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# Modulation of redox potential in electron transfer proteins: Effects of complex formation on the active site microenvironment of cytochrome $b_5$

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The reduction potential of cytochrome  $b_5$  is modulated *via* the formation of a complex with polylysine at the electrode surface (Rivera *et al.*, *Biochemistry*, 1998, **37**, 1485). This modulation is thought to originate from the neutralization of a solvent exposed heme propionate and from dehydration of the complex interface. Although direct evidence demonstrating that neutralization of the charge on the heme propionate contributes to the modulation of the redox potential of cytochrome  $b_5$  has been obtained, evidence demonstrating that water exclusion from the complex interface plays a similar role has not been conclusive. Herein we report the preparation of the V45I/V61I double mutant of rat liver outer mitochondrial membrane (OM) cytochrome  $b_5$ . This mutant has been engineered with the aim of restricting water accessibility to the exposed heme edge of cytochrome  $b_5$ . The X-ray crystal structure of the V45I/V61I mutant revealed that the side chain of Ile at positions 45 and 61 restricts water accessibility to the interior of the heme cavity and protects a large section of the heme edge from the aqueous environment. Electrochemical studies performed with the V45I/V61I mutant of cytochrome  $b_5$ , and with a derivative in which the heme propionates have been converted into the corresponding dimethyl ester groups, clearly demonstrate that dehydration of the heme edge contributes to the modulation of the reduction potential of cytochrome  $b_5$ . In fact, these studies showed that exclusion of water from the complex interface exerts an effect ( $\sim 40$  mV shift) that is comparable, if not larger, than the one originating from neutralization of the charge on the solvent exposed heme propionate ( $\sim 30$  mV shift).

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Pioneering work demonstrating facile heterogeneous electron exchange between cytochrome  $c$  and modified electrodes was reported by Eddowes and Hill<sup>1</sup> and Yeh and Kuwana.<sup>2</sup> Eddowes and Hill proposed that 4,4'-bipyridyl adsorbs on the surface of gold electrodes, hence preventing the adsorption and concomitant denaturation of cytochrome  $c$ . Yeh and Kuwana reported the reversible cyclic voltammetry of cytochrome  $c$  at clean indium oxide electrodes. These two seminal reports stimulated a large effort aimed at understanding important aspects related to protein electrochemistry, including the role played by electrode modifiers in promoting rapid heterogeneous electron transfer, molecular recognition between electrode and protein surfaces, and the nature of the association between electrode and protein. These research efforts carried out by many investigators have been recently reviewed.<sup>3–6</sup>

In contrast to cytochrome *c*, several electron transfer proteins possess localized patches of negatively charged residues on the surface near their active sites. The direct cyclic voltammetry of such proteins has been achieved by implementing a variety of different experimental schemes. For example, thiols with general structures of the type HS-R-X have been extensively used as electrode modifiers.<sup>7-9</sup> In these type of modifiers the thiol group acts like a surface-active functional group that interacts with the electrode (typically gold or platinum) to form self-assembled monolayers. The role of the functional group X is to communicate with the electroactive protein *via* the formation of electrostatic or hydrogen bond interactions. R is typically an organic structure linking the surface-active thiol and the protein "sensing" functionality. Examples of this type of surface modifier include 2-aminoethane<sup>10</sup> and peptides such as (Lys-Cys)<sub>2</sub>,<sup>8</sup> which were used to promote the reversible voltammetry of plastocyanin and cytochrome *b*<sub>5</sub>, respectively. An alternative approach to obtain the reversible cyclic voltammetry of negatively charged proteins consists of promoting electrochemical communication between negatively charged proteins and negatively charged pyrolytic graphite electrodes by addition of multivalent ions such as Ca<sup>2+</sup>, Mg<sup>2+</sup> or [Cr(NH<sub>3</sub>)<sub>6</sub>]<sup>3+</sup> to the solution contained in the electrochemical cell.<sup>11-14</sup>

When the electrochemistry of rat liver outer mitochondrial membrane (OM) cytochrome *b*<sub>5</sub> was studied at polycrystalline gold electrodes modified with β-mercaptopropionate, it was observed that the reduction potential of this protein shifted cathodically as a function of increasing polylysine concentration.<sup>15</sup> Polylysine was added to the electrochemical cell in order to promote interactions between the negatively charged β-mercaptopropionate electrode-surface and the negatively charged surface on cytochrome *b*<sub>5</sub>. It was initially postulated that the formation of a complex between cytochrome *b*<sub>5</sub> and polylysine would neutralize charges on the surface of the protein, including those originating from the heme propionates, hence resulting in the observed cathodic shift of reduction potential.<sup>15</sup>

Subsequently, electrochemical and NMR spectroscopic studies were carried out with OM cytochrome *b*<sub>5</sub> and a derivative of this protein, where the heme propionates are esterified to the corresponding dimethyl ester (DiMe cytochrome *b*<sub>5</sub>). These studies were performed with the aim of elucidating the role played by surface and heme propionate charges in modulating the reduction potential of cytochrome *b*<sub>5</sub>.<sup>16</sup> Potentiometric studies performed with cytochrome *b*<sub>5</sub> demonstrated that the reduction potential of this protein, measured in the presence of polylysine, is significantly more positive than the reduction potential measured in the absence of polylysine. In contrast, potentiometric measurements demonstrated that the reduction potential of DiMe cytochrome *b*<sub>5</sub> is independent of the presence of polylysine.<sup>16</sup> These findings indicated that electrostatic interactions between polylysine and the heme propionates in cytochrome *b*<sub>5</sub> modulate the reduction potential of the protein. Furthermore, the fact that the reduction potential of DiMe cytochrome *b*<sub>5</sub> (measured potentiometrically) is independent of the concentration of polylysine also indicates that neutralization of acidic residues on the surface of the protein exerts a minimum influence in the reduction potential of cytochrome *b*<sub>5</sub>. The latter is in agreement with studies demonstrating that eliminating, even reversing the charge of these residues by site-directed mutagenesis, does not have an effect on the reduction potential of cytochrome *b*<sub>5</sub>.<sup>17</sup>

NMR experiments carried out with cytochrome *b*<sub>5</sub> containing heme labeled with <sup>13</sup>C at the heme propionate groups conclusively demonstrated that only one of the heme propionates in cytochrome *b*<sub>5</sub> undergoes electrostatic interactions with polylysine.<sup>16</sup> In fact, the NMR experiments demonstrated that the heme propionate capable of interacting with polylysine is located on the solvent exposed heme edge of cytochrome *b*<sub>5</sub>. The solvent exposed heme propionate is part of a highly localized patch of negative electrostatic potential surrounding the heme, which is delineated by Glu-44, Glu-48, Glu-56, Asp-60, and the solvent exposed heme propionate. It is noteworthy that these residues<sup>18</sup> and the solvent-exposed heme propionate<sup>19-21</sup> have been implicated in the binding of mitochondrial cytochrome *b*<sub>5</sub> to cytochrome *c*. The formation of this interprotein complex precedes the oxidation of ferrous cytochrome *b*<sub>5</sub> by ferric cytochrome *c*.<sup>19,22</sup>

