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# Control of Cytochrome *c* Redox Potential: Axial Ligation and Protein Environment Effects

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***Abstract:*** Axial iron ligation and protein encapsulation of the heme cofactor have been investigated as effectors of the reduction potential ($E^{\circ\prime}$) of cytochrome *c* through direct electrochemistry experiments. Our approach was that of partitioning the $E^{\circ\prime}$ changes resulting from binding of imidazole, 2-methyl-imidazole, ammonia, and azide to both cytochrome *c* and microperoxidase-11 (MP11), into the enthalpic and entropic contributions. *N*-Acetylmethionine binding to MP11 was also investigated. These ligands replace Met80 and a water molecule axially coordinated to the heme iron in cytochrome *c* and MP11, respectively. This factorization was achieved through variable temperature $E^{\circ\prime}$ measurements. In this way, we have found that (i) the decrease in $E^{\circ\prime}$ of cytochrome *c* due to Met80 substitution by a nitrogen-donor ligand is almost totally enthalpic in origin, as a result of the stronger electron donor properties of the exogenous ligand which selectively stabilize the ferric state; (ii) on the contrary, the binding of the same ligands and *N*-acetylmethionine to MP11 results in an enthalpic stabilization of the reduced state, whereas the entropic effect invariably decreases $E^{\circ\prime}$ (the former effect prevails for the methionine ligand and the latter for the nitrogenous ligands). A comparison of the reduction thermodynamics of cytochrome *c* and the MP11 adducts offers insight on the effect of changing axial heme ligation and heme insertion into the folded polypeptide chain. Principally, we have found that the overall $E^{\circ\prime}$ increase of approximately 400 mV, comparing MP11 and native cytochrome *c*, consists of two opposite enthalpic and entropic terms of approximately +680 and −280 mV, respectively. The enthalpic term includes contributions from both axial methionine binding (+300 mV) and protein encapsulation of the heme (+380 mV), whereas the entropic term is almost entirely manifest at the stage of axial ligand binding. Both terms are dominated by the effects of water exclusion from the heme environment.

## Introduction

Inner-sphere coordination effects, solvation, and electrostatics at the metal−protein interface have been found to control the reduction potential ($E^{\circ\prime}$) in metalloredox proteins.[1] The understanding of how this control is exerted is a long-standing biophysical problem that has been tackled by several theoretical and experimental approaches.[2−16] However, despite recent advances in this field following from an increasing knowledge of the structural[17,18] and redox properties of these species,[19] and as a result of improvements in calculation procedures for metal−protein interaction energies,[2,5,7,20] the "big picture" has not yet been clearly defined.

Cytochromes *c* (cytc), thanks to their availability and the large degree of structural and functional (redox) characterization, lie center-stage in studies directed toward this problem. The $E^{\circ\prime}$

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values of class I cytc vary from approximately +200 to +350 mV (vs SHE).[21−23] It is now established that a significant contribution to these high redox potentials arises from the $\pi$-electron-acceptor character of the thioether sulfur atom of the axially bound methionine to iron, which stabilizes the ferrous over the ferric state.[24−28] This selective stabilization is further enhanced by the poor accessibility of the heme to solvent, and the burial of the heme within a hydrophobic pocket.[21−23,28] The electrostatic effects of polar and charged groups on $E^{\circ\prime}$ depend on several factors, such as the heme−charge distance and the dielectric properties of the intervening medium. The latter effect may vary for each particular interaction and involves contributions from both the solvent and the protein.[4,11−13,29] The hydrophobicity of the heme environment enhances the electrostatic interactions between the redox center and the polar and charged groups within the protein, whereas the electrostatic effect of the net or fractional charges on the protein surface is quenched by the high dielectric constant of water and by enthalpy−entropy compensation phenomena due to solvent reorganization effects.[9,30] Indeed, entropic terms are important effectors of the relative stabilization of the two oxidation states and, consequently, of the redox potential of cytc which originate from variations in dynamic and solvation properties between the two states.[23,30−33] These contributions can be determined with an electrochemical approach,[23,30,32,33] but their molecular determinants are still largely uncharacterized.

At neutral pH and room temperature, exogenous nitrogen-donor ligands, such as imidazole (Im), azide, and ammonia, can replace the methionine as the axial ligand to iron in oxidized cytc.[25−27,34−50] The same ligands can also bind to the second axial position for heme iron in microperoxidases, which is occupied by a water molecule.[28,51,52] Microperoxidases are hemepeptides obtained from the proteolytic digestion of oxidized cytc, differing in the length of the polypeptide chain (between 6 and 11 residues). In these species, the thioether bonds between the two cysteine residues (Cys 14 and 17) of the parent protein and the porphyrin ring are conserved, as well as iron ligation by the proximal histidine (His18).

Spectroscopic studies of the imidazole and *N*-acetylmethionine derivatives of microperoxidases demonstrate that they are good models for the active site of both the bis(imidazole)-ligated cytochromes and class I cytochromes *c*, respectively.[28] A comparison of their reduction potentials, relative to those of the native proteins, provided the first quantitative information on the influence of axial ligands and the protein matrix on the $E^{\circ\prime}$ of cytochrome *c*. These data indicated that substitution of imidazole by methionine as ligand to heme iron gives rise to a 150 mV increase in $E^{\circ\prime}$, whereas protein folding around the heme induces an increase in $E^{\circ\prime}$ by approximately 240 mV.[28] These hemepeptides, with different bound exogenous axial ligands, are also valuable systems for investigating the changes in redox properties of cytc following changes in axial ligation during such processes as protein unfolding, which involves axial ligation of the heme iron by two histidines, and in the alkaline transition of cytc in which the axial Met80 is replaced by a surface lysine. It is also well established that in vitro folding of cytc relies on the stabilizing interactions of the polypeptide chain with the heme group, which indeed include axial ligation as a major feature.[28,53−63]

It has been shown that valuable insights on the correlation between $E^{\circ\prime}$ and structural properties of metalloredox proteins can be gained from the factorization of the corresponding enthalpic ($\Delta H^{\circ\prime}_{rc}$) and entropic ($\Delta S^{\circ\prime}_{rc}$) components, determined from analysis of the temperature dependence of the reduction potential.[9,10,23,30,32,33,64,65] Here, we have applied this approach to studies of derivatives of cytc and microperoxidase-11 (MP11 hereafter). The latter is an 11-residue peptide obtained from hydrolysis of horse heart cytc, that contains the heme group and amino acids 11−21, and where one axial coordination site to iron is occupied by an exogenous nitrogen-donor ligand. Our


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goal was to obtain further insight into the effects of changing either axial heme iron ligation, or protein encapsulation, on the reduction thermodynamics of cytc. This work complements previous investigations carried out on similar systems, which focused essentially on the changes in $E^{\circ\prime}$.[28] In particular, we report the $\Delta H^{\circ\prime}_{rc}$ and $\Delta S^{\circ\prime}_{rc}$ values for four derivatives of cytc in which the axial methionine has been replaced by imidazole, 2-methyl-imidazole, ammonia, and azide. Also studied were the corresponding derivatives of MP11, including those with *N*-acetylmethionine, alanine, and phenyalanine ligands. The analysis of these data, and their comparison with those previously reported for the native and alkaline form of cytc,[23,30] allows the first quantitative evaluation and comparison of the enthalpic and entropic contribution of the polypeptide matrix to the reduction potential of class I cytochromes *c*. Moreover, it provides further insight into the modulation of the thermodynamics of protein reduction by the properties of the heme axial ligand.

