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Evaluation of Heme Peripheral Group Interactions in Extremely Low-Dielectric Constant Media and Their Contributions to the Heme Reduction Potential.

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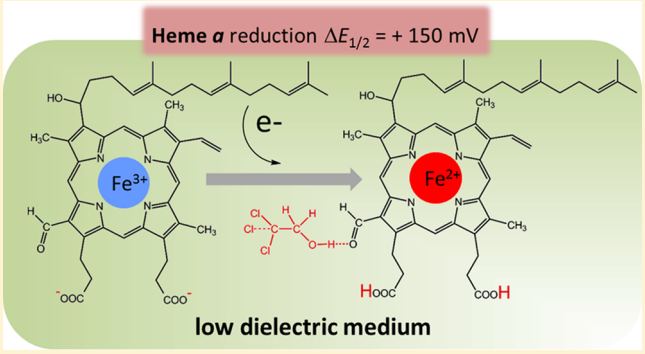
Supporting Information

ABSTRACT: In this study, we measured the contributions of the ionization of the heme propionates to the reduction potentials of heme *b* and heme *a* (bis)*N*-methylimidazole complexes in various low-dielectric constant conditions. Additionally, we measured the effects of H-bond to the heme *a* formyl group on the reduction potential of the heme. The performed electrochemical measurements show that ionization of the heme propionates lead to the largest redox change in dichloromethane with no electrolyte. The measured reduction potential changes for heme *b* and heme *a* were −55 and −47 mV (±10 mV) per ionized propionate, respectively. For heme *a*, the study demonstrates how the dielectric constant of the medium is important in the magnification of the ΔpK_a upon redox-linked ionization of the heme propionates and their roles in the proton pump of cytochrome *c* oxidase.



INTRODUCTION

Heme proteins perform a wide range of key biological functions. Remarkably, these proteins have the ability to utilize the same cofactor for various activities. For example, heme *b* (shown in Figure 1) serves in such roles as oxygen storage and transport, electron transfer, oxygenase, catalase, peroxidase, and gas sensing. A subject of continuing interest is the impact of specific protein interactions with the heme peripheral groups (vinyl, propionate, formyl, and farnesyl groups on heme *a*) and their effects on the function of a particular heme protein. These interactions are important in ligand discrimination and signaling transduction in heme-based sensors.[1−3] They are important in controlling the stability of the heme in the protein, in the modulations of the heme reduction potentials and electron transfer (ET),[4,5] and in the involvement of proton pumping in cytochrome *c* oxidase (CcO).[6,7]

The functioning of the proton pump in CcO is a source of controversy because of its complexity; CcO contains two hemes, heme *a* and heme a_3 (the oxygen-binding heme). Recently, it has been suggested that the heme *a* peripheral groups are involved in the proton pumping mechanism.[8] Also, several studies have shown the involvement of the heme *a* peripheral groups in heme−protein interactions.[9,10] In order to understand the role of heme peripheral group interactions in the stability of the heme, designed proteins have been used to study the affinity between hemes and proteins.[11,12] However, quantification of a redox-linked interaction between the protein and a specific heme peripheral group is difficult because the protein itself is involved in various interactions (hydrogen bonding, electrostatic attraction/repulsion, $\pi-\pi$ stacking, and hydrophobic stabilization) with the heme. In order to study a particular interaction, a model heme must be devoid of the other interactions. Quantification can be performed on a heme model compound in an aprotic solvent, such as dichloromethane (CH_2Cl_2) or benzene, with a ligand that can singularly interact with one of its peripheral groups. The low-dielectric constants (ϵ_r) of these solvents, 8.9 and 2.3 for CH_2Cl_2 and benzene, respectively, make them ideal media in which to study hemes because the interior of proteins can have dielectric constant values as low as 2.5.[13,14]

The properties of these solvents facilitate the evaluation of hydrogen bonding and electrostatic interactions between ligands and the peripheral groups of hemes. Small heme-ligand interactions, perhaps too small to be measured in commonly used solvents, can be magnified in benzene and dichloromethane.[15] Using this approach, we carried out an electrochemical study on the effects of the ionization of the heme propionates (propionic acids) on the reduction potentials of heme *b* and heme *a* in low-dielectric constant media. We have also measured the electrochemical contribution of the H-bond to the formyl group on the reduction potential of heme *a*.