Voltammetric measurements performed with gold disk electrodes modified with β-mercaptopropionate showed that the reduction potential of both, cytochrome *b*<sub>5</sub> and DiMe cytochrome *b*<sub>5</sub>, is modulated by the concentration of polylysine in the electrochemical cell. Although the modulation of the reduction potential of cytochrome *b*<sub>5</sub> was expected (see above), the modulation of the reduction potential of DiMe cytochrome *b*<sub>5</sub> was striking, because the potentiometric studies described above showed that the reduction potential of DiMe cytochrome *b*<sub>5</sub> is indepen-

dent of the concentration of polylysine. On the basis of these findings we proposed that the reduction potential of cytochrome  $b_5$  is modulated by the formation of a complex between cytochrome  $b_5$  and polylysine at the electrode surface. The formation of this complex (a) neutralizes the charge on the heme propionate located on the exposed heme edge and (b) lowers the dielectric of the exposed heme microenvironment by excluding water from the complex interface. These two factors act synergistically to destabilize the positive charge of the ferric heme with respect to the neutral ferrous heme, hence encouraging a cathodic shift in the reduction potential of cytochrome  $b_5$ .

The neutralization of the solvent-exposed heme propionate upon formation of a complex between cytochrome  $b_5$  and polylysine was conclusively demonstrated on the basis of NMR spectroscopic experiments performed with cytochrome  $b_5$  containing  $^{13}\text{C}$ -labeled heme.<sup>16,21</sup> In an attempt to demonstrate the effect of the dielectric constant on the reduction potential of cytochrome  $b_5$ , we prepared the corresponding V45L/V61L double mutant.<sup>16</sup> The reduction potential of the mutant protein, measured potentiometrically, was shown to be 50 mV more negative than that of the wild-type protein. Furthermore, X-ray crystallographic studies demonstrated that the heme in the double mutant is more accessible to water (increased dielectric of the heme microenvironment), hence providing indirect support for the idea that the reduction potential of cytochrome  $b_5$  is also modulated by dehydration of the exposed heme-edge upon formation of a transient complex with the electrode surface.<sup>16</sup>

Herein we report the preparation of a mutant OM cytochrome  $b_5$  in which the exposed heme edge has been closed by engineering hydrophobic contacts between Ile residues at position 45 and 61 and the heme edge. Evidence obtained from X-ray crystallographic and electrochemical studies carried out with the V45I/V61I double mutant provide conclusive evidence demonstrating that dehydration of the exposed heme edge upon formation of a transient complex with polylysine at the electrode surface does indeed contribute to modulate the reduction potential of cytochrome  $b_5$ .

## Experimental procedures

Protocols utilized for standard procedures such as plasmid isolations, transformations, ligation and restriction endonuclease reactions were those published by Sambrook *et al.*<sup>23</sup> Recombinant rat liver outer mitochondrial membrane (OM) cytochrome  $b_5$  was expressed in *E. coli* and purified as described previously.<sup>24</sup> The dimethyl ester of OM cytochrome  $b_5$  was prepared utilizing previously reported methodology.<sup>25,26</sup> Polylysine with an average MW of 3970, was purchased from Sigma and was used without further purification. The pET 11a plasmid and BL21(DE3) *E. coli* cells were purchased from Novagen, Madison WI. All reagents were purchased from Aldrich or Sigma and were used as received. Single-stranded oligonucleotides were synthesized by the Recombinant DNA/Protein Facility at Oklahoma State University.

### Cyclic voltammetry

Cyclic voltammetry was carried out with the aid of a BAS CV50W potentiostat (Bioanalytical Systems, West Lafayette, IN). Glass slides (2.50 cm  $\times$  2.50 cm) coated on one side with indium-doped tin oxide (ITO) semiconductor films exhibiting a typical resistance of less than 10  $\Omega$  were purchased from Delta Technologies (Stillwater, MN) and used as working electrodes. A platinum wire auxiliary electrode and a Ag/AgCl reference electrode containing a fiber junction were purchased from Cypress Systems (Lawrence, KS). The electrochemical cell used for the studies reported herein has been previously described.<sup>27</sup> The ITO working electrode was conditioned by sonicating for 30 min in each of the following solutions: 1% alconox in deionized water, ethanol, and deionized water. The clean electrodes were subsequently dried with a stream of nitrogen and used immediately. The solution in the electrochemical cell, typically 300  $\mu\text{L}$ , was deaerated by bubbling high-purity nitrogen for 30 min and subsequently blanketed with nitrogen to maintain anaerobicity. Protein concentrations, typically 100  $\mu\text{M}$ , were determined with the aid of UV-vis spectrophotometry using an extinction coefficient ( $\epsilon = 130 \text{ mM}^{-1} \text{ cm}^{-1}$ ) previously reported for ferric cytochrome  $b_5$ .<sup>28</sup> The concentration of polylysine was obtained from the weight of substance and the molecular weight provided by Sigma.

## Spectroelectrochemistry

Transmission mode spectroelectrochemical titrations were carried out with a custom-made spectroelectrochemical cell constructed from a glass cuvette (1.0 cm path-length) equipped with a platinum-foil working electrode and a Ag/AgCl reference electrode. The cell design was modeled after that reported previously by Stankovich.<sup>29</sup> The potentiometric titrations were performed by adding the appropriate volumes of a 10 mM solution of sodium dithionite to a solution consisting of the appropriate protein (15  $\mu$ M) and the appropriate redox mediators. For potentiometric titrations carried out with the V45I/V61I double mutant of OM cytochrome  $b_5$  the solution contained 200  $\mu$ M  $[\text{Ru}(\text{NH}_3)_6]\text{Cl}_3$ , 25  $\mu$ M 2,5-dihydroxy-*p*-benzoquinone ( $E^{\circ'} = -0.060$  V vs. NHE),<sup>30</sup> and 25  $\mu$ M anthraquinone-2,6-disulfonate ( $E^{\circ'} = -0.184$  V vs. NHE).<sup>30</sup> Solutions used for potentiometric titrations of the DiMe ester derivative of the V45I/V61I double mutant contained 200  $\mu$ M  $[\text{Ru}(\text{NH}_3)_6]\text{Cl}_3$ , 25  $\mu$ M 2,5-dihydroxy-*p*-benzoquinone ( $E^{\circ'} = -0.060$  V vs. NHE).<sup>30</sup>