These data indicate that stabilization of the reduced heme in cytc through axial S(Met) iron ligation and heme encapsulation into the polypeptide matrix is a result of purely enthalpic effects. Notably, entropic effects due to water exclusion from the environment of the heme tend to disfavor protein reduction and are exerted for the most part upon binding of the heme iron by the axial ligand. This work demonstrates that a detailed analysis of thermodynamic parameters contributing to $E^{\circ\prime}$ is crucial for understanding the influence of protein medium and axial ligation on the redox properties of metalloproteins.

## Experimental Section

**Materials.** Horse heart cytochrome *c* and microperoxidase-11 were purchased from Sigma. The former was further purified by cation exchange chromatography (SP-Sepharose HP). All chemicals were of reagent grade. Nanopure water was used throughout. The Met80-substituted adducts of oxidized cytc were prepared by adding increasing amounts of concentrated solutions of imidazole (Sigma), 2-methyl-imidazole (Fluka), sodium azide (Sigma), and ammonium sulfate (Carlo Erba) in phosphate buffer (0.1 M, pH 7) to a 0.3 mM cytc solution made up in the same buffer. The replacement of the methionine-80 as axial ferriheme ligand was monitored by UV−vis spectroscopy by following the disappearance of the sulfur to Fe(III) charge-transfer band at 695 nm.

The free amino groups of MP11 were acetylated as previously reported.[66,67] Acetylation of the N-terminus inhibits aggregation that would severely influence ligand binding and the attainment of a well-behaved electrochemistry. The adducts of *N*-acetyl-microperoxidase-11 (AcMP11) with *N*-acetylmethionine, imidazole, 2-methyl-imidazole, $NH_3$, L-alanine, and L-phenylalanine (Sigma) were prepared by adding increasing amounts of concentrated ligand solutions dissolved in 0.1 M phosphate buffer at pH 7 or pH 8 to a 0.02 mM AcMP11 solution made up in the same buffer (this concentration is the highest at which aggregation of AcMP11 due to stacking interactions does not occur).[28,66] Ligand binding to Fe(III) was monitored by UV−vis spectroscopy by following the changes in the Soret band, where a shift to longer wavelength is indicative of formation of a six-coordinate low-spin Fe(III).

**Electrochemical Measurements.** Square-wave voltammetry (SWV) experiments were carried out under argon with a potentiostat/galvanostat PAR model 273A, using a cell designed for small sample volume ($V = 0.5$ mL), a scan rate of 0.01 V $s^{-1}$, a frequency of 5 Hz, and a pulse amplitude of 0.025 V. A 2 mm diameter gold disk or an edge-cleaved pyrolitic graphite (PGE) disk were used as working electrodes, and a Pt sheet and a saturated calomel electrode (SCE) were used as counter and reference electrode, respectively. Electrical contact between the SCE and the working solution was obtained with a Vycor set. Potentials were calibrated against the $MV^{2+}/MV^{+}$ couple ($E^{\circ\prime} = -0.446$ vs SHE) (MV = methyl viologen). All reduction potentials reported in this paper are referenced to the standard hydrogen electrode (SHE). The best electrochemical response for the Met80-substituted derivatives of cytc was obtained with a 4-mercaptopyridine surface-modified gold electrode. A pyrolytic graphite electrode was used for studies of AcMP11 and its derivatives. The cleaning procedure of the working electrode is crucial to the voltammetric response. The gold electrode was first treated with anhydrous ethanol for 10 min, then polished with an alumina (BDH, particle size of about 0.015 $\mu$m) water slurry on cotton wool for 5 min, and finally the electrode was treated in an ultrasonic pool for about 10 min. Modification of the electrode surface was performed by dipping the polished electrode into a 1 mM solution of 4-mercaptopyridine for 30 s, then rinsing it with Nanopure water. The PGE electrode was subject to the same cleaning procedure in which the duration of each step was halved and its surface modified by dipping the polished electrode into a 1 mM solution of polylysine for 30 s, then rinsing it with Nanopure water. All measurements were made in 0.1 M phosphate buffer pH 7, except those on the glycine, phenylalanine, and ammonia derivatives of AcMP11 which were carried out at pH 8. The Met80-substituted cytc adducts and six-coordinate AcMP11-ligand derivatives were freshly prepared before use. Sample concentration was checked spectrophotometrically and varied from 0.1 to 0.3 mM and from 0.01 to 0.02 mM for cytc and AcMP11 derivatives, respectively. Equilibrium reduction potentials $E^{\circ\prime}$ were calculated from the peak potential Ep of the square-wave voltammograms using the Parry−Osteryoung equation.[68]

The temperature dependence of the reduction potential was determined with a "nonisothermal" cell[69] in which the reference electrode is kept at constant temperature (21 ± 0.1 °C), while the half-cell containing the working electrode and the Vycor junction to the reference electrode is under thermostatic control with a water bath. The temperature was varied from 5 to 50 °C. With this experimental configuration, the reaction entropy for reduction of the oxidized protein ($\Delta S^{\circ\prime}_{rc}$) is given by:[69,70]

$$\Delta S^{\circ\prime}_{rc} = S^{\circ\prime}_{red} - S^{\circ\prime}_{ox} = nF\,(dE^{\circ\prime}/dT) \qquad (1)$$

Thus, $\Delta S^{\circ\prime}_{rc}$ was determined from the slope of the plot of $E^{\circ\prime}$ versus temperature which turns out to be linear under the assumption that $\Delta S^{\circ\prime}_{rc}$ is constant over the limited temperature range investigated. With the same assumption, the enthalpy change ($\Delta H^{\circ\prime}_{rc}$) was obtained from the Gibbs−Helmholtz equation, as the negative slope of the $E^{\circ\prime}/T$ versus $1/T$ plot. The nonisothermal behavior of the cell was carefully checked by determining the $\Delta H^{\circ\prime}_{rc}$ and $\Delta S^{\circ\prime}_{rc}$ values of the ferricyanide/ferrocyanide couple.[69−71] For each species, the experiments were performed at least two times, and the reduction potentials were found to be reproducible within ±2 mV.