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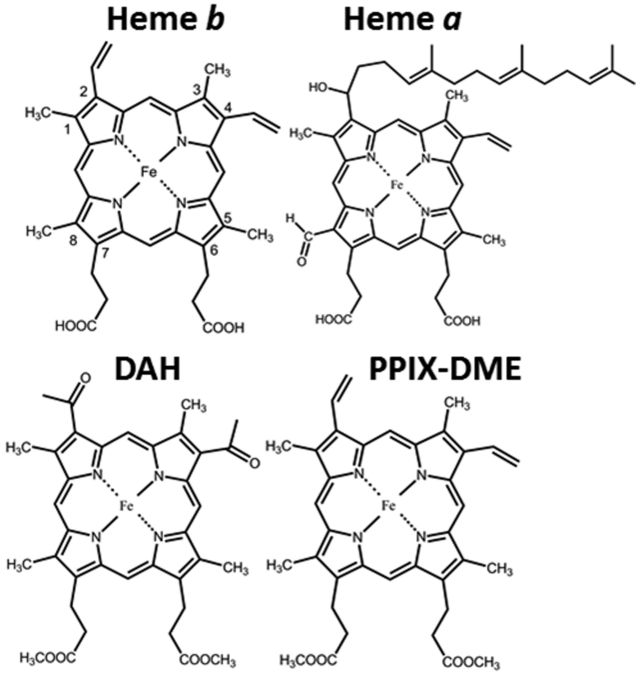

Figure 1. Heme model compounds. Heme *a* and heme *b* are naturally existing hemes, whereas (Fe^{3+}) 2,4-diacetyl deuteroporphyrin IX dimethyl ester (DAH) and (Fe^{3+}) protoporphyrin IX dimethyl ester (PPIX-DME) are artificial hemes used in this study. These hemes were studied as (bis)*N*-methylimidazole complexes.

RESULTS AND DISCUSSION

Cyclic Voltammetry in CH_2Cl_2 and Benzene with Electrolyte. Table 1 displays the ferric-to-ferrous heme reduction potentials of the (bis)*N*-methylimidazole complexes for the hemes in Figure 1. The reduction potentials were determined by using cyclic voltammetry (CV) with a 1.6 mm diameter glassy carbon disk electrode and with a 10 μm diameter glassy carbon ultramicroelectrode (UME) for low-current voltammograms. The use of UME permits the measurement of the reduction potential of an electroactive solute in nonpolar solvents, even without the presence of an electrolyte.[16,17] The *iR* distortion due to the highly resistive solvent is minimized with the use of a 10 μm diameter disk electrode, allowing accurate electrochemical measurements of the redox species. Additional experimental details and voltammograms are available as Supporting Information. The heme reduction potentials ($E_{1/2}$) in CH_2Cl_2 with tetrabutylammonium hexafluorophosphate (TBAHFP) as the electrolyte were calculated from the average values between the cathodic and anodic peak potentials, in millivolts vs ferrocene, from the measurements with the 1.6 mm diameter disk electrode. Addition of excess 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), which has been shown to cause deprotonation of the heme propionates (propionic acids)[18] in acetonitrile, also deprotonates the heme propionates in CH_2Cl_2 and benzene (Supporting Information). The addition of excess DBU produces a reduction potential change ($\Delta E_{1/2}$) of heme *b* and heme *a* of −19 mV in the presence of 1 M dimethyl sulfoxide (DMSO). On the other hand, DBU addition to iron(III) 2,4-diacetyl deuteroporphyrin IX dimethyl ester (DAH) and PPIX-DME, which instead have methyl propionic esters, has negligible effects ($\Delta E_{1/2}$ are less than ±5 mV) on the reduction potentials of these hemes. In the absence of DMSO, DBU addition to heme *b* causes a further 10 mV decrease in $E_{1/2}$, which results in a $\Delta E_{1/2}$ of −29 mV. This shows that removal of the DMSO, which decreases the polarity of the solution, increases the electrostatic effects of the ionization of the propionates on the electrochemical properties of heme *b*.

