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Cation-Promoted Cyclic Voltammetry of Recombinant Rat Outer Mitochondrial Membrane Cytochrome b_5 at a Gold Electrode Modified with β -Mercaptopropionic Acid[†]

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ABSTRACT: Reversible cyclic voltammetry of recombinant rat outer membrane (OM) cytochrome b_5 was observed at a gold electrode modified with β -mercaptopropionic acid. Electron transfer between the negative electrode surface and the negatively charged OM cytochrome b_5 was promoted by the addition of divalent metal ions such as Mg^{2+} or Ca^{2+} and by the positively charged species poly-L-lysine. The titration of OM cytochrome b_5 (0.1 mM) with poly-L-lysine resulted in a gradual positive shift of the $E_{1/2}$ value which leveled off at +8 mV *vs* NHE when the poly-L-lysine:cytochrome b_5 ratio reached a value of 2:1. Since the further addition of poly-L-lysine had no effect on the $E_{1/2}$ value of the protein, it was concluded that a complex is formed in which two molecules of poly-L-lysine bind to each molecule of OM cytochrome b_5 . When the OM cytochrome b_5 –poly-L-lysine complex (0.1 mM) was titrated with Mg^{2+} or Ca^{2+} ions, the $E_{1/2}$ value shifted gradually in the negative direction and leveled off at –40 mV *vs* NHE when the concentration of divalent ions reached 85 mM. When the voltammetric response of 0.1 mM cytochrome b_5 was promoted by Mg^{2+} or Ca^{2+} ions, the minimum concentration of divalent cation necessary to produce a reversible voltammogram was 40 mM and the observed $E_{1/2}$ was –46 mV *vs* NHE. On the other hand, only 0.2 mM $[Cr(NH_3)_6]^{3+}$ was necessary to promote the reversible electrochemistry of 0.1 mM cytochrome b_5 . The half-wave potential observed under these conditions was –78 mV *vs* NHE. This indicates that there is a large dependence of the reduction potential of cytochromes b_5 on the kind and concentration of multivalent ions in solution. A reduction potential of –102 mV *vs* NHE was obtained for OM cytochrome b_5 (0.60 mM) by spectroelectrochemical titration in the presence of 0.4 mM $[Ru(NH_3)_6]^{3+}$ and 1 mM methyl viologen, pH 7.0, $\mu = 0.1$ M. This value is approximately 100 mV more negative than the reduction potentials reported for microsomal cytochromes b_5 obtained from other sources under the same conditions. The binding interactions between OM cytochrome b_5 and poly-L-lysine or Mg^{2+} ions were probed by investigating the isotropically shifted 1H NMR resonances arising from the heme in the OM cytochrome b_5 . The NMR spectroscopic studies showed that the Mg^{2+} ions are chelated by the heme propionate in position f, namely, heme propionate 6 in isomer A and heme propionate 7 in isomer B, and by the carboxylate groups of the cation binding sites on the surface of the protein. Poly-L-lysine was found to interact only with the cation binding sites on the surface of the OM cytochrome b_5 . The differences in reduction potential that were observed for the OM cytochrome b_5 in the presence of $MgCl_2$ or poly-L-lysine are believed to be due to modification of surface charge near the heme brought about by the binding of $MgCl_2$ or poly-L-lysine. The results indicate that the large density of positive charge introduced by the binding of two molecules of poly-L-lysine results in a positive shift of reduction potential, while the binding of Mg^{2+} to the surface of the protein does not reverse the negative charge on the surface of the OM cytochrome b_5 . These results also suggest that the reduction potential of OM cytochrome b_5 may be modulated by physiological concentrations of Ca^{2+} or Mg^{2+} ions and by complex formation with complementarily charged physiological partner proteins.

Microsomal cytochrome b_5 is a small (15-kDa) heme protein that is composed of two parts: a heme-containing (water-soluble) domain and a hydrophobic domain that is used to anchor the protein to the microsomal membrane (Strittmatter *et al.*, 1974). The water-soluble portion of this protein has been the subject of intensive investigation, and as a result, there is a wealth of information regarding microsomal cytochromes b_5 . The structure of the water-soluble lipase-cleaved fragment of bovine liver microsomal cytochrome b_5 has been reported to a resolution of 2 Å (Mathews &

Strittmatter, 1969; Mathews, 1980; Mathews *et al.*, 1979). The structure has been further refined to a resolution of 1.5 Å and is available from the Brookhaven Protein Data Bank. Genes coding for the tryptic fragment of the microsomal rat cytochrome b_5 (Bodman *et al.*, 1986) and for the lipase-cleaved microsomal bovine cytochrome b_5 (Funk *et al.*, 1990) have been synthesized and expressed in *Escherichia coli*. In contrast, the outer mitochondrial membrane cytochrome b_5 (OM cytochrome b_5) has been studied less extensively. The gene that codes for the tryptic fragment of outer mitochondrial membrane (OM) cytochrome b_5 from rat liver has been synthesized and expressed in *E. coli* (Rivera *et al.*, 1992). The OM cytochrome b_5 has been shown to exhibit the same spectroscopic (UV-visible, EPR, and NMR) properties presented by the microsomal proteins (Rivera *et al.*, 1992).

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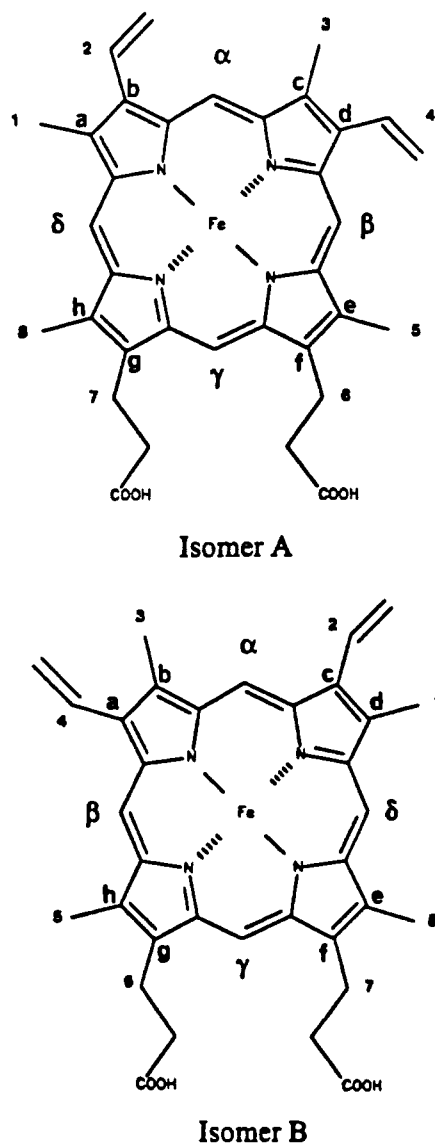


FIGURE 1: Schematic representation of the two heme orientations which differ by a 180° rotation about the α - γ -meso axis which results in heme disorder in the outer mitochondrial membrane cytochrome b_5 and other microsomal cytochromes b_5 . Letters a-h refer to the labeling system that is defined by the heme position and is independent of heme orientation.

The OM cytochrome b_5 has also been shown to consist of two isomeric forms which differ by a 180° rotation of the heme about the α - γ -meso axis (Figure 1). The equilibrium ratio of these two isomers was found to be 1.0 ± 0.1 (Rivera *et al.*, 1992).

The rat OM cytochrome b_5 has been shown to participate in the outer mitochondrial membrane rotenone-insensitive NADH-cytochrome c reductase system that is not coupled to oxidative phosphorylation (Sottocasa *et al.*, 1967). Furthermore, it has been shown that OM cytochrome b_5 is a physiological partner of cytochrome c in rat liver mitochondria, where cytochrome c acts as an electron shuttle between OM cytochrome b_5 in the outer membrane and cytochrome oxidase in the inner membrane (Bernardi & Azzone, 1981).