## Results

**Electrochemical Properties and Reduction Thermodynamics for Met80-Substituted Cytochrome *c* Adducts.** Consistent with previous reports,[21−23,30,71−73] native cytc yields a

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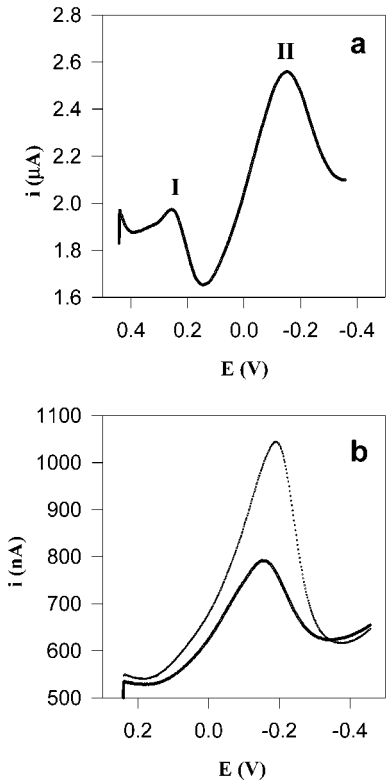


***Figure 1.*** Square-wave voltammograms of (a) 0.1 mM horse heart cytc in 0.1 M phosphate buffer, pH 7, in the presence of 0.1 M imidazole, obtained on a 4-mercaptopyridine surface-modified Au electrode (negative scan from +500 to −370 mV): wave I and wave II refer to free cytc and cyt-Im adduct, respectively. (b) 0.02 mM AcMP11 (black) and 0.02 mM AcMP11 in the presence of 2 mM imidazole (gray) obtained on a PGE-polylysine electrode at pH 7. $T = 25$ °C. Frequency, 5 Hz; pulse amplitude, 0.025 V. Potentials are versus SHE.

one-electron, reversible, and diffusion-controlled electrochemical wave (wave I) with an $E^{\circ\prime}$ value of +0.263 mV (vs SHE) at pH 7. Addition of the nitrogen-donor ligand to native cytc results in all cases in the observation of a new well-behaved electrochemical wave (wave II) at negative potentials, due to the cytc-ligand adduct, whose intensity increases with increasing concentration of added ligand (with no changes in $E^{\circ\prime}$) with concomitant loss of the wave from free cytc. For example, Figure 1a shows the results for Im ligated cytc obtained on a 4-mercaptopyridine surface-modified Au electrode at pH 7. These electrochemical properties unequivocally show that electron transfer (ET) between both forms of cytc and the Au electrode is fast and does not involve protein adsorption and/or denaturation on the electrode surface. That is, we are measuring the electrochemistry of the $Fe^{3+}/Fe^{2+}$ equilibrium of the heme iron in the native protein and in its Met80-substituted derivatives.

At pH 7, $E^{\circ\prime}$ values of −127, −145, −137, and −133 mV (vs SHE) were obtained for cytc adducts with $NH_3$, $N_3^-$, imidazole, and 2-methyl-imidazole, respectively (Table 1). The potential for the imidazole adduct was found to be higher than that obtained previously through spectroelectrochemistry (−178 mV).[44] This difference is most likely due to the fact that the present value has been determined using an Im/cytc molar ratio much lower than that in the previous study. Indeed, it is known that the $E^{\circ\prime}$ value of cytc decreases with increasing ionic strength.[32,44,74−76] We note that the present value is in good agreement with that of −148 mV obtained under comparable conditions by SWV for the same adduct of yeast iso-1 cytc.[77]

***Table 1.*** Thermodynamic Parameters for Reduction of Beef Heart Ferricytochrome *c* (cytc) and *N*-Acetyl-microperoxidase-11 (AcMP11), and Their Adducts with a Variety of Exogenous Axial Ligands[a]

| species | pH | $E^{\circ\prime}$ [b] (mV) | $\Delta S^{\circ\prime}_{rc}$ (J mol$^{-1}$ K$^{-1}$) | $\Delta H^{\circ\prime}_{rc}$ (kJ mol$^{-1}$) | $T\Delta S^{\circ}_{rc}/F'$[b,c] (mV) | $-\Delta H^{\circ}_{rc}/F'$[b,c] (mV) |
|---|---|---|---|---|---|---|
| native cytc[d] | 7 | +263 | −44 | −38 | −136 | +394 |
| alkaline cytc[d] | 8.3 | −75 | −50 | −9 | −154 | +93 |
| cytc-Im | 7 | −137 | −49 | −1 | −151 | +10 |
| cytc-2-$CH_3$-Im | 7 | −133 | −46 | −1 | −133 | +10 |
| cytc-$NH_3$ | 7 | −127 | −52 | −3 | −161 | +31 |
| cytc-$N_3^-$ | 7 | −145 | −61 | −4 | −188 | +41 |
| AcMP11 | 7 | −134 | +49 | +27 | +151 | −280 |
| AcMP11-AcMet | 7 | −67 | −29 | −2 | −89 | +21 |
| AcMP11-Im | 7 | −189 | −18 | +13 | −56 | −135 |
| AcMP11-2-$CH_3$-Im | 7 | −187 | −34 | +8 | −105 | −83 |
| AcMP11-$NH_3$ | 8 | −149 | +72 | +36 | +222 | −373 |
| AcMP11-Gly | 8 | −170 | −43 | +3 | −133 | −31 |
| AcMP11-Phe | 8 | −245 | −30 | +14 | −93 | −145 |

[a] All values obtained in 0.1 M phosphate buffer. Average errors on $\Delta H^{\circ\prime}_{rc}$ and $\Delta S^{\circ\prime}_{rc}$ values are ±2 kJ mol$^{-1}$ and ±6 J mol$^{-1}$ K$^{-1}$, respectively. [b] The reduction potentials are measured at 25 °C and are referred to SHE. [c] The sum $(-\Delta H^{\circ}_{rc}/F' + T\Delta S^{\circ}_{rc}/F')$ often does not exactly match $E^{\circ\prime}$ since the $\Delta H^{\circ\prime}_{rc}$ and $\Delta S^{\circ\prime}_{rc}$ values are rounded to the closest integer, as a result of experimental error. [d] From ref 23.