Table 1. Heme Reduction Potentials ($E_{1/2}$) in the Presence of DBU

(bis)*N*-methylimidazole heme compound[a]	$E_{1/2}$ (mV vs ferrocene), no DBU	$\Delta E_{1/2}$ (mV) with DBU
CH_2Cl_2/DMSO/0.25 M TBAHFP		
heme *b*	−791	−19
heme *a*	−648	−19
DAH	−593	+2
PPIX-DME	−751	+2
CH_2Cl_2/0.2 M TBAHFP		
heme *b*	−781	−29
PPIX-DME	−756	+2
benzene/0.2 M THAPC[b]		
heme *b*		−67
CH_2Cl_2 (no electrolyte)		
heme *b*	−825	−110 (−55/propionate)
heme *a*	−711	−97 (−47/propionate)
DAH	−653	0
PPIX-DME	−831	−2

[a]All measurements were performed with 1 M *N*-methylimidazole. The error was ±10 mV. [b]$\Delta E_{1/2}$ was measured by using DAH as a reference.

The effect of the solvent polarity was further studied by replacing CH_2Cl_2 with benzene and 0.20 M tetrahexylammonium perchlorate (THAP) as the electrolyte. Under these conditions, addition of excess DBU to heme *b* produced a $\Delta E_{1/2}$ of −67 mV (Supporting Information), which represents a 2-fold change in $\Delta E_{1/2}$, relative to the change in CH_2Cl_2 with 0.20 M TBAHFP. The role of benzene in the magnification of the electrostatic effects of the charged propionates on the heme reduction potential confirms a previous finding on the study of the thermodynamic and UV−vis spectral properties of flavin analogues in benzene solvent.[15] In that study, it was found that hydrogen bonding to a synthetic flavin was maximized in benzene, relative to the interaction in CH_2Cl_2. The increase in $\Delta E_{1/2}$ upon deprotonation of the heme *b* propionates in benzene can also be attributed to a decrease in the dissolved-ion concentration of THAP in benzene, most likely due to its incomplete dissociation.[16]

Low-Current Voltammograms in CH_2Cl_2 with No Electrolyte. The contributions of the heme peripheral group interactions to the $E_{1/2}$ were studied in the absence of electrolyte. The $E_{1/2}$ values of the (bis)*N*-methylimidazole complexes of all the studied hemes (Figure 1) were determined in CH_2Cl_2 without electrolyte. Their $\Delta E_{1/2}$ values upon DBU addition are reported in Table 1. These measurements were performed by using a 10 μm diameter UME. The low-current voltammograms, which produce sigmoidal current−voltage curves[17] due to the steady-state reduction of the heme, are shown in Figure 2a, and their first-order derivatives (dI/dV) are shown in Figure 2b. Before addition of DBU, a substoichiometric amount of trifluoroacetic acid (TFA), about 0.1−0.2 equiv, was added to the hemes to ensure complete formation of propionic acids before deprotonation. Addition of excess DBU (5 equiv) to heme *b* and heme *a* in CH_2Cl_2 in the absence electrolyte shifted the heme reduction potentials by −110 and −97 mV, respectively. After the addition of the excess DBU,