Given the physiological relevance of the electron transfer reactions of OM cytochrome b_5 , further characterization of its physical and chemical properties is important in order to understand why there are two cytochromes b_5 in the same cell and what the differences are, if any, between the microsomal and mitochondrial proteins. The present investigation de-

scribes the behavior of OM cytochrome b_5 at a gold electrode modified with β -mercaptopropionate using linear staircase cyclic voltammetry. This voltammetric technique is based on the reversible electron transfer that takes place between redox proteins in solution and the surface of a modified electrode or an oxygen functionalized carbon electrode. [For a review see Armstrong *et al.* (1988)]. Although there are many types of modified electrodes that have been used to observe the reversible cyclic voltammetry of cytochrome c , which is a positively charged species (Tarlov & Bowden, 1991; Bagby *et al.*, 1990; Armstrong *et al.*, 1989; Bond *et al.*, 1990), the number of electrodes reported to promote reversible electrochemistry of negatively charged proteins is relatively small. In order to observe reversible cyclic voltammetry of a negatively charged protein such as cytochrome b_5 , gold disk electrodes have been modified with peptides such as Cys-Lys-Cys, Arg-Cys, and His-Cys (Bagby *et al.*, 1988). Other approaches include the mixed modification of gold electrodes with β -mercaptopropionic acid and 2-aminoethanethiol (Hill & Lawrence, 1989) or the modification with 2,2'-dithiobis[ethanamine] and (pyridinylmethylene)hydrazine carbothioamides (Hill *et al.*, 1985). The surface modifier in these electrodes is electroinactive in the potential window where the metalloproteins are electroactive. The role of the modifier seems to encompass the introduction of electrode surface hydrophilicity in order to avoid the denaturation of the metalloproteins at the metal electrode surface. The more successful modifiers so far usually have a mercapto group which is used to bind the surface modifier to the electrode surface and a charged functional group such as an amine or a carboxylate which extends out into the solution (Hill *et al.*, 1985; Bagby *et al.*, 1988; Hill & Lawrence, 1989). These charged functional groups impart electrostatic recognition motifs to the electrodes to which metalloproteins bind electrostatically prior to the electron transfer between electrode and protein. The complexes that are formed between the electrode and the metalloproteins can be thought to resemble the transient complexes formed by certain metalloproteins and their redox partners before electron transfer takes place (Ng *et al.*, 1977; Rodgers *et al.*, 1988; Whitford *et al.*, 1991; Mauk *et al.*, 1991).

Metalloproteins also give reversible electrochemistry at pyrolytic carbon electrodes (Armstrong *et al.*, 1984a), although the response is very sensitive to the exposed face of the graphite and the presence of surface oxidized functional groups. If the edge plane of the pyrolytic graphite electrode is oriented toward the solution, these electrodes give a stable and well-defined response, in contrast with the ill-defined response observed for these electrodes when the basal planes of the pyrolytic graphite are oriented toward the solution (Armstrong *et al.*, 1984b). The reversible electrochemistry of negatively charged proteins at edge pyrolytic carbon electrodes has been observed when multivalent ions such as Mg^{2+} or $Cr(NH_3)_6^{3+}$ are added to the solution (Armstrong *et al.*, 1984a).

The electrode used in the present study, a gold disk electrode modified with β -mercaptopropionate, can be thought of as a way of combining the thiol-modified electrodes and the charged oxygen-rich functionalities of the pyrolytic graphite electrodes. The addition of multivalent cations, such as Mg^{2+} or poly-L-lysine, promotes the reversible electrochemistry of the negatively charged OM cytochrome b_5 . As will be described in detail below, titration of the OM cytochrome b_5 with poly-L-lysine shifts the potential in the positive direction, while the addition of Mg^{2+} or Ca^{2+} ions to the poly-L-lysine-cytochrome b_5 complex shifts the potential back in the negative direction.

^1H NMR spectroscopic investigation of the isotropically shifted resonances was used to gain insight into the sites on the surface of OM cytochrome b_5 to which poly-L-lysine or the Mg^{2+} ions bind.

EXPERIMENTAL PROCEDURES

Recombinant rat outer mitochondrial membrane cytochrome b_5 was expressed in *E. coli* and purified as described previously (Rivera *et al.*, 1992). Bovine erythrocyte cytochrome b_5 was obtained as described previously (Walker *et al.*, 1988). $[\text{Cr}(\text{NH}_3)_6]\text{Cl}_3$ was synthesized as described before (Oppegard & Bailar, 1950). All other reagents were Aldrich or Sigma Quality.

Linear staircase cyclic voltammetry was carried out using a Cypress Systems computer-controlled potentiostat. A miniature 1-mm-diameter gold disk working electrode (Cypress Systems), platinum gauze counter electrode, and silver-silver chloride miniature reference electrode with an internal filling solution of 3 M KCl saturated with silver chloride (Cypress Systems) were used in a glass cell of approximately 700 μL . Before each experiment the working electrode was polished using 0.3- μm alumina on cotton wool and then thoroughly washed with double-distilled water and sonicated in double-distilled water. Surface modification of the electrode was achieved by dipping the polished gold working electrode into a 10 mM solution of β -mercaptopropionic acid for 10 min, followed by rinsing with double-distilled water. The modified gold electrode was then immediately immersed in the deaerated protein solution. A stream of humidified nitrogen was blown gently across the surface of the protein solution in order to maintain the solution anaerobic. Solutions were typically 0.1 mM in OM cytochrome b_5 and contained 0.1 M K-HEPES [potassium (*N*-2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid], pH 7.0, as the supporting electrolyte and buffer. Titrations with MgCl_2 , $\text{Ca}(\text{NO}_3)_2$, poly-L-lysine, or $[\text{Cr}(\text{NH}_3)_6]\text{Cl}_3$ were carried out by adding aliquots of 3 M MgCl_2 , $\text{Ca}(\text{NO}_3)_2$, or 30 mM poly-L-lysine (degree of polymerization 16, approximate molecular weight 3400) or 0.70 M $[\text{Cr}(\text{NH}_3)_6]\text{Cl}_3$ stock solutions. Aliquots were added with the aid of a 10- μL Hamilton syringe.

Spectroelectrochemical titrations were carried out in cell bodies constructed of Lucite that had a gold minigrid optically transparent thin-layer electrode (OTTLE) (200 wires/inch, 70% transmittance, Buckbee Mears Co., St. Paul, MN), quartz windows, a gold minigrid counterelectrode, and a compartment for the reference electrode (Ag/AgCl with internal filling solution of 3 M KCl saturated with silver chloride). The details of the spectroelectrochemical cell and titrations have been reported (Walker *et al.*, 1988). Spectra were recorded at the following potentials vs Ag/AgCl: -390, -370, -350, -330, -310, -290, -270, -250, -230, and -70 mV. The spectra of fully reduced and fully oxidized protein were obtained at -550 and -70 mV (vs Ag/AgCl), respectively. The OM cytochrome b_5 titration solutions contained 0.4 mM $[\text{Ru}(\text{NH}_3)_6]\text{Cl}_3$, 1 mM methyl viologen, and 0.6 mM OM cytochrome b_5 in phosphate buffer, pH 7.0, $\mu = 0.1$.