The temperature dependences of the $E^{\circ\prime}$ values and the $E^{\circ\prime}/T$ versus $1/T$ plots for these adducts at pH 7 are reported in Figure 2a and b, respectively. In all cases, the reduction potentials decrease linearly with increasing temperature from 4 to 60 °C. In parallel, the intensity of wave II (due to the complex) decreases, whereas that of wave I (due to native cytc) increases, indicating that complex formation is an exothermic process, consistent with previous reports.[47]

The thermodynamic parameters for protein reduction, determined from these nonisothermal experiments, are reported in Table 1. The $\Delta S^{\circ\prime}_{rc}$ values for the various adducts are negative and do not differ sensibly from that of native cytc, whereas the $\Delta H^{\circ\prime}_{rc}$ terms are much higher (less negative) than the value obtained for the native protein. This behavior closely parallels that observed for the alkaline forms of mitochondrial and bacterial class I cytochromes *c* (see Table 1).[23] With the same electrochemical approach, Feinberg et al. determined the thermodynamic parameters for the Phe82His variant of yeast iso-1 cytc, in which the imidazole group of the endogenous His82 coordinates to the ferriheme.[77] In this case, protein reduction results in restoration of native Met80 axial coordination to the $Fe^{2+}$ ion. Consequently, the overall reaction includes a ligand rearrangement, and so these thermodynamic data are not directly comparable to those presented here.

**Electrochemical Properties and Reduction Thermodynamics for AcMP11 and Its Adducts.** To avoid aggregation of microperoxidase as a result of either intermolecular ligation of the N-terminus to Fe(III) or $\pi-\pi$ stacking of the heme groups, all electrochemical measurements were performed on

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(76) It is interesting to point out that the effect of ionic strength on the $\Delta E^{\circ\prime}$ values between cytc and its derivatives with neutral ligands is small, since the slope of the $E^{\circ\prime}$ versus $f$(I) plot for the two species, which share the same charge, is the same.
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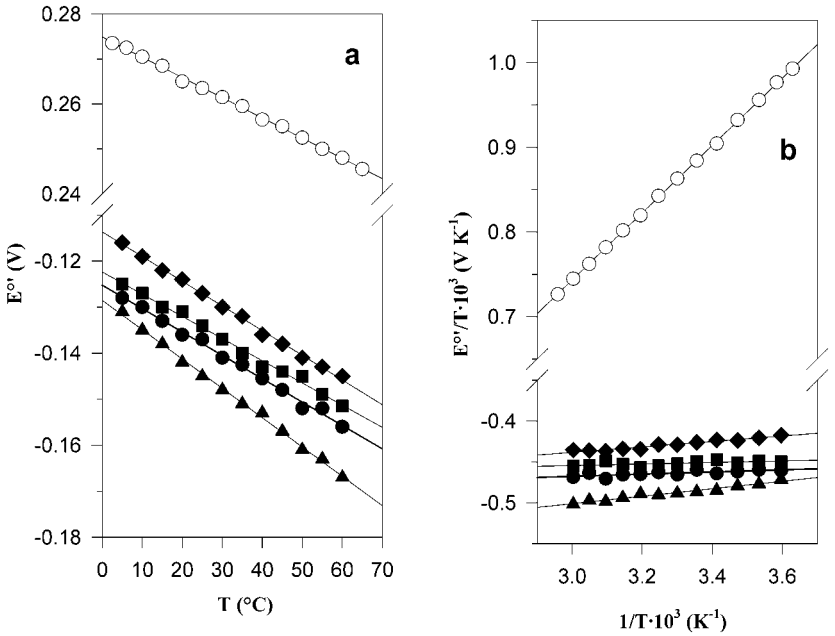


***Figure 2.*** (a) $E^{\circ\prime}$ vs $T$ plots for Met80-substituted cytc adducts. (b) $E^{\circ\prime}/T$ vs $1/T$ plots. (◆) $NH_3$; (▲) $N_3^-$; (●) Im; (■) 2-methyl-Im; (○) free cytc (from ref 23). Solid lines are least-squares fits to the data points.

*N*-acetyl-MP11 (AcMP11) in which both the free amino group of Val11 (N-terminal) and that of the side chain of Lys13 had been acetylated.[66,67] Both forms of AcMP11, either free or axially bound with the exogenous ligand, yield a reversible and diffusion-controlled electrochemical response. In particular, ligand addition to free AcMP11 induces a shift in the peak potential. The potential of the adduct is taken at ligand/microperoxidase molar ratios that ensure whole complex formation,[52] under conditions where further addition of ligand caused no significant change in the peak potential (Figure 1b). The reduction potential of AcMP11 ($E^{\circ\prime} = -134 \pm 2$ mV vs SHE) differs to some extent from those determined previously for the nonacetylated form by either cyclic voltammetry on a glassy carbon electrode ($-160 \pm 8$ mV),[78] or spectroelectrochemistry on gold-plated reticulated vitreous carbon electrode.[79] The modest difference may be attributed to aggregation effects (as noted elsewhere)[51] and to the different ionic composition of the solution, which in the latter case was made up in 0.1 M perchlorate and 0.02 M phosphate buffer. We note that perchlorate is known to be a chaotropic agent capable of altering the intramolecular H-bonding network, and is also a potential heme iron ligand.[80] In the present case, the electrochemical behavior of AcMP11 was found to be independent of sample concentration, indicating that no effects of microperoxidase aggregation are observed.

At pH 7, $E^{\circ\prime}$ values of $-67$, $-189$, $-187$,$-149$, $-170$, and $-245$ mV (vs SHE) were obtained for the AcMP11 adducts with *N*-acetylmethionine, imidazole, 2-methyl-imidazole, $NH_3$, glycine, and phenylalanine, respectively (Table 1). The $E^{\circ\prime}$ value for the imidazole adduct is identical to that ($-190.5$ mV) determined previously through spectroelectrochemistry on bare and gold-plated reticulated vitreous carbon electrodes.[79,81] The $E^{\circ\prime}$ versus $T$ and the $E^{\circ\prime}/T$ versus $1/T$ plots for these adducts at pH 7 are shown in Figure 3a and b, respectively, and the resulting thermodynamic parameters are listed in Table 1. The $\Delta H^{\circ\prime}_{rc}$ and $\Delta S^{\circ\prime}_{rc}$ values for free AcMP11 are both positive. Lower reduction enthalpy and entropy values (i.e., less positive or even negative) are observed for all the various AcMP11 adducts, with the exception of the $NH_3$ adduct, which shows the opposite behavior.

## Discussion

**Effects of Exogenous Axial Ligand Binding on Cytochrome *c*.** Protein reduction in class I cytochromes *c* is invariably accompanied by negative enthalpy and entropy changes.[23,30,32,77] The former effect is mainly related to the stabilization of the $Fe^{2+}$ state by ligand binding interactions (particularly due to the thioether S ligation of the axial methionine), to the hydrophobic environment of the heme, and to the limited accessibility of the heme to the solvent. The entropy loss following reduction is most likely determined by differences in solvation properties between the two redox states,[23,30,31,82,83] which Bertrand et al. have suggested to be mostly localized at the heme propionates, with the degree of solvent accessibility apparently serving as a key effector of $\Delta S^{\circ\prime}_{rc}$ in *c*-type cytochromes.[28] However, a contribution to the negative $\Delta S^{\circ\prime}_{rc}$ values from other oxidation state dependent changes in the hydration sphere of the protein, also possibly related to the greater conformational flexibility of the oxidized relative to the reduced form,[84−88] cannot be excluded.

