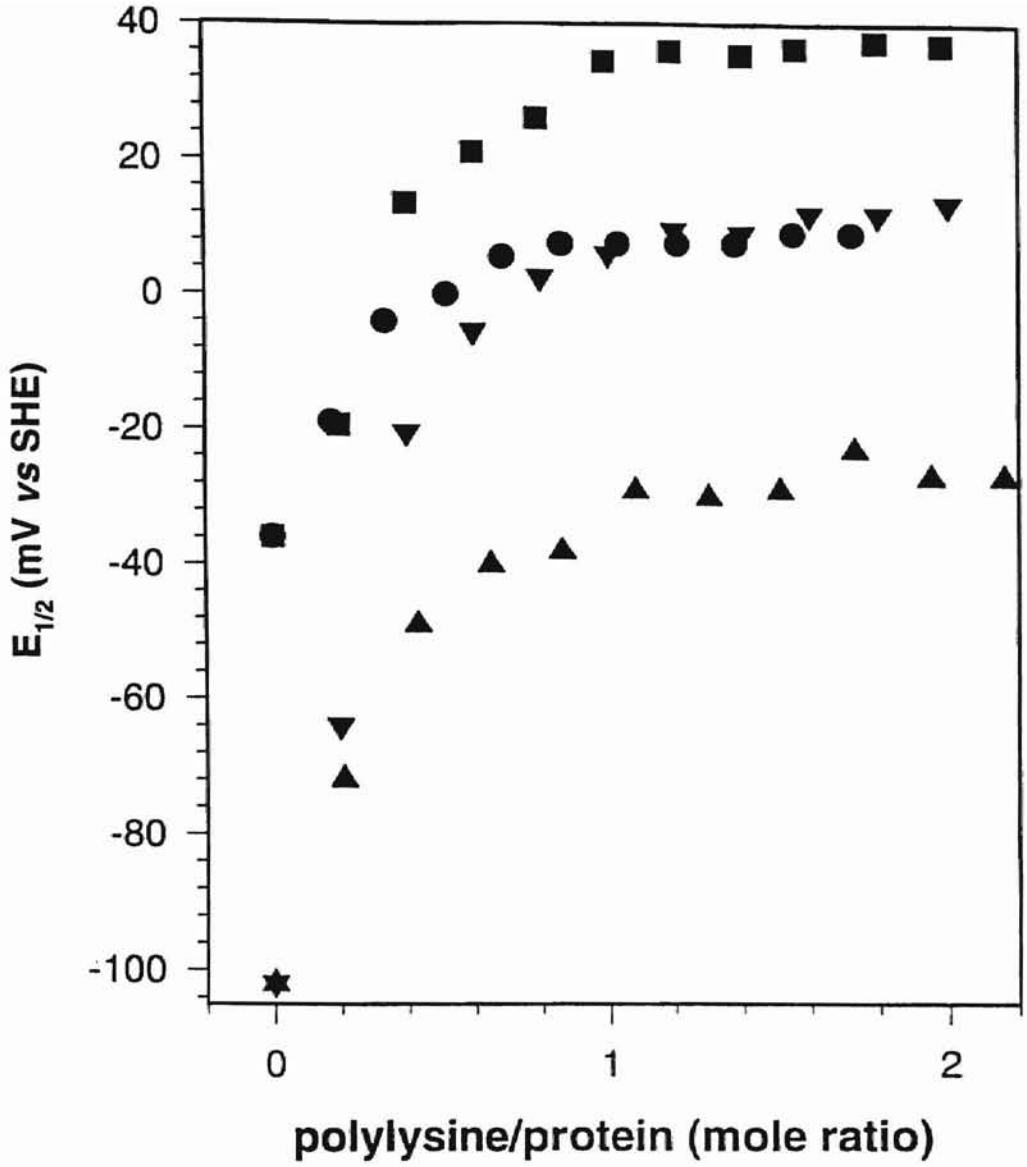


Fig. 3.20 Titration of OM cyt. b_5 with polylysine (MW = 3970) (▲) and with polylysine (MW = 9000) (▼). Titration of OM cyt. b_5 DiMe ester with polylysine (MW = 3970) (●) and with polylysine (MW = 9000) (■). The first point in each titration curve "(polylysine/protein mole ratio = 0) corresponds to the reduction potential measured by potentiometry in the absence of polylysine.