## Site-directed mutagenesis

The recombinant plasmid MRL1<sup>24</sup> and the transformer site-directed mutagenesis kit (Clontech) were used to obtain a gene coding for the V45I/V61I double mutant of OM cytochrome  $b_5$ . The sequences corresponding to the selection primer (AflIII to BglII) and mutagenic primers designed to introduce the V45I and V61I mutations follow: 5'-GGGGATAACGCAGGAAAGAAGATCTGAGCAAAAAGGCC-3'; 5'-CACCCGGGCGGCGAAGAAATCCTGCTGGAACAGGCGGGC-3'; 5'-GCGACCGAATCTTTCGAAGATATCGGCCACTCTCCGGATGCGCG-3'. The underlined codons represent mismatches introduced to generate the mutations. The PBS + plasmid harboring the gene coding for the V45I/V61I double mutant was excised by digesting with the restriction endonucleases BamHI and NdeI and then purified by electrophoresis. The 300-base pair gene was then cloned into the expression plasmid pET11a,<sup>31</sup> and the recombinant pET plasmid was transformed into BL21(DE3) *E. coli* cells for subsequent protein overexpression.

## X-ray crystallography

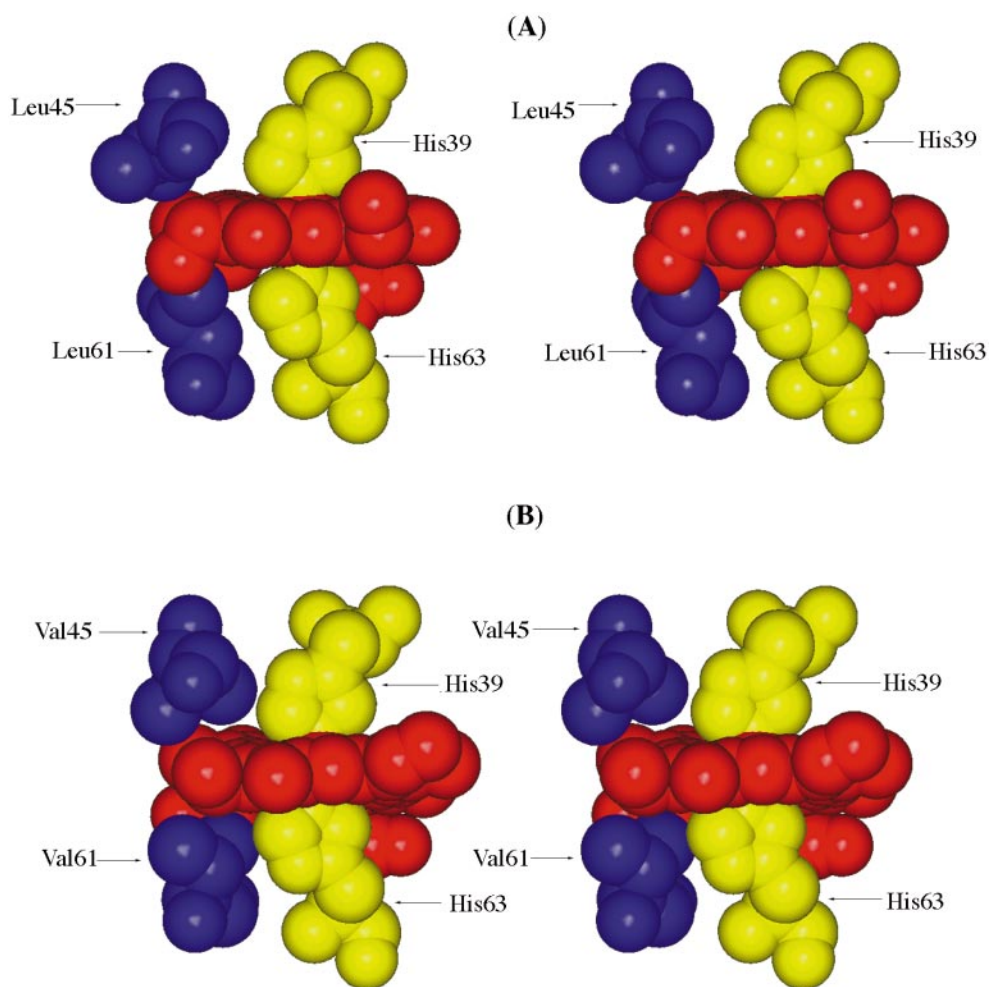
X-ray quality crystals of the V45I/V61I double mutant of OM cytochrome  $b_5$  were grown using the vapor diffusion method applying conditions identical to those used in the crystallization of the wild-type protein<sup>21</sup> and the V45L/V61L double mutant.<sup>16</sup> In short, the protein ( $\sim 20$  mg ml<sup>-1</sup>) was mixed 1 : 1 by volume with precipitant solution [20% (w/v) polyethylene glycol (MW 8,000), 0.2 M magnesium acetate, and 0.1 M PIPES, pH 6.8] in 8  $\mu$ L drops. The drop was allowed to equilibrate overnight with 500  $\mu$ L of precipitant solution in vapor diffusion chambers and then micro-seeded with cytochrome  $b_5$  crystals. Crystals grew to a typical size of  $0.5 \times 0.2 \times 0.2$  mm<sup>3</sup> in a few days. The crystal belongs to space group P2<sub>1</sub>2<sub>1</sub>2<sub>1</sub> and diffracts X-rays to 1.8 Å resolution.

## Results and discussion

### Protein engineering

Research carried out in our laboratories demonstrated that the reduction potential of cytochrome  $b_5$  is modulated by the formation of a transient complex with polylysine at the electrode surface. The modulation of reduction potential stems from the degree of exposure of the heme in cytochrome  $b_5$  to the aqueous environment. In fact, one edge of the heme forms part of the molecular surface, and consequently is in contact with the aqueous environment. This particular feature in the structure of cytochrome  $b_5$ , together with the results obtained from previously reported<sup>16</sup> electrochemical studies carried out with this protein, led us to postulate that formation of a transient complex with polylysine at the electrode surface modulates its reduction potential.<sup>16</sup> The modulation mechanism was proposed to originate from neutralization of the charge on the solvent exposed heme propionate and from dehydration of the protein surface surrounding the exposed heme edge. Direct evidence for the role played by the heme propionate in modulating the reduction potential of cytochrome  $b_5$  upon complex formation was obtained from a combination of electrochemical and NMR spectroscopic experiments. By comparison, evidence demonstrating that sequestering the exposed heme edge from water modulates the reduction potential was indirect.