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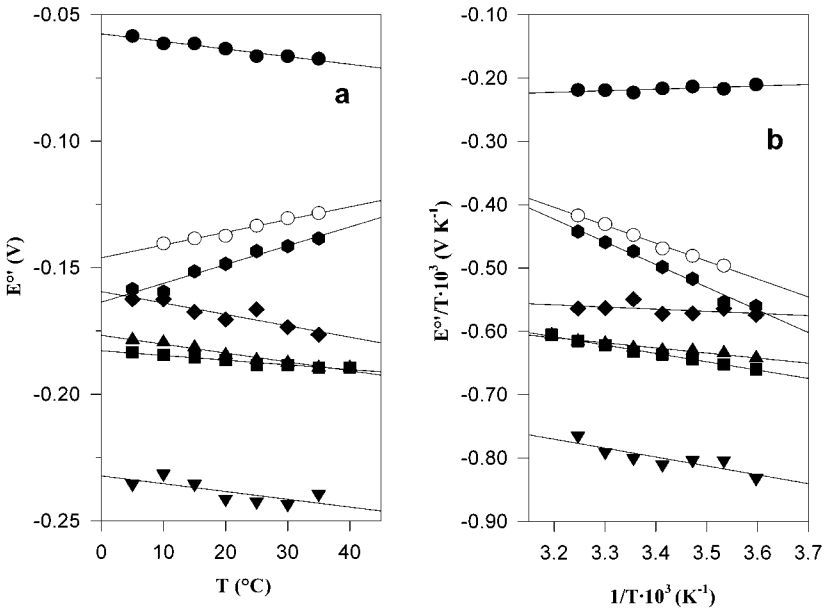


***Figure 3.*** (a) $E^{\circ\prime}$ vs $T$ plots for AcMP11 adducts with different ligands (L). (b) $E^{\circ\prime}/T$ vs $1/T$ plots. (●) L = *N*-acetylmethionine; (hexagon) L = $NH_3$; (■) L = Im; (▲) L = 2-methyl-Im; (◆) L = Gly; (▼) L = Phe; (○) free MP-11. Solid lines are least-squares fits to the data points.

The $E^{\circ\prime}$ and the $\Delta S^{\circ\prime}_{rc}$ and $\Delta H^{\circ\prime}_{rc}$ values of the Met80-substituted adducts are all negative and fully comparable (Table 1), indicating that the thermodynamics of protein reduction are only slightly affected by the differences among the nitrogenous ligands. The most notable difference with the reduction thermodynamics of native cytc is a significant increase in reduction enthalpy. The reduction entropy appears to be scarcely affected by ligand substitution (except for azide binding, where it exerts a barely appreciable contribution to the $E^{\circ\prime}$ change). In particular, the average enthalpic and entropic contributions to $E^{\circ\prime}$ for the adducts obtained at pH 7 and 25 °C are $-\Delta H^{\circ\prime}_{rc}/F = +23$ mV and $T\Delta S^{\circ\prime}_{rc}/F = -160$ mV, relative to values of +394 and −136 mV, respectively, for the native form. This clearly indicates that the remarkable lowering of the $E^{\circ\prime}$ of the Met80-substituted cytc derivatives, relative to native cytc (average $\Delta E^{\circ\prime} = E^{\circ\prime}_{adduct} - E^{\circ\prime}_{cytc} = -399$ mV), is mainly enthalpic in origin and stems from the enhanced stabilization of the ferriheme by the nitrogenous ligands, which are much stronger electron donors, relative to the thioether sulfur of the axial methionine. Accordingly, imidazole and azide have been found to bind much more strongly to oxidized than to reduced cytc.[49] The differences in reduction thermodynamics for the adduct complexes versus native cytc are reported in Table 2. An analogous effect has been observed for the alkaline form of class I cytochromes *c* (Tables 1 and 2) and attributed to the presence of an $\epsilon$-amino group of a lysine axially bound to the heme Fe(III).[23,89] However, it is noteworthy that the average enthalpic stabilization of the oxidized form in the Met80-substituted adducts, relative to native cytc [$-\Delta\Delta H^{\circ\prime}_{rc}/F = -(\Delta H^{\circ\prime}_{rc(N-adduct)} - \Delta H^{\circ\prime}_{rc(cytc)})/F = -370$V], is significantly greater than that observed for the alkaline form ($-\Delta\Delta H^{\circ\prime}_{rc}/F = -[\Delta H^{\circ\prime}_{rc(alkcytc)} - \Delta H^{\circ\prime}_{rc(cytc)}]/F = -300$ mV) (Table 2). This difference is somewhat surprising given the low sensitivity of the redox thermodynamics of the Met80-substituted adducts to the nature of the nitrogenous axial ligands noted above. One determinant of this difference may be related to the widening of the distal site of the heme needed to accommodate the exogenous ligand, as clearly shown by the recent solution NMR structure of the imidazole complex of horse heart cytc[90] and by previous NMR studies,[45] which may result in an increased interaction of the heme with internal water molecules relative to the alkaline conformer. The recently solved X-ray structure of the $NH_3$-adduct of *Rps. palustris* cyt $c_2$[91] shows indeed the presence of eight buried water molecules in

***Table 2.*** Changes in the Thermodynamic Parameters for Reduction of Beef Heart Ferricytochrome *c* (cytc) and *N*-Acetyl-microperoxidase-11 (AcMP11) Following Binding of Exogenous Ligands[a]

| species | $\Delta E^{\circ\prime}$ [b] (mV) | $T\Delta\Delta S^{\circ}_{rc}/F'$ [b,c] (mV) | $-\Delta\Delta H^{\circ}_{rc}/F'$ [c] (mV) |
|---|---|---|---|
| (cytc-Im)−($cytc_{(N)}$) | −400 | −15 | −383 |
| (cytc-2-$CH_3$-Im)−($cytc_{(N)}$) | −396 | −3 | −383 |
| (cytc-$NH_3$)−($cytc_{(N)}$) | −390 | −25 | −363 |
| (cytc-$N_3^-$)−($cytc_{(N)}$) | −408 | −52 | −352 |
| ($cytc_{(A)}$)−($cytc_{(N)}$) | −338 | −18 | −300 |
| (AcMP11-AcMet)−(AcMP11) | +67 | −240 | +301 |
| (AcMP11-Im)−(AcMP11) | −55 | −207 | +145 |
| (AcMP11-2-$CH_3$-Im)−(AcMP11) | −53 | −256 | +197 |
| (AcMP11-$NH_3$)−(AcMP11) | −15 | +71 | −93 |
| (AcMP11-Gly)−(AcMP11) | −37 | −284 | +249 |
| (AcMP11-Phe)−(AcMP11) | −112 | −244 | +135 |