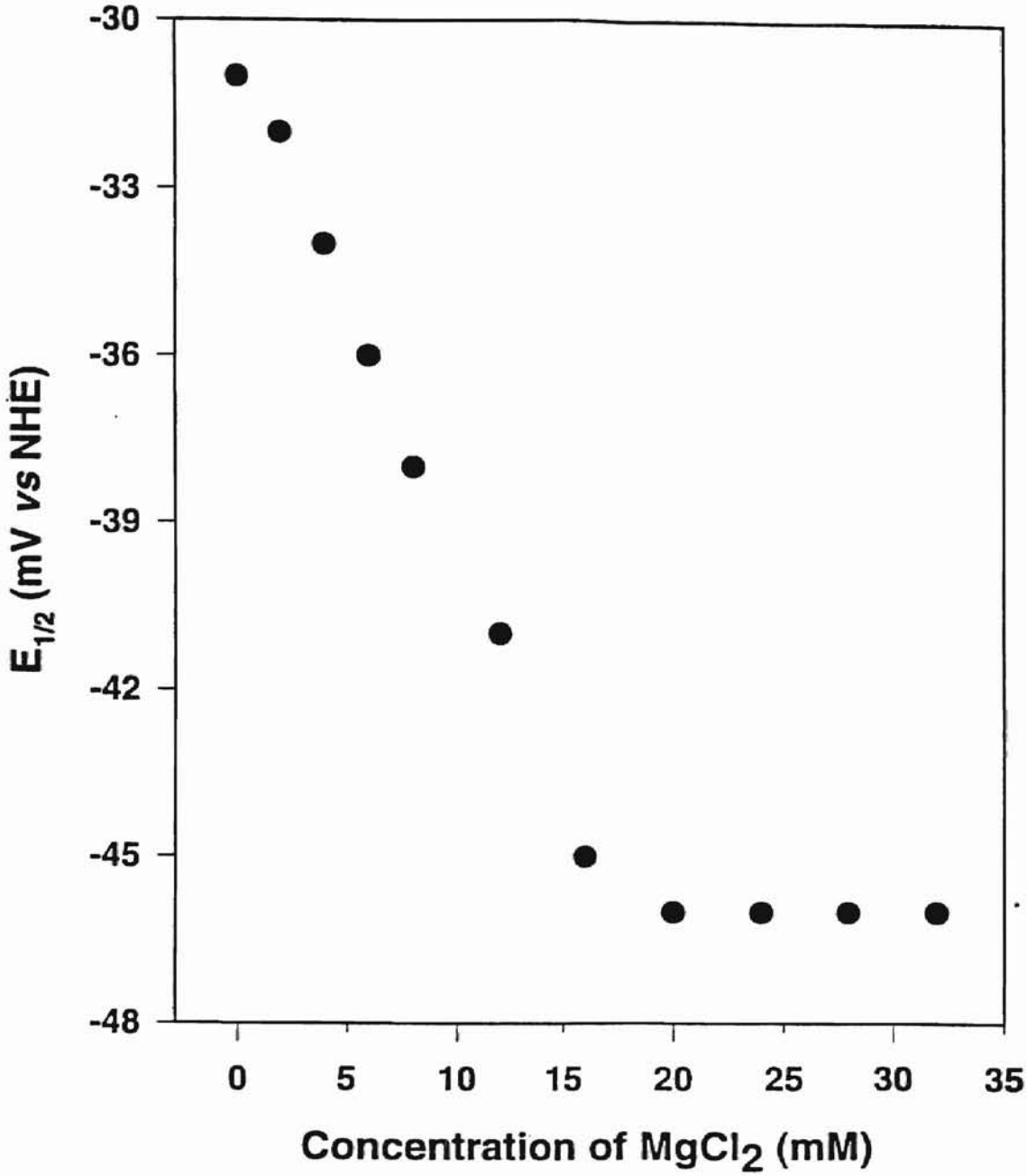


Fig. 3.21 Titration of the polylysine-cyt. b_5 complex (polylysine/cyt. b_5) ratio = 2.0 with $MgCl_2$.