^1H NMR spectra of protein solutions in D_2O were recorded at 27 $^\circ\text{C}$ on a Bruker AM-500 spectrometer operating in the quadrature detection mode with a proton frequency of 500.13 MHz. Typical spectra were acquired with a spectral window of 25 kHz and 16K data points. Spectra were collected with presaturation of the residual HDO peak during the relaxation delay (400 ms). The data sets were processed using an exponential multiplication window function with a line broadening of 4 Hz. Solutions of OM cytochrome b_5 , 0.9

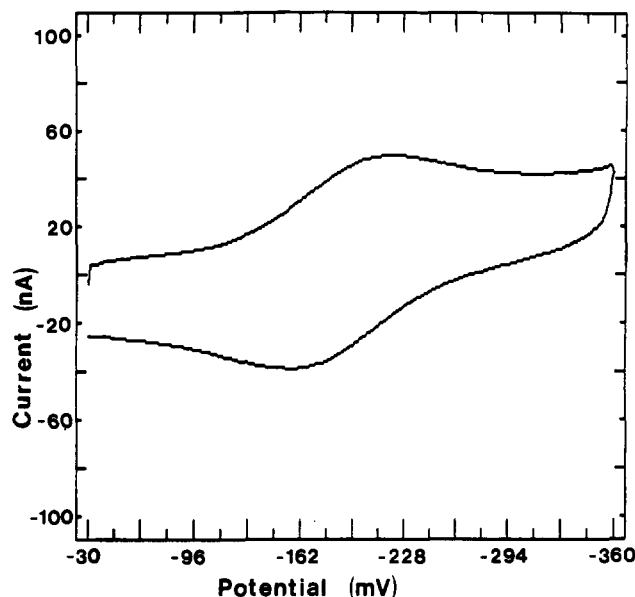


FIGURE 2: Cyclic voltammogram of the rat OM cytochrome b_5 (0.1 mM) in 100 mM K-HEPES, pH 7.0. The gold disk electrode was modified with β -mercaptopropionic acid and the voltammogram was obtained in the presence of 0.2 mM poly-L-lysine (degree of polymerization 16). The potential shown is vs a Ag/AgCl reference electrode with an internal filling solution of 3 M KCl saturated with AgCl ($E^\circ = +197$ mV vs NHE). Sweep rate = 30 mV/s. The peak to peak separation is 67 mV. Similarly shaped voltammograms, with similar peak to peak separations but progressively different values of $E_{1/2}$, were obtained for OM cytochrome b_5 when Mg^{2+} was added to the solution containing the protein.

mM in D_2O , pH* 7.2 (pH* uncorrected for the deuterium isotope effect), were titrated with 2 M MgCl_2 or 30 mM poly-L-lysine and the ^1H NMR spectra of the resulting solutions were recorded after each aliquot.

RESULTS AND DISCUSSION

Effects of Mg^{2+} , Ca^{2+} , and Poly-L-lysine on the Reduction Potential of OM Cytochrome b_5 . A reversible cyclic voltammetric wave arising from the OM cytochrome b_5 was observed at a gold electrode modified with β -mercaptopropionate in the presence of multivalent cations. Electron transfer between the negatively charged electrode and the negatively charged OM cytochrome b_5 was promoted by the addition of Mg^{2+} , Ca^{2+} , or poly-L-lysine. A typical trace for the cyclic voltammogram of the protein in the presence of poly-L-lysine is shown in Figure 2. In the absence of any of these multivalent cations no Faradaic response was observed, indicating that the negative electrode surface repels the negatively charged OM cytochrome b_5 . A plot of the square root of the scan rate vs current was found to be linear for scan rates up to and greater than 150 mV/s (data not shown). The addition of multivalent cations which can bind to the protein and the surface simultaneously allow the negatively charged protein to approach the negatively charged electrode as shown schematically in Figure 3. This phenomenon was termed "ion gating" by Takehara *et al.* (1992). In the case studied by this group, the addition of Ba^{2+} ions to a solution of $[\text{Fe}(\text{CN})_6]^{3-}$ resulted in the reversible electrochemistry of the latter at a glutathione-modified gold electrode. It was postulated that the ion gating response was due to the decreased electrostatic repulsion between the carboxylate groups of glutathione and the $[\text{Fe}(\text{CN})_6]^{3-}$ anions because of the coordination of Ba^{2+} ions by the glutathione carboxylates. The reversible electrochemistry of negatively charged proteins at the edge planes

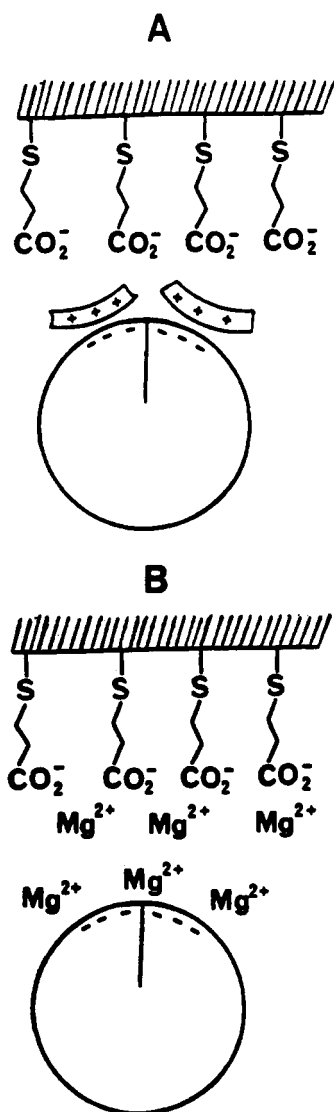


FIGURE 3: Schematic representation of the gold electrode modified with β -mercaptopropionic acid. The cation-promoted reversible electrochemistry of the negatively charged OM cytochrome b_5 at the negative surface of the modified electrode is thought to take place due to the ion gating effect (Takehara *et al.*, 1992) introduced by (A) poly-L-lysine or (B) Ca^{2+} or Mg^{2+} ions.

of pyrolytic graphite electrodes has also been observed when divalent or trivalent ions were added to the solution (Armstrong *et al.*, 1984a,b, 1987, 1988). The oxidized carbon functional groups located at the edges of pyrolytic graphite were implicated in providing discrete areas of negative charge where the multivalent cations can bind, thus promoting the reversible Faradaic response.

In the present study, when a gold electrode modified with β -mercaptopropionic acid was used to monitor the cyclic voltammetry of OM cytochrome b_5 , it was found that the $E_{1/2}$ values observed depend on the poly-L-lysine/cytochrome b_5 ratio. In fact, when the titration of OM cytochrome b_5 with poly-L-lysine was carried out in 100 mM K-HEPES, the $E_{1/2}$ values became more positive as a function of the concentration of poly-L-lysine added, as shown in Figure 4. When the molar ratio (poly-L-lysine/cyt b_5) reached a value of 2.0, the $E_{1/2}$ values leveled off at +8 mV *vs* NHE, thus suggesting that a very stable complex is formed in which 2 molecules of poly-L-lysine bind/molecule of cytochrome b_5 . The possible sites on the surface of OM cytochrome b_5 where poly-L-lysine could bind will be discussed below.

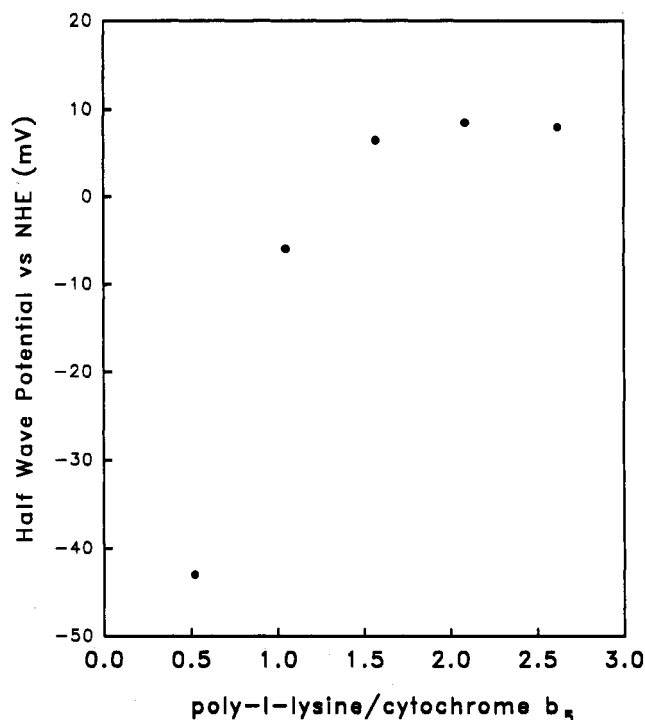


FIGURE 4: Titration of OM cytochrome b_5 (0.1 mM) with poly-L-lysine. The titration curve is shown as a function of the poly-L-lysine/OM cytochrome b_5 ratio and show that 2 molecules of poly-L-lysine bind with high affinity/molecule of cytochrome b_5 . Cyclic voltammograms were obtained using 0.1 mM OM cytochrome b_5 in 100 mM K-HEPES, pH 7.0. A gold disk electrode modified with β -mercaptopropionic acid was used to measure the $E_{1/2}$. The shape of the voltammograms throughout the titration was identical to the one shown in Figure 2. The peak to peak separation of the cyclic voltammograms throughout the titration was 67 ± 7 mV.