Consequently, an important aim of the study presented here was to engineer a cytochrome  $b_5$  mutant in which the exposed heme edge would be sequestered from water by hydrophobic interactions between the heme edge and polypeptide side chains. The availability of a mutant protein exhibiting the desired structural properties is expected to furnish direct experimental information regarding the role played by the exposed heme edge in modulating the reduction potential of cytochrome  $b_5$ . Molecular models constructed to guide the site-directed mutagenesis experiments suggested that the V45I/V61I double mutant of OM cytochrome  $b_5$  is likely to possess the desired structural features. The rationale for constructing a gene coding for this double mutant is described in what follows. The X-ray crystal structure of wild type OM cytochrome  $b_5$ <sup>21</sup> shows that one of the methyl groups ( $C_{\gamma 1}$ ) in the side chains of Val-45 and Val-61 pack against the top and bottom of the heme (Fig. 1B). The same methyl groups in Val-45 and Val-61 also make hydrophobic interactions with His-39 and His-63, respectively. These hydrophobic interactions



**Fig. 1** Cross sectional stereo views obtained from the X-ray crystal structures of (A) V45L/V61L double mutant of OM cytochrome  $b_5$  (PDB ID code: 1AWP) and (B) wild type OM cytochrome  $b_5$  (PDB ID code: 1B5M). The stereo view shown in B demonstrates that the isopropyl methyl groups ( $C_{\gamma 1}$ ) in Val-45 and Val-61 pack against His-39 and His-63, respectively. The stereo view shown in A demonstrates that replacement of Val for Leu at positions 45 and 61 leaves the position previously filled by the  $C_{\gamma 1}$  carbon of Val, vacant. The voids introduced by the conformation of the Leu side chains result in increased water accessibility to the interior of the heme binding cavity and in a  $-50$  mV shift in the reduction potential of the mutant with respect to the wild type protein.<sup>16</sup>

taken together create a hydrophobic wall that prevents access of water into the heme binding pocket (Fig. 1B). When Val-45 and Val-61 were replaced by Leu, the X-ray crystal structure of the double mutant<sup>16</sup> showed that the longer Leu side-chains adopt a conformation that leaves the volume previously occupied by the C<sub>γ1</sub> carbon in Val-45 and Val-61 unoccupied (Fig. 1A). The effect of replacing Val for Leu at positions 45 and 61, therefore, is to create water cavities in the interior of the heme binding pocket. This observation underscores the idea that subtle (conserved) changes in amino acid composition can have significant changes in structure that ultimately exert an effect in protein function. For instance, the water cavities created in the interior of the heme binding pocket of the V45L/V61L mutant increase the dielectric constant of the heme micro-environment, therefore resulting in a -50 mV shift in the reduction potential (function) of OM cytochrome *b*<sub>5</sub>.<sup>16</sup>

In order to engineer a mutant cytochrome *b*<sub>5</sub> displaying a heme edge with restricted water accessibility, it is desirable to create hydrophobic interactions between amino acids lining the exposed heme edge and this part of the heme cofactor. To accomplish this task, however, it is not only necessary to introduce the desired hydrophobic interactions at the heme edge, but it is also important to avoid the creation of voids in the interior of the molecule. Molecular models built with coordinates from the X-ray crystal structure of wild type and V45L/V61L cytochrome *b*<sub>5</sub>, suggested that placing Ile at positions 45 and 61 is likely to accomplish the task of closing the heme edge, while maintaining packing integrity inside the heme binding pocket. In fact, a model of the V45I/V61I double mutant indicated that the sec-methyl in the Ile side chains is likely to occupy the volume normally filled by the isopropyl methyl in Val-45 and Val-61 in the wild type protein. Furthermore, the model also indicated that sec-propyl side chains at positions 45 and 61 are likely to shield the heme edge from the aqueous environment. A gene coding for the V45I/V61I double mutant was therefore constructed and the corresponding protein expressed in *E. coli* and then purified to homogeneity. The protein was subsequently studied by X-ray crystallography and electrochemistry.

### X-ray crystallography

X-ray diffraction data were collected at room temperature using a RAXIS IV image plate system (MSC, Woodlands, Texas). Crystallographic data are available in .cif format as Electronic Supplementary Information (CCDC reference number 1355/1).<sup>†</sup> The data were processed with the HKL program package.<sup>32</sup> Since the unit cell parameters of the V45I/V61I crystal form are marginally different from those of the previously reported V45L/V61L crystal form (ID: 1AWP), less than 2% in any given dimension, the initial phases of the new structure were calculated from the model of the V45L/V61L structure. There are two identical cytochrome *b*<sub>5</sub> molecules in a crystallographic asymmetric unit, with a  $V_M$  of 2.6 Å<sup>3</sup> Da<sup>-1</sup>. Refinement was carried out with the program CNS (V. 1.0),<sup>33</sup> and 4.6% of the total reflections were randomly selected to monitor the free-*R* factor. Non-crystallographic symmetry restraint was not applied since the number of observations exceeds the degrees of freedom. Simulated annealing (from 5000 K) was used at the initial stage of the refinement to reduce bias for the template model. Solvent molecules were introduced after the *R*-factor dropped below 23% and were all modeled as water molecules. The average *B* factor of the Ile-45 side chain is 34 Å<sup>2</sup> and that of Ile-61 is 43 Å<sup>2</sup>. In both cases molecule B displays higher *B* factors than molecule A (there are two cytochrome *b*<sub>5</sub> molecules per crystallographic asymmetric unit). The side-chain methylene groups in Ile-45 and Ile-61 are well adopted and show good electron density. In three of the four Ile residues of interest (Ile-45 and Ile-61 in molecules A and B) the sec-methyl carbon displays a slightly lower *B* factor than the other side-chain atoms beyond C<sub>β</sub>. Bulk solvent correction and correlated *B*-factor refinement were used in the final stage of refinement. The statistics of the diffraction data and refinement results are summarized in Table 1.