In Eq. 4.1, k_s is the heterogeneous electron transfer rate constant, and in Eq. 4.2, k_f and k_b are the forward and backward second-order electron transfer rate constants. The presence of polylysine in solution results in the selective reduction of ferricytochrome b_5 as shown schematically in Fig. 3.5-I. The reduction of positively charged Fe(II) 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 (Fig. 3.5-II). If the ratio of k_f/k_b is large, a pre-peak separates from the main peak [24]. 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 Fig. 3.5-III. In the return scan, the peak observed is due to the oxidation of ferrocytochrome b_5 at the electrode surface (Fig. 3.5-IV). These results are consistent with the reported cyclic voltammetric behaviour of cytochrome c in the presence of plastocyanin at gold electrodes modified with bis(4-pyridyl)bisufide, reported by Barker *et. al.*[24]. They observed pre-shoulders to both reduction and reoxidation current peaks. This was interpreted 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. 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 Fig.

3.4, *i. e.*, a pre-peak preceding the reduction current peak if the electrode is selective to the negatively charged cytochrome b_5.

As further experimental evidence supporting the above interpretation of the cyclic voltammogram shown in Fig. 3.4, an experiment was performed in which the first step involved obtaining a cyclic voltammogram 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 (Fig. 3.6A). When polylysine is added to the solution containing cytochrome c, no Faradaic current is observed (Fig. 3.6B) 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 Fig. 3.6C 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 Eqs. 4.1 and 4.2 and schematically shown in Fig. 3.5. 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 (Fig. 3.6D).

Titration of cytochrome c with a solution of cytochrome b_5 and polylysine-

When ferricytochrome c was added stepwise to a solution of cytochrome b_5 and polylysine, while monitoring the titration by cyclic voltammetry (Fig. 3.7), 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 obtained (Fig. 3.8A). The absence of the pre-peak in Fig. 3.8A 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 (Fig. 3.8B).

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 experiments involving mitochondrial cytochrome b_5. This voltammogram (Fig. 3.9) 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. 4.3), which reacts with another species P in solution to regenerate O and produce Q (Eq. 4.4), the net effect is catalysis of the reduction of P by the O/R couple, thus giving rise to the EC mechanism:

$$O + e^- \overset{k_s}{\rightleftharpoons} R \tag{4.3}$$

$$R + P \underset{k_b}{\overset{k_f}{\rightleftharpoons}} O + Q \tag{4.4}$$

The EC mechanism requires that the reduction of P at the electrode is much slower than the homogeneous redox reaction between P and R so that the reduction of P at the electrode can be neglected [25]. 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 Fig. 3.5. This implies that in Eqs. 4.3 and 4.4, O = ferricytochrome b_5, R = ferrocytochrome b_5, P = ferricytochrome c, and Q = ferrocytochrome c. Ferricytochrome c 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. 4.1 and 4.2.

In order to have a better understanding of the events that are occurring, digital simulation of the proposed EC reaction scheme has been carried out in order to further substantiate the above interpretation of the cyclic voltammogram shown in Fig. 3.4. 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 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.