When the gold electrode modified with β -mercaptopropionic acid was used to monitor the cyclic voltammetric response of the OM cytochrome b_5 -poly-L-lysine complex as a function of the concentration of Ca^{2+} or Mg^{2+} ions, it was observed that the $E_{1/2}$ values depend on the concentration of divalent ions in the solution. The titration of the OM cytochrome b_5 -poly-L-lysine complex (1:2) with MgCl_2 or $\text{Ca}(\text{NO}_3)_2$ resulted in the gradual negative shift of the $E_{1/2}$ of the cytochrome. The $E_{1/2}$ leveled off at -40 mV *vs* NHE when the concentration of Mg^{2+} or Ca^{2+} ions was approximately 85 mM, as shown in Figure 5. The behavior of the $E_{1/2}$ values observed during the titration of the OM cytochrome b_5 -poly-L-lysine complex with MgCl_2 or $\text{Ca}(\text{NO}_3)_2$ is identical. Furthermore, the $E_{1/2}$ values observed in the presence of MgCl_2 or $\text{Ca}(\text{NO}_3)_2$ are the same, thus indicating that the anions (Cl^- or NO_3^-) do not play a role in altering the $E_{1/2}$ values observed for the OM cytochrome b_5 -poly-L-lysine complex.

Upon addition of higher concentrations of multivalent cations, the characteristics of the cyclic voltammograms (current and peak shape) in the titrations described above remained unchanged from the one shown in Figure 2, with the exception of the half-wave potential, which changed at every point, as shown in Figures 4–6. The peak to peak separation in all the cyclic voltammograms was 67 ± 7 mV, thus indicating that the heterogeneous electron transfer is reversible at all points in the titrations.

Reversible electrochemistry of OM cytochrome b_5 can also be observed in the presence of MgCl_2 or $\text{Ca}(\text{NO}_3)_2$ alone when the concentration of either of these ions is 40 mM or higher in 100 mM K-HEPES at pH 7.0. In this case the $E_{1/2}$ value observed is -46 mV. It should also be noted that no

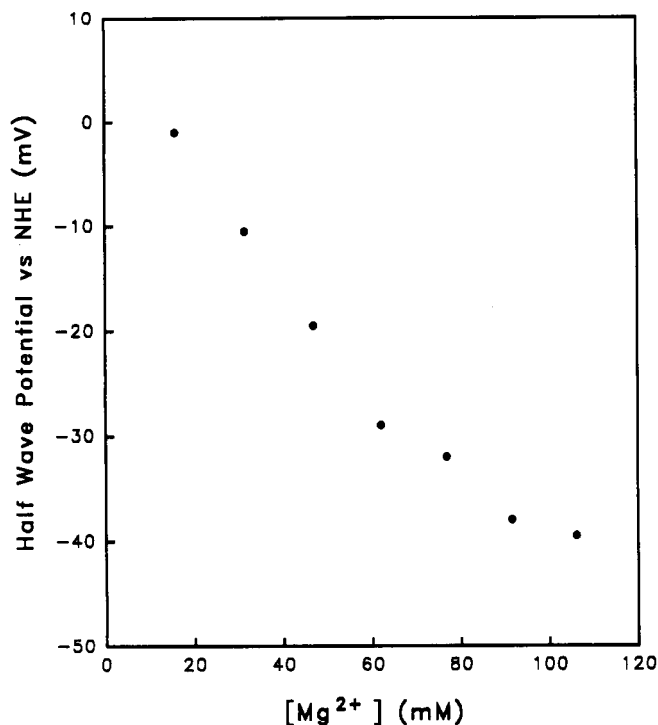


FIGURE 5: Titration of the 2:1 poly-L-lysine-OM cytochrome b_5 complex (0.1 mM) with MgCl_2 . A similar titration curve is obtained by the addition of $\text{Ca}(\text{NO}_3)_2$. Cyclic voltammograms were obtained using 0.1 mM OM cytochrome b_5 in 100 mM K-HEPES, pH 7.0. A gold disk electrode modified with β -mercaptopropionic acid was used to measure the $E_{1/2}$. The shape of the voltammograms throughout the titration was identical to the one shown in Figure 2. The peak to peak separation of the cyclic voltammograms throughout the titration was 69 ± 5 mV.

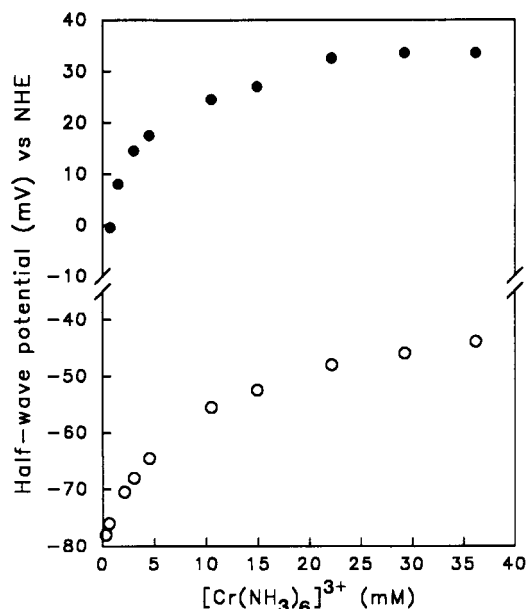


FIGURE 6: Titration of (●) microsomal erythrocyte beef cytochrome b_5 and (○) rat OM cytochrome b_5 with $[\text{Cr}(\text{NH}_3)_6]^{3+}$. The titrated solutions consisted of 0.1 mM protein in phosphate buffer, pH 7.0, $\mu = 0.1$. A gold disk electrode modified with β -mercaptopropionic acid was used to measure the $E_{1/2}$. The cyclic voltammograms throughout the titration were identical to the one in Figure 2. The peak to peak separation throughout the titrations was 67 ± 6 mV.

Faradaic response was observed for OM cytochrome b_5 in K-HEPES in the presence of KCl (0–100 mM). This demonstrates that monovalent ions are not capable of promoting the reversible electrochemistry of cytochromes b_5

at gold modified electrodes.

These results may be interpreted as being due to modification of the surface charge on cytochrome b_5 by chelation of divalent cations to the carboxylates of the heme and those on the surface of the protein and, when the 2:1 poly-L-lysine complex is present, to displacement of poly-L-lysine by Mg^{2+} or Ca^{2+} . The absence of a Faradaic response in the presence of monovalent ions such as sodium or potassium must be due to the inability of surface carboxylate side chains to chelate these ions. On the other hand, divalent ions, such as calcium or magnesium, are known to be chelated by carboxylate ligands of acidic amino acids such as glutamic or aspartic acid (Shmidbaur *et al.*, 1986).