[a] Reported here are the differences in parameters for reduction of the adducts and the free species. The terms $cytc_{(N)}$ and $cytc_{(A)}$ stand for native and alkaline cytc, respectively. The sum ($-\Delta\Delta H^{\circ}_{rc}/F' + T\Delta\Delta S^{\circ}_{rc}/F'$) often does not exactly match $\Delta E^{\circ\prime}$ since the $\Delta H^{\circ\prime}_{rc}$ and $\Delta S^{\circ\prime}_{rc}$ values are rounded to the closest integer, as a result of experimental error. [b] At 25 °C. [c] At 25 °C, the magnitude of the factor $T = 298$ K means that the error associated with the parameter $T\Delta S^{\circ}_{rc}/F'$ is about ±20 mV. The error associated with $\Delta H^{\circ}_{rc}/F'$ is comparable. Thus, they are both much larger than the experimental error in estimating $E^{\circ\prime}$ (±2 mV). Hence, in the present work, only differences in enthalpic and entropic terms which are greater than 40 mV are taken to be significant.

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the adduct, versus three in the native protein. Of the five additional water molecules, three lie in the space cleared by Met 93, and two are close to a heme propionate. The distinct conditions of pH and ionic strength under which the thermodynamic parameters were determined for the adducts described here, relative to conditions for the alkaline form,[23] may also contribute to the above difference.

The substantial invariance of the reduction entropy for the cytc adducts, relative to the native cytc conformer (Tables 1 and 2), indicates that ligand replacement at the sixth (axial) coordination position does not influence oxidation-state induced effects, such as changes in solvation properties (possibly at the heme propionates)[30] or the conformational flexibility in the vicinity of the heme. Consistently, the NMR data and the structural information available on cytc adducts with Im, $NH_3$, and $N_3^-$ show that the structural changes arising from ligand binding are highly localized in the distal site containing the replaced methionine ligand. In particular, the conformational changes observed in the imidazole adduct of horse heart cytc and *Rb. Sphaeroides* cyt $c_2$[90,92] turn out to involve 5−6 residues adjacent to the Met ligand, which in the eukaryotic protein show an increase in flexibility and move away from the heme. In the cyt $c_2$-$NH_3$ adduct, the changes are more localized and involve only the replaced Met and two adjacent residues.[91] While the latter species shows an extensive reorganization of H-bonding, the total strength of the H-bonding network in the heme environment appears to be roughly conserved. The small, yet significant difference in reduction entropy induced by azide binding cannot be explained unambiguously, since the NMR studies of the cyc-$N_3^-$ derivative[93] show the occurrence of conformational changes localized at the Met80 site that appear to be comparable to those induced by imidazole binding.[90] The negative charge of azide most likely plays a role in this effect.

**Effects of Exogenous Axial Ligand Binding on Microperoxidase-11.** The negative reduction potential of AcMP11 has a totally enthalpic origin (Table 1), and may be attributed to the stabilization of the oxidized over the reduced state by axial water coordination, and to exposure of the heme to solvent. The entropy change instead favors reduction. The positive $\Delta S_{rc}^{\circ\prime}$ value most likely originates from the decrease in the positive charge of AcMP11 following reduction (from +1 to 0), which would decrease the ordering of water molecules in proximity to the heme relative to that realized in the oxidized state. Such solvent structuring effects provide the most widely accepted explanation for the positive reduction entropies shown by $M^{3+}/M^{2+}$ aquo couples and their complexes with simple monodentate ligands and planar macrocyclic systems in aqueous solution.[69,94−96] Accordingly, the reduction entropy of hemin chloride in mixed water/*N*,*N*′-dimethylformamide (DMF) and water/*N*,*N*′-dimethylacetamide (DMA) solutions, determined through potentiometric titrations, were found to increase with increasing water content.[97] Water indeed replaces DMF and DMA in axial heme iron coordination.[97,98] The thermodynamics of $M^{3+}/M^{2+}$ redox reactions in M-TPP complexes (M = Co, Fe) in nonaqueous coordinating solvents (DMSO, pyridine, DMF, butyronitrile) are dominated by solvent binding/dissociation at the axial positions of the metal.[99] It is interesting to note that, analogous to previous observations for all cytochromes *c* and for most electron transport proteins, the enthalpy and entropy terms of the free energy change of the reduction reaction lead to opposing contributions to $E^{\circ\prime}$. In particular, for AcMP11, both $\Delta H_{rc}^{\circ\prime}$ and $\Delta S_{rc}^{\circ\prime}$ have an opposite sign as compared to cytc, but again the entropic term is smaller than the enthalpic one.

With the exception of $NH_3$, substitution of the axial water molecule by the nitrogen-donor ligands results in a decrease in reduction enthalpy, which indicates stabilization of the ferrous state. This effect is paralleled by a compensative decrease in reduction entropy, which instead favors the oxidized over the reduced state (Tables 1 and 2). The enthalpy effect can be at least qualitatively explained by (i) the more or less pronounced ability of the exogenous ligands to accept back-donation from the $Fe^{2+}$ to their $\pi$-orbital system, which would stabilize the ferrous state to a greater extent as compared to the presence of water as axial ligand, and (ii) the at least partial exclusion of solvent from the heme environment (i.e., an increase in hydrophobicity) due to the presence of the axial ligand. It is not surprising that the greatest decrease in reduction enthalpy is indeed observed for *N*-acetylmethionine (AcMet) binding, due to the strong $\pi$-acceptor character of the thioether sulfur and the bulkiness of its side chain. Imidazole, 2-methyl-imidazole, and the amino group of glycine and phenylalanine bind more strongly to the ferric heme than does water,[52] and their $\pi$-acceptor character is much lower than that of the thioether sulfur of AcMet. Therefore, in this case the effect of water exclusion from the immediate environment of the heme should play an important role in the decrease in $\Delta H_{rc}^{\circ\prime}$. On the contrary, ammonia binding should not appreciably alter the hydration sphere of the heme; thus the modest increase in reduction enthalpy observed by $NH_3$ binding is likely to be related to the greater affinity of ammonia for the ferric heme iron as compared to water.[52] Because the affinity constants for the various ligands toward AcMPs are available almost only for the ferric form, no correlations can be considered for the change of $E^{\circ\prime}$, relative to the selective stabilization of one of the two redox states of the heme iron as a result of axial ligand binding.