Experimental and simulated cyclic voltammograms obtained with solutions containing OM cytochrome b_5 (0.050 mM) and horse heart cytochrome c (0.10 mM); 0.10 mM OM cytochrome b_5 and 0.050 mM horse heart cytochrome c, are shown in Figs. 3.12 a, 3.12 b. The cyclic voltammogram depicted in Fig. 3.12 a obtained with a cytochrome b_5/cytochrome c ratio = 0.5 is consistent with the model and 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 (Fig.3.12 b) 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 [30]. At low ionic strengths, 2 mM, the kinetic behaviour was reported to be consistent with electron transfer within a 1:1 complex formed between microsomal cytochrome b_5 and horse heart cytochrome c. Increasing the ionic strength to approximately 20 mM decreases the stability of the complex and the second-order rate constants decrease rapidly as the ionic strength is increased. 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 3.5). 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) at different ionic strengths.

Experimental and simulated cyclic voltammograms obtained at μ=130 mM and 230 mM are depicted in Figs. 3.13 a, 3.13 b. 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/cytochrome c complex [31]. Eley & Moore found that the binding constant for the formation of the cytochrome b_5/cytochrome c complex is 4 X 10^6 M^{-1} at μ = 1 mM and 8 X 10^4 M^{-1} at μ = 10 mM. Subsequently, Eley and Moore (1983) [32] measured the binding constant at μ = 40 mM and reported a value of 9.6 X 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 a 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 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 [7, 8]. 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 [12 a], 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.

Comparison study of the reduction potential values measured by Voltammetry and Spectroelectrochemistry:

When the reduction potential of cytochrome b_5 was measured with the aid of

several different surface modified electrodes that function based on electrostatic interactions with the protein, the resultant values have been consistently more positive (40-100 mV) than the reduction potentials measured with potentiometric methods.

It was therefore apparent that there was something unique about the interaction between cyt. b_5 and modified electrodes that resulted in reduction potentials that were significantly more positive than the reduction potential values measured with potentiometric techniques. The electrostatic recognition between modified electrode and protein, which precedes heterogeneous electron transfer in voltammetric experiments, has been compared to the electrostatic interactions that occur between physiological partner proteins just before electrons are transferred from one protein to another in homogeneous solution [34]. For this reason, the significant anodic shift observed in the reduction potential of cyt. b_5 when it is measured at surface-modified electrodes that rely upon electrostatic interactions may be indicative of important structure-function relationships in this heme protein.

Rivera *et al.*[9] reported that, when the cyclic voltammogram of cyt. b_5 was obtained at a β-mercaptopropionate-modified gold electrode in the presence of polylysine as a facilitator, the reduction potential of the cyt. shifted in the positive direction as the concentration of polylysine was increased. This unusual behaviour was attributed to the formation of a complex between cyt. b_5 and polylysine. When the reduction potential of cyt. b_5 was measured in the presence of polylysine (polylysine/cyt. b_5 mole ratio = 2.0) by spectroelectrochemistry, its reduction potential was 30 mV (polylysine MW = 3970) and 62 mV (polylysine MW = 9000) more positive than the value measured in the absence of

polylysine, respectively (Table 3.6). By comparison, when similar experiments were performed with the dimethyl ester of cyt. b_5, DiMe cyt. b_5, the reduction potentials measured were independent of the presence of polylysine in the spectroelectrochemical cell (Table 3.6). The 66 mV difference between the reduction potentials of OM cyt. b_5 and its DiMe ester can be explained by the stabilization of the positive charge of the ferric heme in OM cyt. b_5 by the heme propionates. This stabilization is no longer possible in the DiMe derivative, and consequently, its reduction potential becomes more positive [32].

An important feature of the spectroelectrochemical experiment is that electrons are transferred from the electrode to the protein via redox mediators, and consequently, the protein is not in intimate contact with the electrode during the oxidation or reduction process. Therefore, the positive shift in the reduction potential of OM cyt. b_5 in the presence of polylysine (Table 3.6) must arise from the formation of a complex between the protein and polylysine. By comparison, the reduction potential of the DiMe derivative is not affected by the presence of polylysine in solution, thus indicating that the shift in reduction potential observed for cyt. b_5 in the presence of polylysine must result from the electrostatic neutralization of the heme propionate located on the exposed heme edge. The values of the reduction potential of DiMe cyt. b_5, which are independent of the concentration of polylysine (Table 3.6), also indicate that the neutralization of the acidic residues on the surface of the protein upon complexation with polylysine does not have a large effect on its reduction potential. Evidence for the formation of a complex between DiMe cyt. b_5 and polylysine is provided by the fact that polylysine promotes the voltammetry of DiMe cyt. b_5, but since the heme propionate on the exposed heme edge of

the ester derivative cannot participate in electrostatic binding, its reduction potential measured by spectroelectrochemistry, is not affected (Table 3.6).