An extensive report (Armstrong *et al.*, 1987) on the ability of metal ions to promote the cyclic voltammetry of negatively charged proteins such as plastocyanin, azurin, rubredoxin, $[\text{4Fe-4S}]$ ferredoxin, and $[\text{2Fe-2S}]$ ferredoxin at an edge plane pyrolytic graphite electrode showed that reversible and diffusion-dominated heterogeneous electron transfer is modulated by the concentrations of redox-inert multivalent cations such as Mg^{2+} and $[\text{Cr}(\text{NH}_3)_6]^{3+}$. Furthermore, these authors also showed that the concentration of multivalent ions necessary for optimum Faradaic response was dependent on the type of protein. The half-wave potential of these proteins, however, was not reported to be dependent on the concentration of multivalent metal ions (Armstrong *et al.*, 1987). In the case of cytochromes b_5 the situation is different, because we have found that the half-wave potential of these proteins is dependent on the concentration of multivalent metal ions, as shown in Figures 4–6.

It has been reported that poly-L-lysine is capable of replacing some surface modifiers from gold surfaces and therefore inhibits the reversible electrochemistry of cytochrome c (Hill *et al.*, 1987). In the case of cytochrome b_5 , it should be possible to observe the reversible electrochemistry of cytochrome b_5 if poly-L-lysine is bound directly to the gold surface. In order to test the possibility of replacement of β -mercaptopropionic acid by poly-L-lysine on the surface of the gold electrode, a clean gold disk electrode was immersed in a solution of poly-L-lysine (30 mM) for 15 min, washed with deionized water, and then immersed in a solution of cytochrome b_5 in 100 mM K-HEPES at pH 7.0. No Faradaic response was observed. Furthermore, when the surface modifier was β -mercaptopropionic acid, the peak to peak separation remained constant even when the mole ratio, poly-L-lysine/cytochrome b_5 , was greater than 2, thus indicating that poly-L-lysine (with degree of polymerization 16) is not capable of replacing β -mercaptopropionic acid at gold surfaces.

Effect on Reduction Potential Brought about by Large vs Small Changes in the Surface Charge of Cytochrome b_5 . It is noteworthy that the addition of positively charged ions such as Mg^{2+} or Ca^{2+} has an effect on the $E_{1/2}$ values opposite to that observed upon addition of the positively charged poly-L-lysine species. In the OM cytochrome b_5 -poly-L-lysine complexes, poly-L-lysine is likely to bind electrostatically to the glutamates and aspartates on the surface of the protein as shown schematically in Figure 3. These acidic residues on the surface of the beef microsomal cytochrome b_5 are arranged in three patches that could serve as three different cation binding sites, as shown in Figure 7 (Whitford, 1992). Nine of the 11 acidic residues that constitute the three different potential cation binding sites in the microsomal protein are conserved in the rat OM cytochrome b_5 (Lederer *et al.*, 1983); Glu 37 and Glu 82 in the microsomal protein have been replaced by Ser and Asn, respectively, in OM cytochrome b_5

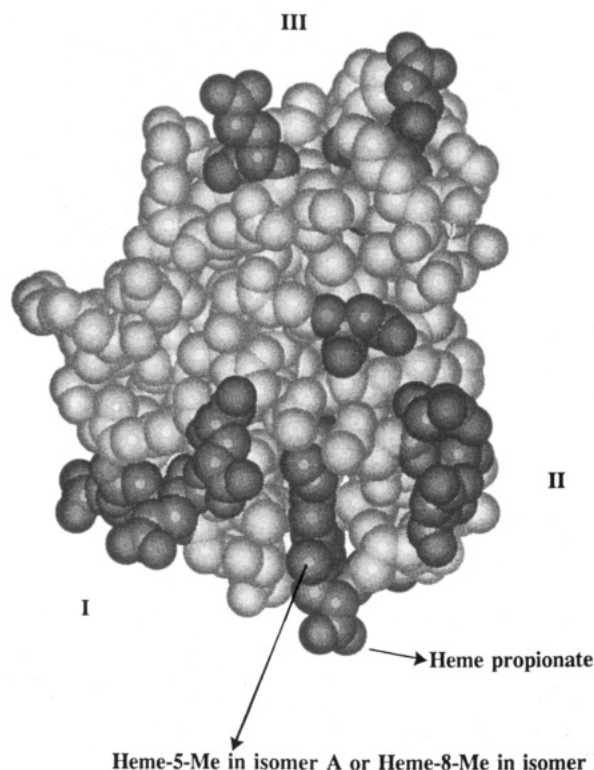


FIGURE 7: Space-filling diagram of the polypeptide backbone of microsomal beef cytochrome b_5 showing the three cation binding sites (Whitford, 1992). The individual sites are defined by the darker glutamates and aspartates on the surface of the protein. The diagram also makes evident that the heme propionate in position f, the heme methyl in position e, and the heme substituent (vinyl or methyl depending on the isomer) in position d are largely exposed to the aqueous medium.

(Glu 37 is part of cation binding site I and Glu 82 is part of cation binding site III). Because of the large degree of homology between these two proteins, OM cytochrome b_5 must have the same three domains of negative charge, and although two of these sites (I and II) are located close to the heme, site I in Figure 7 is the one closest to the prosthetic group (Whitford, 1992). It is very likely that cation binding sites I and II in the OM cytochrome b_5 are involved in binding two molecules of poly-L-lysine, thus explaining the stoichiometry found when OM cytochrome b_5 is titrated with poly-L-lysine (Figure 4). Binding of poly-L-lysine is also expected to change or possibly reverse the charge on the surface of OM cytochrome b_5 , thereby decreasing the stability of the Fe^{3+} state of the protein and shifting the reduction potential of the protein in the positive direction.

The effect of surface charge near the prosthetic group has been documented. For example, it has been shown that the reduction potential of cytochrome c decreased (indicating increased stability of the Fe^{3+} state) with the increase in the number of negatively charged groups on the surface of the protein. This was accomplished by modifying some of the lysine groups with maleate (Aviram *et al.*, 1981). A similar effect has been observed for the cytochrome c_2 from *Rhodobacter capsulatus* (Caffrey *et al.*, 1991) when surface lysines were replaced by glutamates. In this case, the introduction of negative charges in place of positive charges, by means of site-directed mutagenesis, shifted the reduction potential of the protein in the negative direction, thus stabilizing the Fe^{3+} oxidation state. In the Ca^{2+} or Mg^{2+} complexes of cytochrome b_5 , the metal ions are likely to be bound by the heme propionates and carboxylates of the Glu and Asp residues in the three cation binding sites shown in Figure 7. The maximum number

of Mg^{2+} ions that can be chelated by the surface residues of OM cytochrome b_5 cannot be larger than the number of acidic residues on the surface of the protein. Accounting for the Glu-37-Ser and Asp-82-Asn in the cation binding sites I and III, respectively, and assuming that the two heme propionates chelate Mg^{2+} or Ca^{2+} , the maximum number of M^{2+} ions that could be coordinated is 11. Since only one of the positive charges on the metal ion is being neutralized by coordination of a carboxylate, the maximum number of positive charges on the surface of OM cytochrome b_5 is +11. Moreover, it is likely that only one or two Mg^{2+} ions are chelated by each cation binding site, which implies that the charge on the surface of the OM cytochrome b_5 is still negative. On the other hand, in the 2:1 poly-L-lysine:OM cytochrome b_5 complex, the charge of the protein has clearly been reversed, because the maximum number of positive charges introduced by poly-L-lysine (degree of polymerization 16) is +21, assuming that 11 acidic residues on the surface of the protein have been neutralized by poly-L-lysine. The introduction of positive charges on the surface of OM cytochrome b_5 , and especially the positive charges that are in proximity to the heme prosthetic group, may be responsible for decreasing the stability of the ferric state of the protein (positive shift of $E_{1/2}$), whereas when Mg^{2+} replaces poly-L-lysine on the surface of the protein, the density of positive charges close to the heme is largely decreased or possibly reversed, thus stabilizing the ferric oxidation state (negative shift of $E_{1/2}$). Evidence that Mg^{2+} displaces poly-L-lysine from the surface of cytochrome b_5 was obtained from NMR spectroscopic studies and will be discussed in detail in a later section.