Regarding the general decrease in reduction entropy on ligand binding (except for ammonia), we note that the bulkier the axial ligand is, the more pronounced the decrease in reduction entropy; that is, the system more closely resembles native cytc. Hence, this effect turns out to be mainly linked to the exclusion of water molecules from the heme environment. Axial heme ligation would indeed at least partially suppress the electrostatic mechanism that leads to a decreased ordering of the water molecules in the surrounding of the heme on metal reduction. This is in agreement with the decrease in $\Delta S_{rc}^{\circ\prime}$ observed for $M^{3+}/M^{2+}$ aquo couples following substitution of coordinated water by large organic ligands, and ascribed to the increased shielding of the metal cation from the surrounding water.[69,96]

Overall, it appears that axial heme ligation determines the change in reduction potential in a manner that is related both

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to the $\sigma$-donor/$\pi$-acceptor properties of the ligand (affecting the enthalpic term) and to the effect of water exclusion, which exerts a dual enthalpic and entropic role, that results in an increase or decrease in the reduction potential, respectively. As usual, changes in the hydration sphere of the molecule resulting from ligand binding effects give rise to enthalpy−entropy compensation phenomena.[100−102]

In the case of AcMet, the enthalpic effect of axial ligation prevails over the entropic contribution. This results in a 67 mV increase in $E^{\circ\prime}$. This increase in $E^{\circ\prime}$ is lower than that measured previously (+110 mV) for AcMet ligation to AcMP8, a microperoxidase lacking residues 11−13.[28] This difference cannot be explained unambiguously with the data at hand. Of course, it cannot be due to a difference in coordination effects. We note that the charge of the MP8 complex changes from 0 to −1 following reduction; hence, the sign of the reduction entropy (at least for the part due to the solvation effects) should revert, relative to that described above. As a consequence, the effect of methionine binding on the reduction entropy is likely to be different from that exerted on AcMP11. Moreover, this difference in $\Delta E^{\circ\prime}$ may also be attributed to the different ionic composition of the medium, which for AcMP8 contained 0.1 M perchlorate (see discussion above).

For all the adducts with nitrogenous ligands (with the exception of ammonia), the entropic effects prevail over the enthalpic ones. As a consequence, exogenous ligand binding results in a decrease in $E^{\circ\prime}$. In particular, the lowering of the $E^{\circ\prime}$ value of AcMP11 by 55 mV following imidazole ligation is in reasonable agreement with the previous observed 40 mV decrease in $E^{\circ\prime}$.[28,78] Again, the (small) difference may be attributed either to the different entropic effects of ligation and/or to the difference in ionic composition used.

**Comparison between the Reduction Thermodynamics of Cytochrome *c* and AcMP11 Adducts: Influence of the Polypeptide Matrix on the Reduction Thermodynamics of cytc.** The increase in reduction potential of the heme due to methionine coordination (measured above for AcMP11), with the concomitant changes in enthalpy and entropy resulting from both ligand substitution and water exclusion from the heme, provides an estimate of the contribution of axial Met ligation in cytc to the increase in reduction potential relative to the unfolded (bis)imidazole-ligated cytc. We found an increase in $E^{\circ\prime}$ of the order of (55 + 67 =) 122 mV (Table 2), which compares reasonably well with the value of (110 + 40 =) 150 mV determined electrochemically for MP8,[28] and with the value of 168 mV determined for synthetic heme models.[103,104] This increase in potential turns out to be, as would be expected, a mostly enthalpic effect that results from the differences in ligand binding properties between the two ligands. We note in passing that the potential difference between native cytc and its imidazole adduct is much higher (+400 mV) than the above value (Table 2), indicating that the protein environment exerts an important contribution. This can be easily explained by the fact that in the case of the cytc-Im adduct, the heme crevice is almost intact; hence, the hydrophobicity of the environment strengthens the $Fe^{3+}$−Im bond relative to that in the Im-AcMP11 adduct, where the ligand is surrounded by an aqueous environment.

***Table 3.*** Variations in Thermodynamic Parameters for Reduction in cytc and Various Met80-Substituted Adducts Resulting from the Polypeptide Matrix[a]

| species | $\Delta E^{\circ\prime}$ [b] (mV) | $T\Delta\Delta S^{\circ}_{rc}/F'$ [b,c] (mV) | $-\Delta\Delta H^{\circ}_{rc}/F'$ [c] (mV) |
|---|---|---|---|
| $(cytc_{(N)})-(AcMP11)$ | +397 | −287 | +674 |
| $(cytc_{(N)})-(AcMP11\text{-}AcMet)$ | +329 | −47 | +373 |
| (cytc-Im)−(AcMP11-Im) | +51 | −96 | +145 |
| (cytc-2-$CH_3$-Im)−(AcMP11-2-$CH_3$-Im) | +53 | −37 | +93 |
| (cytc-$NH_3$)−(AcMP11-$NH_3$) | +21 | −383 | +404 |

[a] The sum $(-\Delta\Delta H^{\circ}_{rc}/F' + T\Delta\Delta S^{\circ}_{rc}/F')$ often does not exactly match $\Delta E^{\circ\prime}$ since the $\Delta H^{\circ\prime}_{rc}$ and $\Delta S^{\circ\prime}_{rc}$ values are rounded to the closest integer, as a result of experimental error. [b] At 25 °C. [c] At 25 °C, the magnitude of the factor $T$ = 298 K means that the error associated with the parameter $T\Delta S^{\circ}_{rc}/F'$ is about ±20 mV. The error associated with $\Delta H^{\circ}_{rc}/F'$ is comparable. Thus they are both much larger than the experimental error in estimating $E^{\circ\prime}$ (±2 mV). Hence, in the present work, only differences in enthalpic and entropic terms which are greater than 40 mV are taken to be significant.

The effect of protein encapsulation of the heme on the reduction thermodynamics of native cytc can be estimated from a comparison of the data for the intact protein relative to those for the AcMP11-AcMet adduct (Table 3). Apparently the presence of the hydrophobic environment around the heme, provided by the polypeptide chain, increases the potential by approximately 330 mV[105] as a result of an almost totally enthalpic effect. The entropic effect is in the opposite direction, but is much smaller and at the limit of significance due to the experimental error (see footnote c of Tables 2 and 3). It is interesting to compare these data with the difference in parameters obtained for native cytc and AcMP11, which provide the overall change due to axial Met ligation and protein encapsulation (Table 3). It is apparent that most of the entropy-driven decrease in $E^{\circ\prime}$ (by 287 mV), arising from water exclusion from the heme environment on passing from AcMP11 to cytc (Table 3), arises at the stage of axial methionine binding. Encapsulation of the heme with the rest of the polypeptide chain exerts an almost totally enthalpic effect that results in an increase of $E^{\circ\prime}$ by 373 mV, which is comparable to that exerted by axial Met ligation (301 mV). A*ccordingly, since imidazole ligation to AcMP11 exerts a similar entropy-driven decrease in E°′, as a result of the loss of exposure of the heme to the solvent, these data suggest that most water is already excluded from the heme environment during folding of cytochrome c following binding of the second His ligand (His26 or His33)*[54] *to the heme iron, the His being substituted in the last stages of folding by Met80.* Scheme 1 illustrates the changes in $E^{\circ\prime}$ (and their enthalpic and entropic components) following a change in axial heme ligation of MP-11 and its insertion in the folded polypeptide chain of cytc.