Reversible cyclic voltammograms were obtained for OM cyt. b_5 or its DiMe ester at gold electrodes modified with β-mercaptopropionate in the presence of polylysine. The typical cyclic voltammogram obtained for OM cyt. b_5 in the presence of polylysine (polylysine/cyt. $b_5 = 2.0$) is shown in Fig. 3.17. The ratio of the cathodic to anodic peak currents (i_{pc}/i_{pa}) is unity, and the peak to peak separation (ΔE_p) is 62 mV. The cathodic peak current is proportional to the square root of the scan rate (Fig. 3.11), indicating that the electrochemical process is diffusion-controlled. The reduction potential values of cyt. b_5 depend on the concentration and molecular weight of polylysine (Fig. 3.18), but the attributes of the voltammograms (i_{pc}/i_{pa}, ΔE_p, and $v^{1/2}$ *vs* i_{pc}) remain unchanged throughout the titration with polylysine. It is also notewothy that the potential at which the reduction potential becomes independent of the concentration of polylysine (the point at which the potential remains constant) also depends on the molecular weight of the polylysine. The positive shift in the reduction potential may be explained by the formation of a transient complex between cyt. b_5 and polylysine at the electrode surface, in which the negative charge on the propionate located on the exposed heme edge is neutralized.

In order to obtain further evidence for the participation of the exposed heme edge propionate of OM cyt. b_5 in binding to polylysine (MW 3970), and thereby modulating the reduction potential of the protein, NMR titrations were carried out to obtain Fig. 3.19. On adding increments of polylysine, the resonances corresponding to the carbonyl carbon (▲ and ▼) located on the exposed heme edge show a shift, whereas those on the buried heme

edge (■ and •) remain unaffected. Therefore, only one of the heme propionates in cyt. b_5 participates in binding to polylysine. So the electrostatic interaction between cyt. b_5 and polylysine neutralizes the negative charge on the heme propionate located on the exposed heme edge, resulting in an anodic shift in the reduction potential.

However, if neutralization of the heme propionate is the only factor modulating the reduction potential of cyt. b_5, the reduction potential observed for DiMe cyt. b_5, whose heme propionates are esterified, should be independent of the concentration of polylysine, as observed with the potentiometric experiments. The cyclic voltammetric experiments, however, showed that the reduction potential of DiMe cyt. b_5 also shifted in the positive direction as a function of the concentration of polylysine (•and ■ in Fig. 3.20). This indicated that the neutralization of the heme propionate located on the exposed heme edge was not the only factor determining the potential shift observed when the cyclic voltammetry of cyt. b_5 was promoted by the addition of polylysine. An additional factor influencing the anodic shift in the reduction potential of OM cyt. b_5 and its DiMe ester must take place.

For electron transfer to occur at the modified electrode surface, cyt. b_5 or its DiMe ester diffuses toward the electrode surface where a transient complex is formed between polylysine and the negatively charged protein. At the scan rate of 50 mV/s, diffusion of cyt. b_5 toward the electrode surface is evident from the proportionality of the cathodic peak current with square root of the scan rate (Fig. 3.17). In the transient complex formed at the electrode surface, cyt. b_5 is likely to bind with polylysine utilizing its surface area delimited by acidic residues 44, 48, 56, and 60 and the heme propionate located on the