The Reduction Potential of OM Cytochrome b_5 Is 100 mV More Negative than That of the Microsomal Cytochromes b_5 . The reversible cyclic voltammetric response of OM cytochrome b_5 at a gold electrode modified with β -mercaptopropionic acid requires that the concentration of Mg^{2+} be at least 40 mM in 100 mM K-HEPES at pH 7.0, as discussed above. The half-wave potential of OM cytochrome b_5 under these conditions is -46 mV *vs* NHE. The half-wave potential observed for OM cytochrome b_5 when the poly-L-lysine/cytochrome b_5 mole ratio equals 0.5 is -43 mV *vs* NHE (Figure 4). Half-wave potentials at mole ratios less than 0.5 were not obtained because the system is not reversible under these conditions. The plot of $E_{1/2}$ *vs* mole ratio (Figure 4), however, indicates that the true reduction potential of OM cytochrome b_5 in the absence of poly-L-lysine is substantially more negative than -43 mV. In order to more closely estimate the reduction potential of OM cytochrome b_5 , the cyclic voltammetric response of OM cytochrome b_5 at a gold electrode modified with β -mercaptopropionic acid was promoted by the addition of the trivalent cation $[\text{Cr}(\text{NH}_3)_6]^{3+}$. It was found that $[\text{Cr}(\text{NH}_3)_6]^{3+}$ is a more efficient promoter than Mg^{2+} or Ca^{2+} , because a reversible cyclic voltammogram, shaped like the one shown in Figure 2, was obtained with a minimum of 0.2 mM $[\text{Cr}(\text{NH}_3)_6]^{3+}$, whereas a minimum of 40 mM Mg^{2+} or Ca^{2+} was required to produce a reversible cyclic voltammogram. The half-wave potential observed for OM cytochrome b_5 under these conditions (100 μM protein, phosphate buffer, pH 7.0, $\mu = 0.1$, and 0.2 mM $[\text{Cr}(\text{NH}_3)_6]^{3+}$) is -78 mV *vs* NHE.

To compare the reduction potential of OM cytochrome b_5 with other cytochromes b_5 , titration curves were obtained for the recombinant rat OM cytochrome b_5 and the trypsin-cleaved microsomal bovine liver cytochrome b_5 using a β -mercaptopropionic acid derivatized gold electrode and $[\text{Cr}(\text{NH}_3)_6]^{3+}$ as the titrant (Figure 6). It is seen from these curves that the

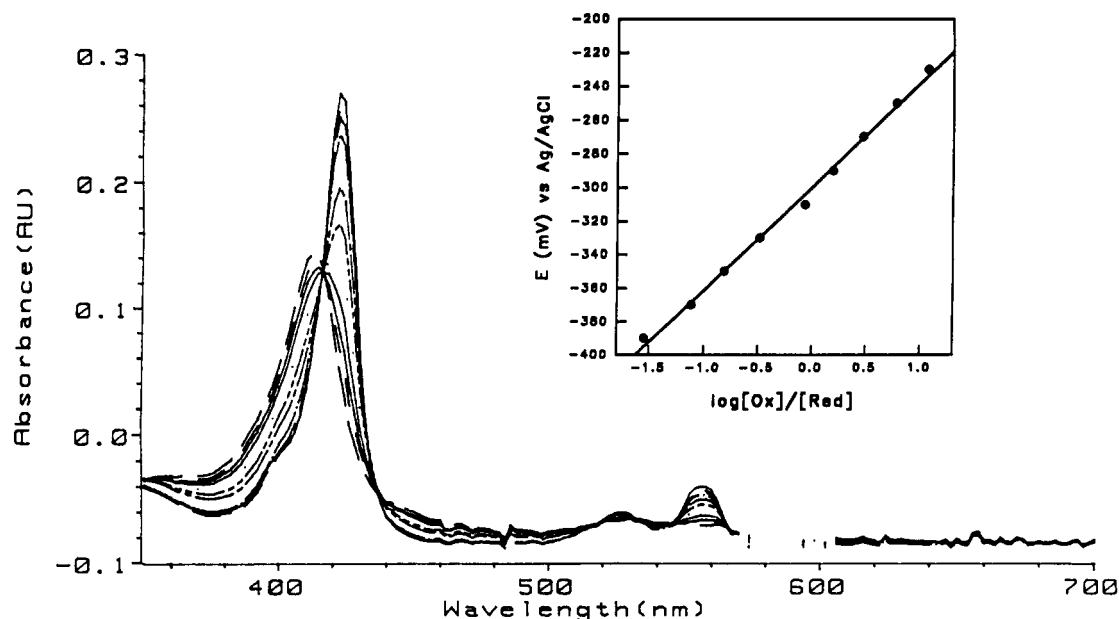


FIGURE 8: Spectra obtained from spectroelectrochemical titration of OM cytochrome b_5 in the presence of $[\text{Ru}(\text{NH}_3)_6]\text{Cl}_3$ and methyl viologen as mediators. Phosphate buffer, pH 7.0, $\mu = 0.1$, was used for this experiment. Inset: Nernst plot of the same data. The midpoint potential calculated from this plot is -102 mV *vs* NHE. The Nernst slope is 62.5 mV. The Nernst plot was made on the basis of the absorbance at 422 nm.

reduction potential of both microsomal and outer mitochondrial membrane cytochromes b_5 is dependent on the concentration of the multivalent cation. Their dependency on the concentration of $[\text{Cr}(\text{NH}_3)_6]^{3+}$ is almost identical; thus the half-wave potential of OM cytochrome b_5 is always approximately 70 mV more negative than that of the microsomal beef liver cytochrome b_5 .

Spectroelectrochemical titrations of the recombinant OM cytochrome b_5 in phosphate buffer, pH 7.0 and $\mu = 0.1$, in the presence of two mediators, $[\text{Ru}(\text{NH}_3)_6]^{3+}$ (0.4 mM) and methyl viologen (1 mM), as reported previously for bovine microsomal cytochrome b_5 (Walker *et al.*, 1988), resulted in a reduction potential of -102 mV *vs* NHE (Figure 8). This value is substantially more negative than the values reported for the trypsin-cleaved bovine liver microsomal cytochrome b_5 , $E_m = 5.1$ mV (Reid *et al.*, 1982) or $E_m = -1.9$ mV (Walker *et al.*, 1988), or the microsomal recombinant rat liver cytochrome b_5 , $E_m = -7$ mV (Rodgers & Sligar, 1991). This reduction potential is surprisingly low when compared with the reduction potentials of cytochromes b_5 from other sources, whose reduction potentials are all very close to 0 mV. It has been shown that the removal of individual negative surface charges near the active site in the rat microsomal cytochrome b_5 results in small positive shifts (8 – 12 mV) of the reduction potential (Rodgers & Sligar, 1991). The rat OM cytochrome b_5 has two less acidic residues than the microsomal cytochromes b_5 , namely, Glu-37-Ser and Asp-82-Gln (Lederer *et al.*, 1983; Rivera *et al.*, 1992), and therefore, on the basis of the effect of surface charge alone, a reduction potential more positive than 0 should have been observed for this protein. On the other hand, in the case of the cytochrome b_5 domain of yeast flavocytochrome b_2 , the mutation Leu-36-Ile, one of the hydrophobic residues in the heme binding crevice, changed its half-wave potential by -36 mV. This change was in a direction opposite to the one predicted by an earlier postulate on the effect of hydrophobicity on the reduction potential of heme proteins (Kassner, 1979), and it was sufficient to alter the redox cooperativity between the heme and the flavin mononucleotide (FMN) cofactor (Kay & Lippay, 1992). This result

demonstrates that small changes in the reduction potential of an electron transfer protein can have a large impact on its physiological activity and also that the investigation of the factors that modulate these reduction potentials are worth investigating in great detail. Among the factors that influence the reduction potential of heme proteins, the hydrophobicity of the heme binding site, potential solvent channels to the heme, and the presence of hydrophilic and charged residues in the heme pocket have been postulated as the more important (Kassner, 1979; Moore *et al.*, 1986; Varakrajan *et al.*, 1989). In the rat OM cytochrome b_5 amino acid sequence (Lederer *et al.*, 1993), some of the amino acids in the heme pocket are substantially different from their counterparts in microsomal proteins, as pointed out previously (Rivera *et al.*, 1992). For example, Ser 71 in the microsomal proteins has been mutated to a Leu in the OM cytochrome b_5 , and Met is found at positions 23 and 70 only in the rat OM cytochrome b_5 and at position 70 in the human protein. It is possible that the interaction of one or more of these residues in OM cytochrome b_5 may account for the large differences in reduction potential observed between the microsomal and OM cytochromes b_5 . Experiments to probe these factors are currently underway in our laboratories.