In the V45I/V61I mutant (Fig. 2A), the sec-methyl carbon (C<sub>γ2</sub>) in Ile-45 is located 3.84 Å away from the C<sub>ε1</sub> atom in His 39. By comparison, in the wild type protein the isopropyl-methyl carbon (C<sub>γ1</sub>) in Val-45 is 3.85 Å away from the C<sub>ε1</sub> atom in His 39 (Fig. 2B). Consequently, the sec-methyl group of Ile-45 in the double mutant maintains the compact packing that restricts water accessibility into the heme binding pocket. This is also illustrated by the space filling stereo views

<sup>†</sup> Electronic Supplementary Information available. See <http://www.rsc.org/suppdata/fd/b0/b001520m/>

**Table 1** Data collection and refinement statistics

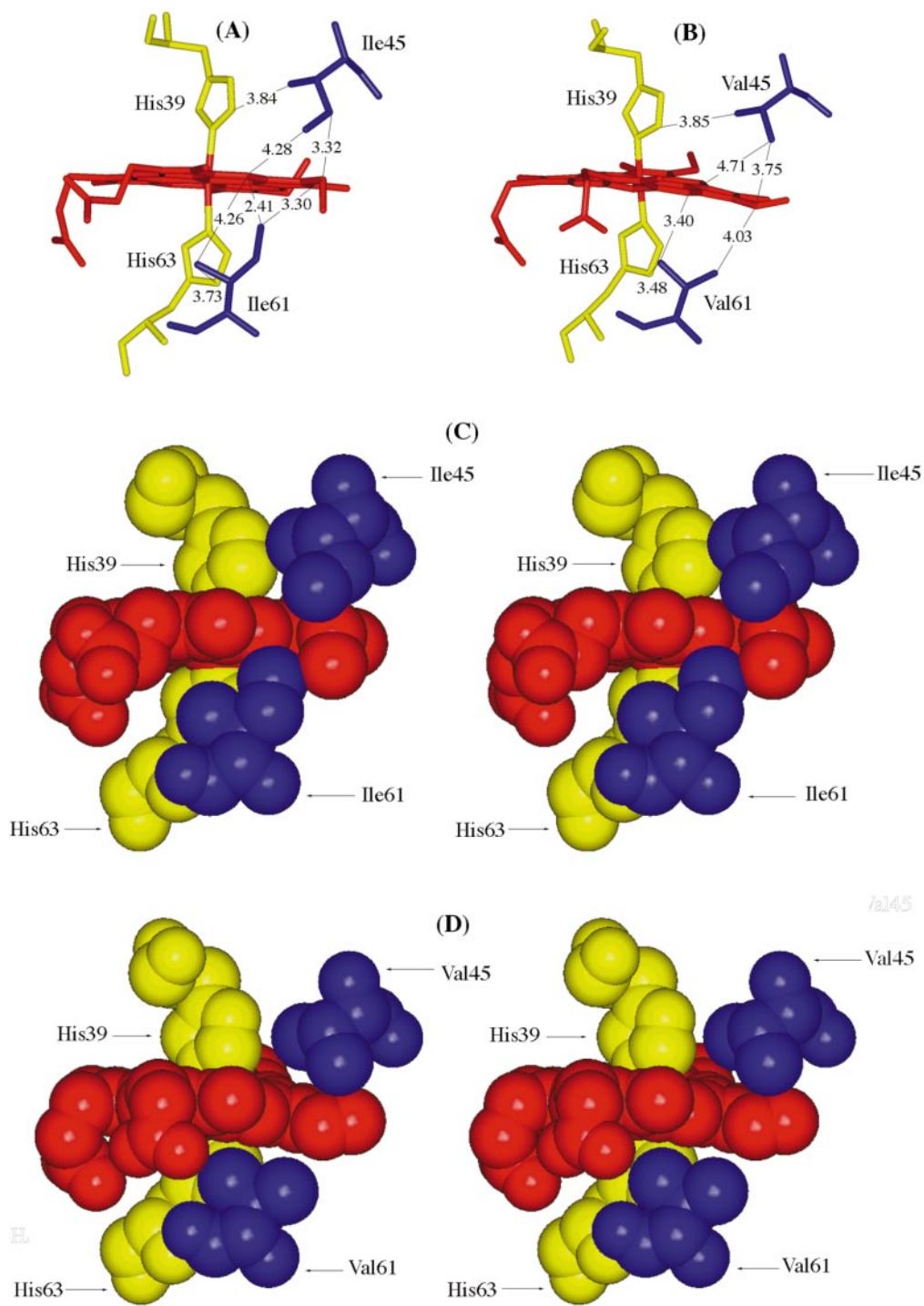
(a) Data statistics		
Space group	P2 <sub>1</sub> 2 <sub>1</sub> 2 <sub>1</sub>	
Unit cell ( <i>a</i> , <i>b</i> and <i>c</i> in Å)	46.2, 70.8 and 72.4	
Resolution/Å	24.2–1.8	
<i>R</i> <sub>merge</sub> (%)	6.5	(48) <sup>a</sup>
No. of reflections	21 091	
Completeness (%)	93.0	(91.8)
$\langle I \rangle / \sigma$	20.6	(1.8)
(b) Refinement statistics		
<i>R</i> <sub>working</sub> (%)	20.8	for 20 051 reflections
<i>R</i> <sub>free</sub> (%)	23.9	for 1040 reflections
No. of non-hydrogen atoms		
Protein	694 × 2	
Heme	43 × 2	
Solvent	78	
Rms deviation from ideal values		
Bond length/Å	0.0056	
Bond angle/degree	1.18	
Average <i>B</i> -factor/Å <sup>2</sup>		
Protein atoms	34	
Heme	37	
Solvent molecules	40	
<sup>a</sup> Numbers in parentheses are the corresponding numbers for the highest resolution shell (1.85–1.80 Å). The highest resolution shell with <i>R</i> <sub>merge</sub> below 20% is that corresponding to 2.13–2.03 Å.		

corresponding to the V45I/V61I mutant (Fig. 2C) and wild type protein (Fig. 2D). In the wild type protein (Fig. 2B and D), methyl carbon C<sub>γ2</sub> in Val-45 is located 4.71 Å and 3.75 Å from the heme-meso and heme vinyl-β carbons, respectively. In the mutant protein, the C<sub>γ1</sub> and C<sub>δ1</sub> carbons in Ile-45 are closer to the heme-meso (4.28 Å) and heme-vinyl-β (3.32 Å) carbons (Fig. 2A and C). The X-ray crystal structure of the mutant also reveals that the sec-methyl carbon (C<sub>γ2</sub>) in Ile-61 is located 3.73 Å from the N<sub>δ1</sub> atom in His-63 (Fig. 2A), therefore occupying a position similar to that taken by the isopropyl C<sub>γ1</sub> carbon of Val-61 (3.48 Å from His-63 N<sub>δ1</sub>) in the wild type protein (Fig. 2B). Consequently, the sec-methyl group in Ile-61 restricts water accessibility to the interior of the heme binding pocket in the V45I/V61I double mutant. Furthermore, the C<sub>γ1</sub> carbon in Ile-61 is located 2.41 Å from the heme-meso carbon and 3.30 Å from the heme-vinyl-β carbon (Fig. 2A and C). The shorter comparable distances in the wild-type protein (Fig. 2B and D) are from Val-61 C<sub>γ1</sub> carbon to heme-meso carbon (3.40 Å) and from Val-61 C<sub>γ2</sub> carbon to heme-vinyl-β carbon (4.03 Å).