exposed side of the heme (Fig. 1.2 a). Consequently, water at the interface between polylysine and the protein may be excluded upon formation of the transient complex, thus decreasing the value of the dielectric experienced by the heme microenvironment. A smaller dielectric around the heme microenvironment may result in a decrease in the stability of the positively charged ferric heme with respect to the neutral ferrous heme, therefore contributing to the anodic shift in reduction potential. When the molecular weight of the polylysine is increased, a more positive saturation potential is observed because the larger polyelectrolyte must be capable of making a larger number of electrostatic interactions with acidic residues on the surface of cyt. b_5 or its DiMe ester, thus resulting in a more efficient dehydration of the exposed heme microenvironment. Furthermore, when Mg^{2+} was added to a solution containing polylysine/cyt. b_5 ratio larger than 2.0 (the point at which potential remained constant in Fig. 3.17), the reduction potential gradually shifted cathodically and remained constant at approximately -47 mV (Fig. 3.22). This indicated that Mg^{2+} competed with polylysine for the heme propionate and acidic residues on the surface of the protein. As the concentration of Mg^{2+} increased, the cyt. b_5-polylysine complex dissociated, and the electrochemistry was likely to be promoted mainly by Mg^{2+} ions, which have been previously shown to promote the electrochemistry of cyt. b_5 at β-mercaptopropionate electrodes [9]. The negative excursion of the reduction potential upon titration of the cyt. b_5-polylysine complex with Mg^{2+} ions maybe due to the increased accessibility of water to the area surrounding the exposed heme edge when the cyt. b_5-polylysine complex was dissociated and the electrochemistry was promoted mainly by Mg^{2+} ions. The reduction potential remaining constant at -47 mV

is likely to result from neutralization of the heme propionate negative charge upon binding to Mg^{2+}.

Chapter V

CONCLUSION

It is important to realize that the investigation of the electrochemistry of redox proteins leads to consideration of the nature of the interface formed between electrode and protein. Further, when protein-protein reactions are studied, we have shown that the presence in solution of a second species reacting by fast second-order electron transfer reaction with the electroactive species can lead to a coupled electrochemical response. The use of a chemically modified electrode interface, in the presence of a polycation, which is selective for one species only facilitates the transfer of an electron to the second species, cytochrome c only through coupled electron transfer reactions in homogeneous solution. The features of the cyclic voltammogram obtained in Fig. 3.4 are accounted for by such a scheme. We have, therefore, been able to combine strong electrochemical selectivity with fast homogeneous electron transfer reactions.

The EC scheme has been simulated adequately by explicit digital methods using Digisim. This mechanism gave rise to the observation of a prepeak. The potential of the main peak shifted only slightly by the occurrence of the chemical reaction that gave rise to the prepeak. This method provided a way in which a prepeak could be noticed as the reaction between two proteins was sufficiently rapid. Further, in the absence of cyt. c, the charge transfer process, Fe(III) $b_5 + e^- \rightleftharpoons$ Fe(II) b_5, gave rise to a reversible cyclic voltammogram.

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. However, 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.* [17] had to use a mixture containing an 8-fold molar excess of cytochrome b_5 to measure the second-order rate constant for the oxidation of ferrocytochrome b_5 by ferricytochrome c. Durham *et al.* [12 b] and Willie *et al.* [30] had to covalently attach a $[Ru(bypyridine)_3]^{2+}$ complex to the surface of cytochrome b_5. The photochemical properties of ruthenium complexes of this type provided a means of generating ferrocytochrome b_5 in the presence of ferrocytochrome c.

The electrochemical method developed as part of the work presented here provides an alternative experimental scheme to selectively reduce a protein possessing a relatively low potential (cytochrome b_5) in the presence of equimolar concentrations of a protein possessing a relatively high potential (cytochrome c). This is a reliable, economic method. The electrochemical signal obtained 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 ferricyotchrome c and a reversible wave corresponding to the reduction and oxidation of cytochrome b_5 in the diffusion layer (Fig. 3.1). Digital simulation of these electrochemical signals obtained under a variety of 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 method can be reliably used to determine second-order rate constants for interprotein electron transfer systems under a variety of experimental conditions.