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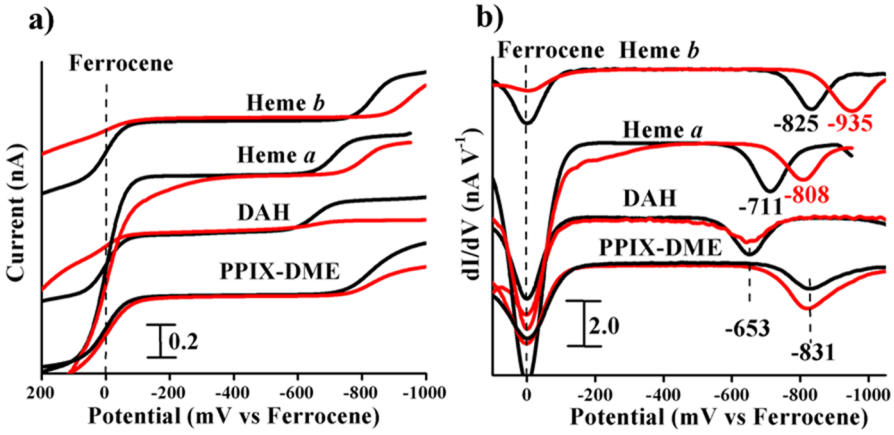

Figure 2. Low-current voltammograms of the reduction of the heme (bis)*N*-methylimidazole complexes in CH_2Cl_2 without electrolyte upon addition of excess DBU (shown in red), panel a. The derivatives of the reductive waves are shown in panel b. Heme *b* and heme *a* concentrations were ∼0.3 and ∼0.15 mM, respectively. DAH and PPIX-DME concentrations were ∼0.3 mM. The added DBU was at least 5 times the heme concentration. The ferrocene concentration was 0.1 mM. The scan rate was 20 mV s^{-1}.

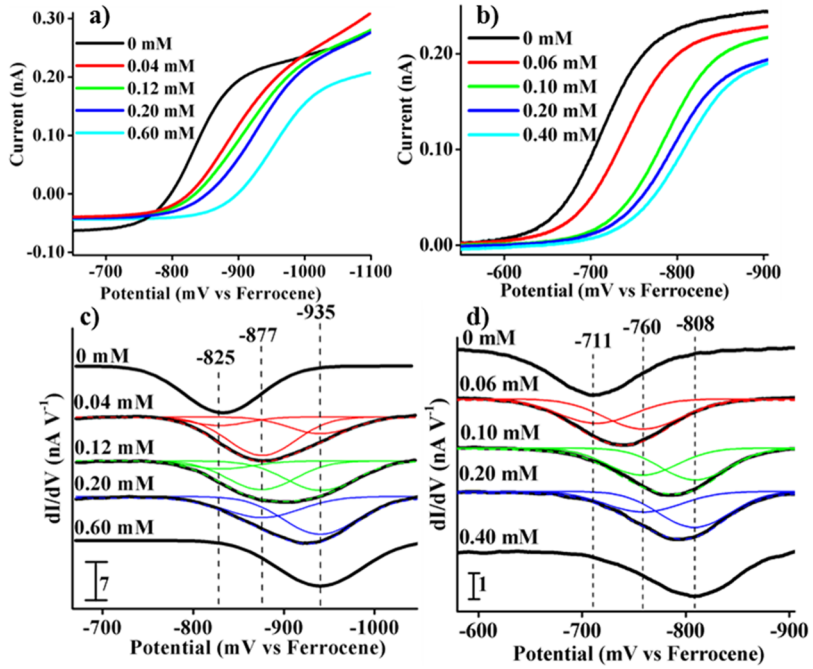

Figure 3. DBU titrations of heme *b* and heme *a*. Low-current voltammograms of heme *b* and heme *a* (bis)*N*-methylimidazole complexes in CH_2Cl_2 without electrolyte. Panel a is the DBU titration of ∼0.3 mM heme *b*. The DBU concentrations at 0.12 and 0.60 mM represent ∼1 and ∼2 equiv per heme *b*, respectively. DBU titration of ∼0.15 mM heme *a* is shown in panel b. Additions of 0.2 and 0.4 mM DBU represent ∼1 and ∼2 equiv per heme *a*. First-order derivatives of the voltammograms of heme *b* and heme *a* titrations are displayed in panels c and d, respectively. The first-order derivative voltammograms were fitted with two or three Gaussian peaks (shown in red, green, and blue). The scan rate was 20 mV s^{-1}.

addition of TFA resulted in an increase in the reduction potentials of heme *b* and heme *a*. Additionally, the UV−vis spectra of these hemes taken after the electrochemical measurements showed no evidence of the presence of ionized propionates. This indicates that deprotonation and protonation of the heme propionates is a reversible process.