We also wish to address the 24 -mV difference in the reduction potential of the recombinant rat OM cytochrome b_5 when it is measured using spectroelectrochemical titration (-102 mV) or a gold electrode modified with β -mercapto-propionic acid (-78 mV). This difference is probably due to the unique nature of electron transfer to or from the electrode in the two techniques. In the cyclic voltammetric technique, in order for heterogeneous electron transfer to occur, it is likely that a transient ternary complex is formed in which polyvalent cations are trapped between the negatively charged protein and the negatively charged electrode. The polyvalent cations in these ternary complexes likely interact with the propionate(s) of the heme and with the acidic residues on the surface of the protein close to the heme (this interaction is discussed in detail in the next section) during the process of electron transfer. These interactions may result in altered

Table 1: Chemical Shift Perturbations of the Heme Substituents Caused by the Addition of Mg^{2+} or Poly-L-lysine to OM Cytochrome b_5

group	$\Delta\delta^a$	group	$\Delta\delta^a$
Cytochrome b_5 + $MgCl_2^b$			
A-2V $_{\alpha}$	0.09	A-2V $_{\beta}$	-0.04, -0.05
A-5Me	0.13	B-3Me	0.11
A-1Me	0.13	B-8Me	0.12
A-3Me	-0.09	B-4V $_{\alpha}$	0.08
Cytochrome b_5 + Poly-L-lysine ^c			
A-2V $_{\alpha}$	0.23	A-2V $_{\beta}$	-0.16, -0.14
A-5Me	0.05	B-3Me	0.30
A-1Me	0.31	B-8Me	-0.03
A-3Me	-0.27	B-4V $_{\alpha}$	0.21

^a Assignments for the resonances corresponding to these heme substituents were obtained from Rivera *et al.* (1992). $\Delta\delta = \delta(\text{cytochrome } b_5 + \text{promoter}) - \delta(\text{cytochrome } b_5)$. ^b OM cytochrome $b_5 = 0.90$ mM, $Mg^{2+} = 12$ mM. ^c OM cytochrome $b_5 = 0.81$ mM, poly-L-lysine = 1.8 mM.

conformations of heme substituents and/or electrostatic interactions with the heme iron. In the spectroelectrochemical experiment, it is not necessary for the protein to be locked in a ternary complex before homogeneous electron transfer occurs between the mediator, $[Ru(NH_3)_6]^{3+}$, and the protein, thus resulting in the different reduction potentials observed with the two methods.

The fact that the half-wave potential of cytochromes b_5 at β -mercaptopropionic acid derivatized electrodes was found to be dependent on the concentration of polyvalent cations (this work), and that the half-wave potential of other negatively charged proteins such as plastocyanin, azurin, rubredoxin, and ferredoxins at edge pyrolytic graphite electrodes was not (Armstrong *et al.*, 1987), suggests that the large degree of exposure of the heme to the aqueous environment in the cytochromes b_5 plays an important role. Exposure of the heme, and especially of the heme propionates, results in electrostatic and binding interactions between these propionates and multivalent cations in solution. These interactions are likely to result in the dependency of the half-wave potential of these proteins on the concentration of multivalent cations in solution.

¹H NMR Spectroscopic Studies of the Interactions between OM Cytochrome b_5 and Mg^{2+} or Poly-L-lysine. The binding interactions between Mg^{2+} or poly-L-lysine and OM cytochrome b_5 were probed using the ¹H NMR hyperfine shifted resonances arising from the heme in ferricytochrome b_5 . These resonances have been used to probe the formation of protein complexes between ferricytochrome b_5 and ferricytochrome c (Eley & Moore, 1983; Miura *et al.*, 1980; Whitford *et al.*, 1990). The binding in the cytochrome b_5 -cytochrome c complex is thought to incorporate complementary salt bridges between negatively charged side chains on cytochrome b_5 and lysyl residues on cytochrome c (Salemme, 1976). This is the same type of binding that is expected to occur between OM cytochrome b_5 and poly-L-lysine, as discussed above. When OM cytochrome b_5 was titrated with $MgCl_2$ or poly-L-lysine, the isotropically shifted heme resonances showed chemical shift perturbations which indicate that the magnetic microenvironment of the heme substituents is affected by complex formation, but in different ways for the two cationic species.

Table 1 shows the ¹H NMR chemical shift perturbations of the hyperfine shifted resonances of the heme brought about by the addition of poly-L-lysine or $MgCl_2$ to a solution of OM cytochrome b_5 . During the titration of OM cytochrome b_5 (0.9 mM in D_2O) with poly-L-lysine, the solution became turbid until the mole ratio poly-L-lysine/cytochrome b_5 was

greater than 1.6, although it was still possible to obtain acceptable NMR spectra. Once this mole ratio had been attained, the solution became clear and it remained clear even when the mole ratio was greater than 2.0. The largest chemical shift perturbations on the isotropically shifted resonances were obtained when the mole ratio poly-L-lysine/cytochrome b_5 was 2.0. Addition of more poly-L-lysine did not have any further effect on the chemical shifts of these resonances. Addition of Mg^{2+} to the cytochrome b_5 -poly-L-lysine complex resulted in a shift of the isotropically shifted resonances toward their values obtained when the protein had been titrated with Mg^{2+} ions alone. When the concentration of Mg^{2+} ions reached 150 mM, the poly-L-lysine in the cytochrome b_5 -poly-L-lysine complex had been almost completely replaced by Mg^{2+} , because the chemical shifts of the isotropically shifted resonances arising from the heme were almost identical to the ones observed in the presence of only Mg^{2+} ions.

The largest chemical shift perturbations observed when OM cytochrome b_5 (0.9 mM) was titrated with $MgCl_2$ alone were obtained when the concentration of Mg^{2+} ions was 12 mM; the addition of more $MgCl_2$ did not further perturb the resonances. Titrations with potassium chloride were also carried out in order to test the effect of changes in the ionic strength on the isotropically shifted heme resonances. The addition of potassium chloride, final concentration 24 mM, did not perturb the isotropically shifted resonances of the cytochrome, thus indicating that the chemical shift perturbations observed when Mg^{2+} ions were added are due to the coordination of the Mg^{2+} by the carboxylate groups on the surface of the protein. This also corroborates the observations made by cyclic voltammetry that monovalent cations and anions such as chloride and nitrate do not bind to OM cytochrome b_5 .

It is apparent from Table 1 that the chemical shift perturbations of the heme resonances of OM cytochrome b_5 caused by the addition of poly-L-lysine are larger than the chemical shift perturbations caused by the addition of Mg^{2+} ions. This suggests that the binding of poly-L-lysine to the OM cytochrome b_5 causes a larger structural perturbation of the protein than does the binding of Mg^{2+} . A consequence of these structural perturbations may be the rearrangement of the helices that carry the residues that line the heme pocket, thus forcing the heme substituents which have close contacts to these side chains to reaccommodate to a new heme pocket topology. It is possible that such reaccommodation of heme substituents may be responsible for the chemical shift perturbations observed in the ¹H NMR titration experiments. Among the bulkiest substituents in the heme are the two vinyl groups, which are likely to respond to a new topology of the heme pocket by changing their conformations by rotating about their β -pyrrole-vinyl σ bonds. The conformation and rotational mobility of the heme vinyl groups has been correlated with the modulation of the reduction potential of heme proteins (Satterlee & Erman, 1983; Reid *et al.*, 1986; Lee *et al.*, 1991). The modulation of the reduction potential is thought to arise from the variable degree of coplanarity between the heme vinyls and the heme π system (Reid *et al.*, 1986; Lee *et al.*, 1986). Extensive ¹H NMR investigations aimed at determining the conformational preference of the vinyl groups in OM cytochrome b_5 and its complexes with Mg^{2+} and poly-L-lysine are underway.