Thus, one of the important features of this method, besides the discriminatory nature of electron transfer at electrodes, is that it throws light on electron transfer rates that could be compared with biological reaction rates. Electrochemistry has been used as a probe of reaction rates of proteins.

Cyclic voltammetric measurements, along with the spectroelectrochemical titrations of cytochrome b_5 and its dimethyl ester (in the presence and absence of polylysine, MWs 3970, 9000), indicate that reduction potential of the protein is largely modulated by its structural properties. Modulation of the reduction potential in the protein results from the formation of a complex between cytochrome b_5 and polylysine, which encompasses the surface area of the protein delineated by the exposed heme propionate and acidic residues 44, 48, 56, 60. The formation of such a complex results in:

(a) Charge neutralization of the heme propionate on the exposed heme edge, which results in the destabilization of the positive charge on the ferric heme, thus partially contributing to the anodic shift.

(b) Exclusion of water from the complex interface, which includes the exposed heme edge that in cytochrome b_5 is part of the protein surface. This may result in a significantly lower value of the dielectric experienced by the exposed heme microenvironment, thus contributing to the observed positive shift. In addition, it is also possible that double layer effects governed by the charged promoter on the electrode surface may contribute to the

modulation of the reduction potential of cytochrome b_5.

It is important to appreciate that the orientation of cytochrome b_5 in the electrostatic complexes formed with modified electrodes appears to be similar to that encountered when it binds physiological partner proteins. Since it is known that the heme propionate located on the exposed heme edge of cytochrome b_5 participates in electrostatic binding to cytochrome c [35], and that the formation of the cytochrome b_5-cytochrome c complex is accompanied by exclusion of water from the complex interface [36 a, b], it is possible that the reduction potential of cytochrome b_5 shifts in the positive direction when it forms a transient complex with cytochrome c before the electron-transfer event. Because the system consisting of cytochrome b_5 and cytochrome c has been a paradigm for the study of electron transfer reactions among redox proteins, the possibility that the reduction potential of cytochrome b_5 is modulated upon formation of a transient complex with cytochrome c is worth is exploring.

Table 3.1 Parameters for Digital Simulation of a Typical Cyclic Voltammogram Obtained from a Mixture Containing Rat OM Cytochrome b_5 and Horse Heart Cytochrome c

Charge Transfer Parameters	
E^o (*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. conc. (P) (M)	0.00010

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

Table 3.2 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

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. conc. (P) (M)	0.00010

Table 3.3 Scan rates and cathodic peak current values obtained in the experimental determination of diffusion coefficient, D_O of OM cytochrome b_5 (Refer Fig. 3.11)

Scan rate, v (V/s)	$(v)^{1/2}$	Cathodic peak current, i_{pc} (A)
0.02	0.1414	5.2×10^{-8}
0.05	0.224	8.6×10^{-8}
0.075	0.274	1.1×10^{-7}
0.1	0.316	1.3×10^{-7}
0.150	0.387	1.6×10^{-7}

Slope of line in Fig. 3.11 = 4.4×10^{-7}

Table 3.4 Values of Ψ and the heterogeneous electron transfer rate constant, k_s

ΔE_p (mV)	Ψ	$k_s = \Psi[D_o \pi \nu (nF/RT)]^{1/2}$, cm/s
103	0.554	0.0086
97	0.636	0.00983
96	0.653	0.0101
89	0.77	0.0119
93	0.747	0.0116
96	0.653	0.0101
91	0.77	0.0119

Table 3.5 Second-Order Rate Constants for the Reduction of Horse Heart Ferricytochrome c by OM Ferrocytochrome b_5

protein conc. (mM)	μ (M)	scan rate (mV/s)	k_f ($M^{-1}s^{-1}$)
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	$2.2 \times 10^8 \pm 0.95$

Each value of k_f is an average of four measurements.