From the above it is evident that the sec-methyl group in the side chains of Ile-45 and Ile-61 occupies the volume taken by the isopropyl methyl groups of Val-45 and Val-61 in the wild type protein, therefore preventing water access to the interior of the heme cavity. In addition, it is also clear that hydrophobic contacts between the heme edge and the side chains of Ile at positions 45 and 61 sequester a large section of the heme edge from the aqueous environment. Consequently, it is anticipated that the structural properties engineered into the V45I/V61I mutant are going to be useful in unraveling the role played by the solvent exposed heme edge in modulating the redox potential of cytochrome *b*<sub>5</sub>. The results of studies aimed at probing this idea are described in the following section.

### Electrochemistry

In previous electrochemical studies carried out with OM cytochrome *b*<sub>5</sub>, cyclic voltammograms of the protein were obtained at gold-disk electrodes modified with β-mercaptothiopyridone.<sup>15,16,34</sup> Interactions between negatively charged protein and electrode surfaces were promoted by the addition of polylysine to the electrochemical cell. For the present study, cyclic voltammograms of OM cytochrome *b*<sub>5</sub> have been obtained with the aid of indium-doped tin oxide (ITO) electrodes.

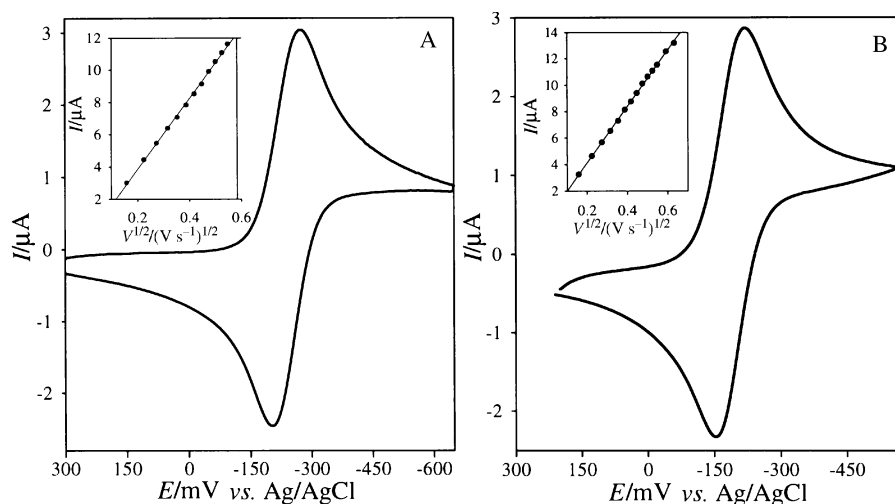


**Fig. 2** The cross sectional views shown in A and B allow the comparison of important distances in the structure of wild type OM cytochrome  $b_5$  (B) with corresponding distances in the structure of the V45I/V61I mutant (A). The space filling stereo-views shown in C and D correspond to the views shown in A and B, respectively. These representations, however, provide visual confirmation that the *sec*-methyl groups in Ile 45 and Ile-61 restrict water accessibility to the interior of the heme pocket. It is also evident that Ile at positions 45 and 61 shield the heme edge from the aqueous environment, whereas Val at the same positions leaves the heme edge highly accessible to water.

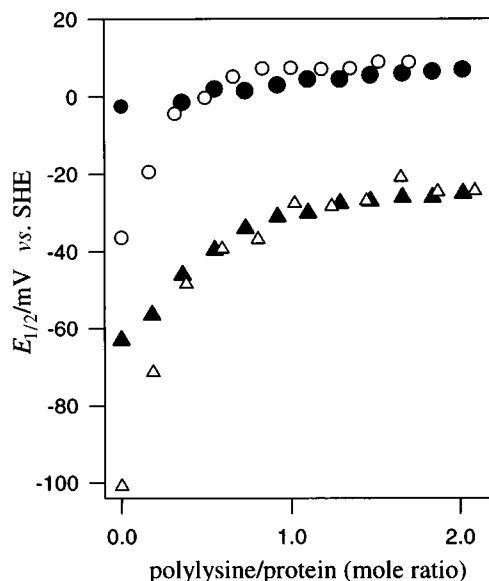
The negative charge on the surface of indium oxide electrodes<sup>35</sup> was initially exploited by Yeh and Kuwana,<sup>2</sup> who used it in order to obtain the unmediated electrochemistry of cytochrome *c*, a positively charged protein. In subsequent studies, the voltammetry of negatively charged proteins has been obtained at indium oxide electrodes by addition of a polycation in order to overcome the electrostatic repulsion between negatively charged protein and electrode surfaces.<sup>27,36,37</sup>

Typical background subtracted cyclic voltammograms obtained from solutions containing a mixture of polylysine and the V45I/V61I double mutant, or polylysine and the DiMe ester derivative of the V45I/V61I double mutant, are shown in Fig. 3A and B. The ratio of the cathodic to anodic peak currents ( $i_{pc}/i_{pa}$ ) is unity and the peak to peak separation ( $\Delta E_p$ ) is 63 mV. Cyclic voltammograms obtained for OM cytochrome  $b_5$  and its corresponding DiMe ester derivative at ITO electrodes possess the same attributes and are essentially identical to those obtained previously at gold electrodes modified with  $\beta$ -mercaptoacrylate<sup>15,34</sup> (data not shown). For all proteins, the cathodic peak current is proportional to the square root of the scan rate, indicating that the electrochemical process is diffusion controlled. In agreement with results previously obtained from electrochemical studies conducted with a gold disk electrode modified with  $\beta$ -mercaptoacrylate,<sup>15,16</sup> cyclic voltammograms obtained at ITO electrodes demonstrate that the reduction potential of OM cytochrome  $b_5$  and that of its DiMe derivative shift cathodically with increasing concentrations of polylysine (Fig. 4a and b). Similar observations were made for the reduction potential of the V45I/V61I double mutant (Fig. 4c). In sharp contrast, the reduction potential of the DiMe ester derivative of the V45I/V61I double mutant, measured by cyclic voltammetry, is nearly independent of polylysine concentration (Fig. 4d).

When cyclic voltammetry is used to measure the reduction potential of OM cytochrome  $b_5$ , reduction potential values shift from  $-102$  to  $-26$  mV (76 mV), as the concentration of polylysine



**Fig. 3** Background subtracted cyclic voltammogram of (A) V45I/V61I double mutant of OM cytochrome  $b_5$  and (B) DiMe ester derivative of the V45I/V61I mutant of OM cytochrome  $b_5$ . The cyclic voltammograms were obtained at an ITO electrode from solutions containing the appropriate protein (100  $\mu$ M) and polylysine (MW 3970), scan rate = 20  $\text{mV s}^{-1}$ . The reduction potential depends on the concentration of polylysine (see Fig. 4) but the attributes for reversibility are independent of the concentration of polylysine when the polylysine/cytochrome  $b_5$  ratio is larger than 0.2.