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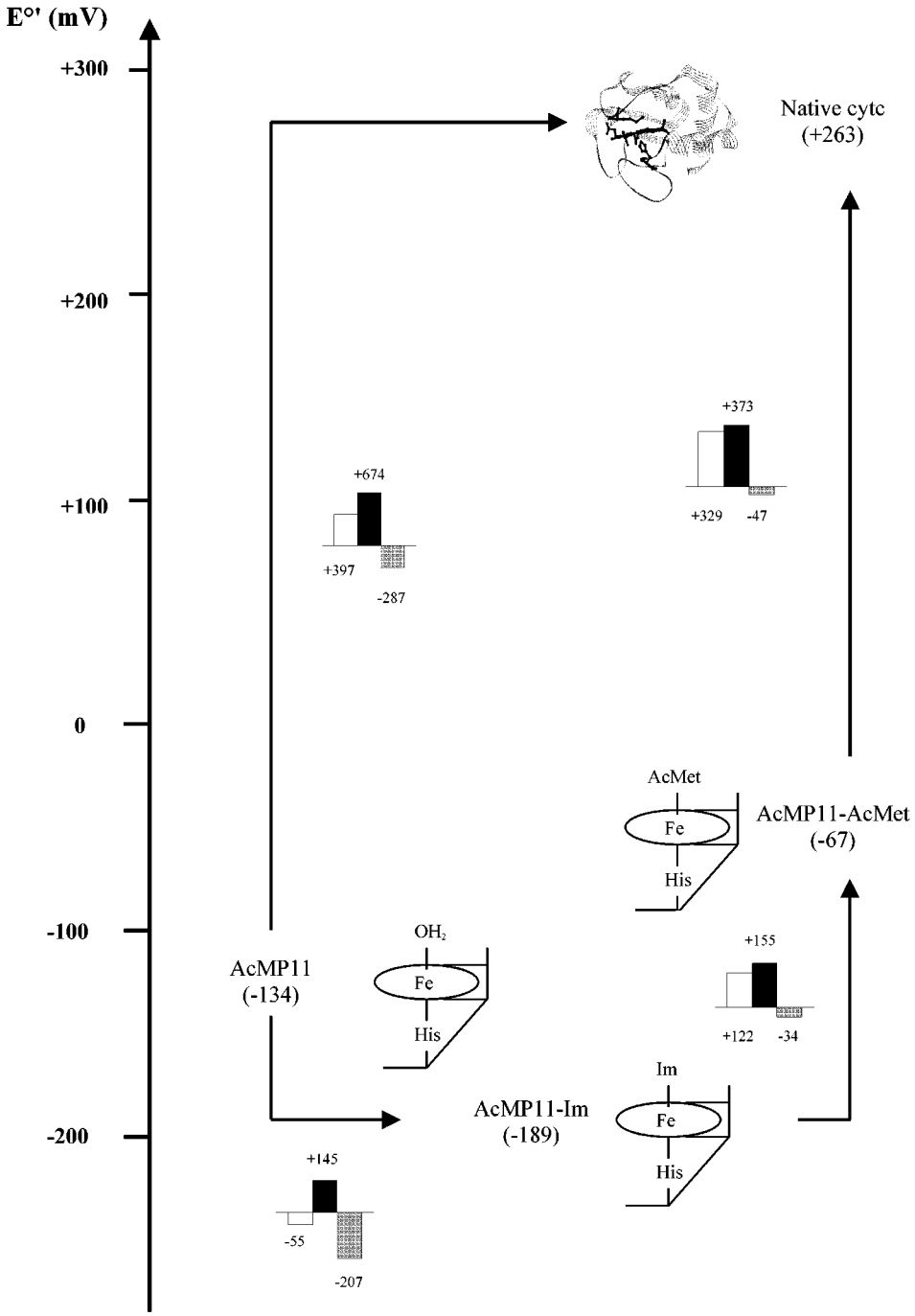
***Scheme 1.*** Cycle Connecting AcMP11 to Native cytc Showing the Changes in the Redox Potential ($\Delta E^{\circ\prime}$, White Column) and Its Enthalpic ($-\Delta\Delta H^{\circ\prime}/F$, Black Column) and Entropic ($T\Delta\Delta S^{\circ\prime}/F$, Gray Column) Components (see Table 3) Arising from Changes in Axial Ligation of AcMP11 Corresponding to Those Occurring during the Folding Process of cytc, and from Protein Encapsulation of the His,Met-Ligated Heme




A similar comparison can be extended to the cytc-ligand complexes relative to the corresponding AcMP11 adducts. The behavior is the same as described above, confirming that protein encapsulation causes an exquisitely enthalpy-based increase in $E^{\circ\prime}$. The only case in which the entropic effect of water exclusion is relevant at this stage is for the ammonia adduct.

It is interesting to note that the enthalpy-based increase in $E^{\circ\prime}$ by 373 mV for the heme due to protein encapsulation in native cytc compares very well with the $\Delta E^{\circ\prime}$ value of +380 mV obtained from the change in electrostatic solvation energy of the heme (with its axial ligands). The latter has been calculated with the linear response approximation, using the solution NMR structures after taking heme from the water to the protein environment.[106] Thus, the difference between the calculated and the experimental $\Delta E^{\circ\prime}$ values appears to be related to the entropic effects of water reorganization that the model does not consider explicitly. We may conclude that these entropic effects, although being much smaller than the electrostatic interaction energies, are likely to be among the factors responsible for the imperfect match often observed between experimental $E^{\circ\prime}$ values and those calculated using purely electrostatic models.

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## Conclusions

In summary, the present study shows that replacement of the native Met80 by exogenous N-donor ligands in cytochrome *c* results in an enthalpy-based decrease in $E^{\circ\prime}$ due to the ligand-induced stabilization of the oxidized state, with no substantial entropic effects. At variance, significant entropic contributions to the $E^{\circ\prime}$ change are observed upon binding of the same ligands (plus *N*-acetylmethionine and some amino acids) to microperoxidase-11, which appear to be related to (at least) partial water exclusion from the heme environment. Comparison of the data for the adducts of cytc and microperoxidase-11 provides information on the enthalpic and entropic components of the $E^{\circ\prime}$ changes associated with some of the main events of cytochrome *c* folding. In particular, it appears that the remarkably higher $E^{\circ\prime}$ value of cytochrome *c*, relative to microperoxidase-11, is totally enthalpic in origin and arises from the effects of axial ligation and protein encapsulation of the heme. The (smaller) entropic term, which provides a negative contribution to $E^{\circ\prime}$, is apparently related to water exclusion from the heme environment at the early stage of bis-imidazole heme coordination.

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