Table 3.6 Reduction Potentials Obtained by Spectroelectrochemistry

protein	polylysine (MW = 3970)	polylysine (MW = 9000)	E° (mV)
cyt. b_5 (47 μM)	-	-	-102
cyt. b_5 (52 μM)	103 μM	-	-70
cyt. b_5 (52 μM)	-	104 μM	-40
DiMe b_5 (52 μM)	-	-	-36
DiMe b_5 (52 μM)	104 μM	-	-33
DiMe b_5 (52 μM)	-	104 μM	-32

cyt. b_5 = OM cyt. b_5. DiMe b_5 = OM cyt. b_5 dimethyl ester.

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APPENDIX

I. List of Abbreviations

OM	Outer Mitochondrial
cyt.	Cytochrome
UV	Ultra-violet
SHE	Standard Hydrogen Electrode
EDTA	Ethylene diamine tetra acetic acid
EC	Electrochemical Chemical
LB	Luria Bertani

II. Definition of Terms:

1. Cyclic voltammetry: A technique that consists of cycling the potential of an electrode, which is immersed in an unstirred solution containing an inert supporting electrolyte, MOPS, 0.10 M, pH 7.0, and measuring the resulting current. The potential of the working electrode is controlled *vs* a reference electrode such as an Ag/AgCl electrode.

2. Spectroelectrochemical Titrations: Two quite different techniques, electrochemistry and spectroscopy, are combined. Oxidation states are changed electrochemically by the addition of electrons at an electrode while spectral measurements on the solution adjacent to the electrode are made simultaneously.

3. Reduction potential: The potential of for the following reaction written as reduction:

$$Fe(III)\ b_5 + e^- \rightleftharpoons Fe(II)\ b_5.$$

4. Electron transfer rate constants: Consider the electron transfer process

$$A + e \rightleftharpoons B$$

Suppose that the rate of the forward reaction (the cathodic or reduction rate) is first-order in A: cathodic rate = $k_s\ C_o(0, t)$, k_s denotes the electron transfer rate constant, C_o, the initial concentration of the species A (*i. e.*, concentration at time t = 0 sec.).

5. Faradaic current: Charge (*e. g.*, electron) transfer across the metal-solution interface that causes oxidation or reduction to occur. Since these reactions are governed by Faraday' s law (i. e., the amount of chemical reaction caused by the flow of current is proportional to the amount of electricity passed), the current is called Faradaic current.

6. Capacitance: The capacitance of a conductor is defined as the ratio of its charge

Q to its potential V, i. e., $C = Q/V$. For a parallel-plate capacitor, $C = A\epsilon_o\epsilon/d$, where A is the area, and ϵ the dielectric constant of the medium between the plates.

7. Diffusion-controlled current: Electron flow or ion or molecule transport occurs through diffusion (transport of mass under the influence of a chemical potential or concentration gradient).

8. Diffusion coefficient: Fick' s law involves the flux, q, which is the number of moles of diffusing substance crossing one square centimeter of an imaginary plane per second. The law states that the flux is proportional to the concentration gradient. The constant of proportionality, D, is called the diffusion coefficient.

$$q(x, t) = D\ \delta C\ (x, t)/\delta x$$

9. EC Reaction (Electrochemical-Chemical): In general an electrode process consists of a series of electron-transfer steps, designated by the symbol E, and chemical steps, designated by C. In an EC Reaction, also called "Following Reaction",

$$O + e \rightleftharpoons R$$

Here, R is an electroactive product formed by the reduction of O. This is an electrochemical reaction (E). This is followed by

$$R \rightarrow Z,$$

which is a chemical reaction involving the conversion of R to Z, an electroinactive species. Hence the name EC reaction.

10. Flash photolysis: In this approach used by Meyer *et al.*, a high-intensity short-duration flash of light reduces the reagent, deazariboflavin, that in turn reduces ferricytochrome b_5, and the absorbance *vs* time curves read.

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