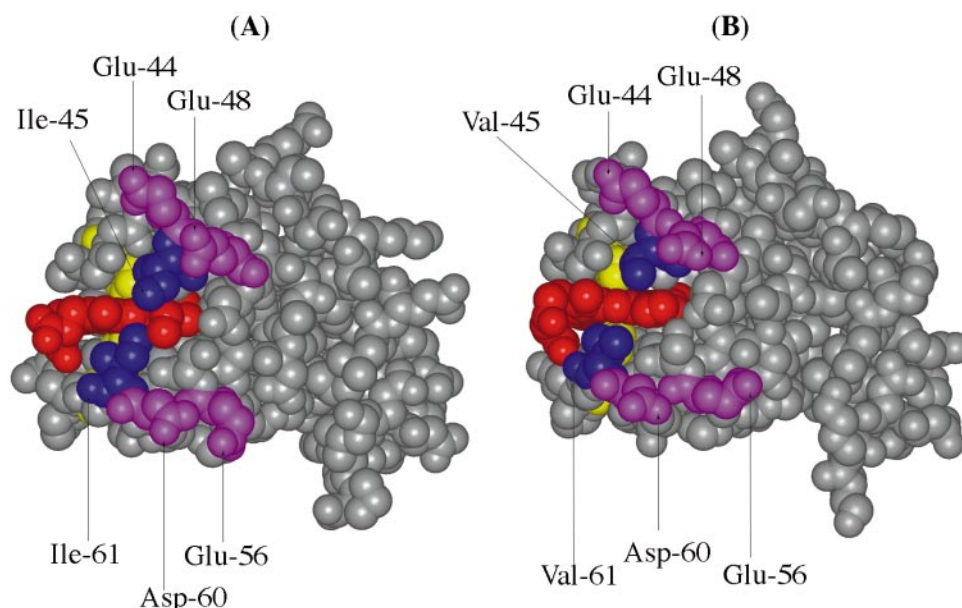


**Fig. 4** Titration of (a) OM cytochrome  $b_5$  ( $\Delta$ ), (b) DiMe cytochrome  $b_5$  ( $\circ$ ), (c) V45I/V61I cytochrome  $b_5$  ( $\blacktriangle$ ), and (d) DiMe V45I/V61I cytochrome  $b_5$  ( $\bullet$ ) with polylysine (MW 3970). The first point in each titration curve, *i.e.* polylysine/cytochrome  $b_5 = 0$ , was obtained from a potentiometric titration, as described in the Experimental procedures section.

is increased (Fig. 4a). This is in good agreement with previous results obtained with the aid of gold-disk electrodes modified with with  $\beta$ -mercapto propionate.<sup>15,16</sup> Since polylysine is also necessary to promote the electrochemistry of cytochrome  $b_5$  at the ITO electrodes, the reduction potentials corresponding to the absence of polylysine in Fig. 4 were obtained from potentiometric (spectroelectrochemistry) titrations. The reduction potential of DiMe OM cytochrome  $b_5$  (measured potentiometrically) is  $-37$  mV *vs.* NHE, a value 65 mV more positive than the corresponding value for the wild type protein. The cathodic shift in reduction potential brought about by esterification of the heme propionates is well documented and has been attributed to the destabilization of the positive charge on the ferric heme upon neutralization of the negative charge on the heme propionates.<sup>26,38–40</sup> When cyclic voltammetry is used to measure the reduction potential of DiMe OM cytochrome  $b_5$ , the values shift from  $-37$  to  $+4$  mV (41 mV), as the concentration of polylysine is increased (Fig. 4b). This behavior corroborates the idea that the neutralization of the solvent exposed heme propionate is not the only factor modulating the reduction potential of OM cytochrome  $b_5$  when this molecule forms a transient complex with polylysine at the electrode surface.

Potentiometric measurements demonstrated that the reduction potential of the V45I/V61I mutant is  $-63$  mV *vs.* NHE. Consequently, the mutant exhibits a reduction potential 40 mV more positive than that displayed by wild type OM cytochrome  $b_5$ . The more positive reduction potential of the mutant protein is in agreement with the fact that its heme experiences a lower dielectric constant because it is more shielded from the aqueous environment, as demonstrated by its X-ray crystal structure (see above). When cyclic voltammetry is used to measure the reduction potential of the V45I/V61I mutant, the values shift from  $-63$  to  $-24$  mV (39 mV), as the concentration of polylysine is increased (Fig. 4c). In contrast, the voltammetric values obtained for the DiMe ester derivative of the V45I/V61I double mutant are nearly independent of the concentration of polylysine (Fig. 4d).

In order to understand the experimental observations described above, it is useful to consider how the structures of the wild type and mutant proteins affect the properties of the transient complex formed with polylysine at the electrode surface. The X-ray crystal structure of the mutant protein clearly shows that as a result of closer packing between the side chains of Ile-45 and Ile-61 and the heme-meso and heme-vinyl groups, the heme cofactor is more protected from the aqueous



**Fig. 5** (A) View of the V45I/V61I double mutant of OM cytochrome  $b_5$  showing the relative positions of the heme (red) Ile-45 and Ile-61 (blue) and acidic residues Glu-44, Glu-48, Glu-56 and Asp-60 (magenta). This view shows that the side chains of Ile at positions 45 and 61 protect the heme-meso and heme-vinyl groups from the aqueous environment. (B) This view of wild type OM cytochrome  $b_5$  demonstrates that the heme edge in this molecule is largely exposed to the aqueous environment.

environment than its counterpart in wild type OM cytochrome  $b_5$ . This is illustrated in the space-filling representations shown in Fig. 2C and 2D and in Fig. 5. The side chain of Ile-61 (Fig. 2C and 5A) packs against the heme-meso and heme-vinyl groups, hence restricting water accessibility to these heme substituents. The methylene group ( $C_{\gamma 2}$ ) in the side chain of Ile-45 packs against the top of the heme-vinyl group; furthermore, the terminal methyl group in the Ile-45 side chain ( $C_{\delta 1}$ ), acts as a hydrophobic barrier that restricts water accessibility to the heme-meso and heme-vinyl groups. By comparison, the shorter side chains of Val at positions 45 and 61 in the wild type protein (Fig. 2D and 5B) impose a structure where the heme-meso and heme-vinyl groups are highly exposed to the aqueous environment.