Table 1 also shows that the addition of poly-L-lysine has only a very small effect on the resonances arising from the heme 5-methyl group in isomer A (A-5Me), or the heme 8-methyl group in isomer B (B-8Me) (see Figures 1 and 7),

each of which is adjacent to a heme propionate. Interestingly, the same resonances have the largest shift when Mg^{2+} ions are added. This can be rationalized as follows: It is clear from Figure 7 that one side of the heme is largely exposed to the aqueous medium and that the groups A-5Me or B-8Me are almost free of interactions with the amino acid residues lining the heme pocket. Rearrangement of the heme pocket, therefore, is not expected to have a large effect on the chemical shifts of the A-5Me or B-8Me groups when Mg^{2+} ions or poly-L-lysine binds to the surface of OM cytochrome b_5 . On the other hand, if poly-L-lysine or Mg^{2+} ions were to interact with heme propionate 6 in isomer A or heme propionate 7 in isomer B, which are adjacent to the A-5Me and B-8Me groups, respectively, the chemical shifts of these methyl groups should be affected. Given the large chemical shift perturbation observed for these two methyl groups in the presence of Mg^{2+} ions and the very small chemical shift perturbation in the presence of poly-L-lysine (see Table I), it can be concluded that the heme propionates adjacent to A-5Me or B-3Me, namely, heme propionate 6 in isomer A and heme propionate 7 in isomer B, bind Mg^{2+} ions. In contrast, poly-L-lysine does not interact with either of these heme propionates but instead interacts with the aspartates and glutamates of sites I and II (Figure 7). In agreement with this, the crystal structure of the beef microsomal cytochrome b_5 (Mathews *et al.*, 1979) shows that the heme propionate in position f in Figure 1 is extended into the solvent with the α - and β -methylene carbon atoms partially exposed and the carbonyl and oxygen atoms fully exposed, whereas the propionate in position g in Figure 1 is almost completely buried. Moreover, the titration of the beef microsomal cytochrome b_5 with $[\text{Ru}(\text{NH}_3)_6]^{3+}$, monitored by the ^1H NMR resonances arising from the heme $\beta\text{-CH}_2$ groups, showed that the heme propionate in position f is largely involved in binding $[\text{Ru}(\text{NH}_3)_6]^{3+}$, whereas the heme propionate in position g does not bind this cation (Rivera-Haynes, 1984). The incremental addition of Gd^{3+} to a solution of microsomal beef ferricytochrome b_5 broadened only the 6-propionate proton resonance, while the 7-propionate proton peak was unaffected (McLachlan *et al.*, 1986).

In the present study, it is not possible to monitor the heme propionate α - and $\beta\text{-CH}_2$ groups by ^1H NMR spectroscopy, because in the rat OM cytochrome b_5 the equilibrium ratio of isomers A and B is 1.0. This means that the number of resonances arising from the diastereotopic α -methylene groups in the heme propionates is doubled (eight rather than four expected resonances). In addition, the resonances of the $\alpha\text{-CH}_2$ groups are closer to each other, thus resulting in severe signal overlap between 15.5 and 16.0 ppm. (This situation is somewhat simplified in the case of the beef microsomal cytochrome b_5 , where the heme orientation ratio A:B is 10:1). Nevertheless, it is important to point out that four independent experiments, the X-ray crystal structure of beef microsomal cytochrome b_5 (Mathews *et al.*, 1979), the titration of beef microsomal cytochrome b_5 with $[\text{Ru}(\text{NH}_3)_6]^{3+}$ (Rivera-Haynes, 1984) and with Gd^{3+} (McLachlan *et al.*, 1986), and the present study, have concluded that the heme propionate in position f is capable of binding to multivalent ions while the heme propionate in position g is not. Furthermore, since in the present study it was found that the heme propionate in position g does not interact with poly-L-lysine, it is possible that this propionate is not involved in the electrostatic binding of OM cytochrome b_5 to its physiological protein partners. The later conclusion is in agreement with the results of Whitford *et al.* (1992), who reported that the exposed propionate in position g in the microsomal beef cytochrome

b_5 is at best only marginally shielded from $[\text{Cr}(\text{en})_3]^{3+}$ by cytochrome c .

CONCLUDING REMARKS

Cytochromes b_5 appear to be unique in their ability to modulate their reduction potentials as a function of the concentration and kind of multivalent cations in solution. Although the cyclic voltammetry of other negatively charged proteins such as plastocyanin, azurin, and ferredoxins has been known to be promoted by multivalent cations (Armstrong *et al.*, 1987), their reduction potentials have not been reported to depend on the concentration of these ions. Among the cytochromes b_5 , the rat liver outer mitochondrial membrane cytochrome b_5 has been found by spectroelectrochemical titration in the presence of two mediators to have a reduction potential 100 mV more negative than the potentials observed for the microsomal proteins, which are very close to 0 mV *vs* NHE under the same conditions. In contrast, the cyclic voltammetric half-wave potentials of the same two proteins under the same conditions of buffer, pH, and ionic strength differ only by -78 mV. The smaller difference in reduction potential when the latter is measured at a modified gold electrode is probably due to the influence of the multivalent cation that is likely to be bound simultaneously to the protein and the electrode. The reason for the much more negative reduction potential of the mitochondrial protein is as yet unknown, although experiments to probe this phenomenon are underway in our laboratories.

The large differences in reduction potential that were observed for the OM cytochrome b_5 in the presence of MgCl_2 or poly-L-lysine can in principle be due in part to the density of positive charge near to the prosthetic group that is produced by the binding of poly-L-lysine to OM cytochrome b_5 . This density of positive charge near the heme decreases the stability of the ferric state (positive shift of $E_{1/2}$). In the Mg^{2+} complexes of OM cytochrome b_5 , the charge of the protein near the heme is likely to be still negative because there may be only 1 Mg^{2+} ion/cationic binding site, therefore stabilizing the ferric state (negative shift of the $E_{1/2}$ values). The modulation of the reduction potential of OM cytochrome b_5 by the possible changes in the shape of the heme binding pocket brought upon by binding of poly-L-lysine or Mg^{2+} to OM cytochrome b_5 may also contribute to the reduction potential of this protein. Experiments to test this hypothesis are currently underway.

Finally, it is noteworthy that the reduction of cytochrome b_5 by NADPH-cytochrome b_5 reductase is strongly inhibited by CaCl_2 and MgCl_2 (Tamura *et al.*, 1988). These authors suggested that it was possible for Ca^{2+} or Mg^{2+} to affect step(s) in the reduction process other than the electrostatic interaction between cytochrome b_5 reductase and cytochrome b_5 . The present report suggests that the reduction potential of cytochrome b_5 can be modulated by changes in surface charge near the heme, by conformational changes brought about by cation binding, and by the formation of electrostatic complexes with physiological partner proteins. It has also been reported that the catalytic properties of horseradish peroxidase are regulated by the binding of 2 mol of Ca^{2+} endogenous to the enzyme (Morishima *et al.*, 1986; Shiro *et al.*, 1986). The calcium ions are thought to maintain the protein structure in the vicinity of the heme to favor the catalytic activity of this enzyme. Hence, the importance of multivalent redox-inactive cations in modulating the *in vivo* electron transfer and/or catalytic reactivity of heme proteins

must be considered carefully in future investigations involving these proteins.

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