For heterogeneous electron transfer to take place at the electrode surface, cytochrome  $b_5$  must diffuse toward the electrode surface, where a transient complex is formed prior to the electron transfer event. Evidence for diffusion control stems from the proportionality of the cathodic peak current with the square root of the scan rate (inset in Fig. 3). In the transient complex formed at the electrode surface, cytochrome  $b_5$  is expected to form electrostatic interactions with polylysine *via* the solvent exposed heme propionate, Glu-44, Glu-48, Glu-56 and Asp-60; the position of these acidic residues with respect to the heme is shown in Fig. 5. The formation of a complex neutralizes the charge on the heme propionate and excludes water from the interface between polylysine and cytochrome  $b_5$ , which is delineated by the above mentioned acidic residues. Exclusion of water from the complex interface, therefore, has a large effect on the dielectric constant experienced by the heme microenvironment. In other words, before the complex is formed the heme edge in the wild type protein is solvated by water, whereas within the complex the heme edge is in a more hydrophobic environment, hence experiences a lower dielectric constant. The reduced value of the dielectric constant is less efficient in stabilizing the positive charge on the ferric heme, therefore destabilizing the ferric oxidation state with respect to the ferrous oxidation state. Consequently, the protein within the complex is more easily reducible, as manifested in the more positive reduction potential.

Direct experimental evidence in support of this argument comes from the fact that the reduction potential of the DiMe ester derivative of the V45I/V61I double mutant is nearly independent of

polylysine concentration (Fig. 4d). The heme edge of the DiMe ester derivative of the V45I/V61I mutant is also delineated by the solvent exposed heme propionate, Glu-44, Glu-48, Glu-56 and Asp-60. In contrast to the wild type protein, however, the heme edge in the mutant is not readily solvated by water (Fig. 5) because of the hydrophobic contacts that it makes with the side chains of Ile-45 and Ile-61. This is evident not only from the X-ray crystal structure of the mutant but also from the fact that the reduction potential of this protein is 40 mV more positive than that of the wild type protein. As a result, the dielectric constant experienced by the heme microenvironment in the V45I/V61I double mutant is not expected to change significantly upon formation of a complex with polylysine. In fact, this is manifested in the nearly independent behavior of the reduction potential of the DiMe ester derivative of V45I/V61I as a function of polylysine concentration. In comparison, when the reduction potential of the V45I/V61I mutant is measured by voltammetry, the values are dependent on polylysine concentration (Fig. 4c). The modulation of reduction potential in this case originates almost exclusively from the neutralization of the negative charge on the solvent exposed heme propionate, upon formation of a complex with polylysine.

#### Relative contributions to the modulation of redox potential

Previous studies carried out with native<sup>26</sup> and mutated<sup>40</sup> bovine microsomal cytochrome *b*<sub>5</sub> and their corresponding DiMe ester derivatives revealed that the proteins with esterified hemes possess a reduction potential 64–67 mV more positive than proteins containing normal heme. Furthermore, studies conducted with cytochrome *b*<sub>5</sub> reconstituted with modified hemes demonstrated that the neutralization of only one heme propionate in cytochrome *b*<sub>5</sub> is typically accompanied by a 27–35 mV cathodic shift in reduction potential.<sup>41</sup> In this context, it is interesting to analyze the magnitude of the shifts observed with the wild type, V45I/V61I mutant, and corresponding DiMe ester derivatives of OM cytochrome *b*<sub>5</sub>. Inspection of Fig. 4 reveals that OM cytochrome *b*<sub>5</sub> undergoes a total shift of 76 mV (Fig. 4a), whereas the reduction potential of the corresponding DiMe ester is modulated by 41 mV (Fig. 4b). Assuming that the shift in the potential of the DiMe ester of OM cytochrome *b*<sub>5</sub> originates primarily from the dehydration of the complex interface, then it is possible to subtract 41 mV from the 76 mV shift displayed by OM cytochrome *b*<sub>5</sub>. This indicates that neutralization of the solvent exposed heme propionate in OM cytochrome *b*<sub>5</sub> by polylysine results in a 35 mV shift in reduction potential. This value is in good agreement with the expected shift resulting from the neutralization of one heme propionate. In a similar manner, inspection of Fig. 4c reveals that the reduction potential of the V45I/V61I mutant is modulated by a total of 39 mV, whereas the reduction potential of the DiMe ester derivative of the mutant protein is modulated by a total of 8 mV. Assuming that the shift observed for the DiMe ester derivative of V45I/V61I originates from a small change in the dielectric constant experienced by the heme environment upon complex formation, then the neutralization of the heme propionate in the mutant protein must modulate the reduction potential by 31 mV. These observations imply that in OM cytochrome *b*<sub>5</sub> the exclusion of water from the complex interface exerts an effect that is comparable, if not larger, than the one originating from neutralization of the charge on the solvent exposed heme propionate.

#### Concluding remarks

It is known that the reduction potential of electroactive cofactors is affected by the dielectric constant of the environment that surrounds them. For instance, it has been reported that the reduction potential of the heme in cytochrome *c* is 240 mV more positive in the folded protein than in its unfolded counterpart.<sup>42</sup> This large shift has been attributed to water exclusion from the heme environment. In fact, the availability of X-ray crystal structures for several different cytochromes *c* allowed these authors to demonstrate that the reduction potential of heme proteins can be tuned by approximately 500 mV, through variations in the exposure of heme to the aqueous environment.<sup>42</sup> The results presented in this report suggest that in addition to the large modulation of reduction potential resulting from embedding the heme cofactor in the interior of a polypeptide, an additional modulation of redox potential may originate from protein–protein interactions. This secondary modulatory mechanism is likely to be more noticeable in proteins where the redox-active cofactor is partially exposed to the aqueous environment. When such

proteins bind a redox partner, immediately prior to electron exchange, the partially exposed cofactor is likely to be buried at the interface of the interprotein complex. The change in dielectric constant experienced by the cofactor, in turn, will impose a shift in the reduction potential of the corresponding protein.

It has also been demonstrated that the binding of cytochrome *c* to cytochrome *b*<sub>5</sub> lowers the reorganization energy of the intramolecular electron transfer reaction between a ruthenium complex appended to cytochrome *b*<sub>5</sub> and the heme center in this protein.<sup>22,43</sup> These investigators proposed that the change in reorganization energy originates from the exclusion of water from the cytochrome *b*<sub>5</sub>-cytochrome *c* complex interface. The modulation of the reduction potential of cytochrome *b*<sub>5</sub> by its exposed heme edge, therefore, is in good agreement with the conclusions obtained from the kinetic experiments of Durham and coworkers.<sup>22,43</sup> In fact, if the electrochemical and kinetic observations are taken together, it is possible to conclude that the reduction potential exhibited by cytochrome *b*<sub>5</sub> is likely to be significantly more positive when it is bound to cytochrome *c* than when it is measured with classical potentiometric techniques. Consequently, in order to attain a detailed description of electron transfer between cytochrome *b*<sub>5</sub> and cytochrome *c* it may be necessary to consider that the formation of an interprotein complex has an effect in the reorganization energy and in the driving force of the reaction.

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