The excess DBU (5 equiv) added to the methyl propionic esters hemes DAH and PPIX-DME resulted in negligible changes in their $E_{1/2}$. There is a 4-fold increase in $\Delta E_{1/2}$ for heme *b* without electrolyte, relative to the change in CH_2Cl_2 with 0.2 M TBAHFP. These results show how removal of the electrolyte has a significant impact on the $\Delta E_{1/2}$ of heme *b* upon ionization of the propionates. This can be explained because of the absence of nonspecific Debye−Hückel screening effect from the electrolyte and its impact on the Gibbs energy of electrostatic interactions.[19,20]

The overall change of about −100 mV of heme *b* and heme *a* is caused by the ionization of both heme propionates. To assess the contribution of the ionization of one heme propionate on $E_{1/2}$, DBU titrations were carried out in CH_2Cl_2 with no electrolyte. These results are displayed in Figure 3. The low-current voltammograms in Figure 3a,b, due to DBU titrations of heme *b* and heme *a*, respectively, show the decrease in $E_{1/2}$ as the concentration of DBU is increased from 0 up to 2 equiv per heme unit. The derivatives of the reductive waves in Figure 3c,d show the heterogeneity in the shapes of these peaks. With the exception of the initial and final peaks, deconvolution of the data into two or three Gaussian peaks resulted in the consistent

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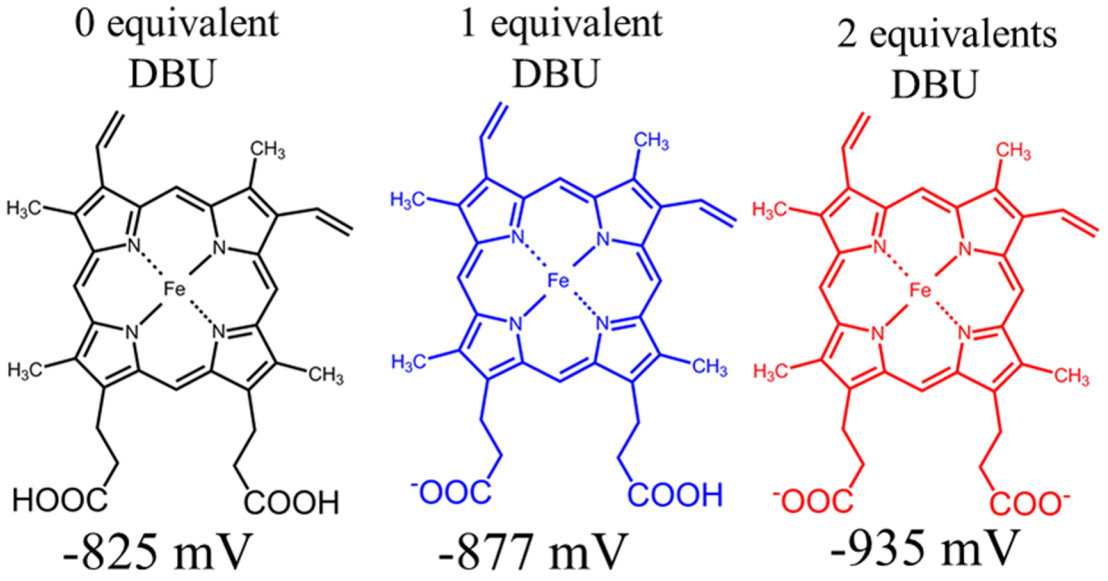
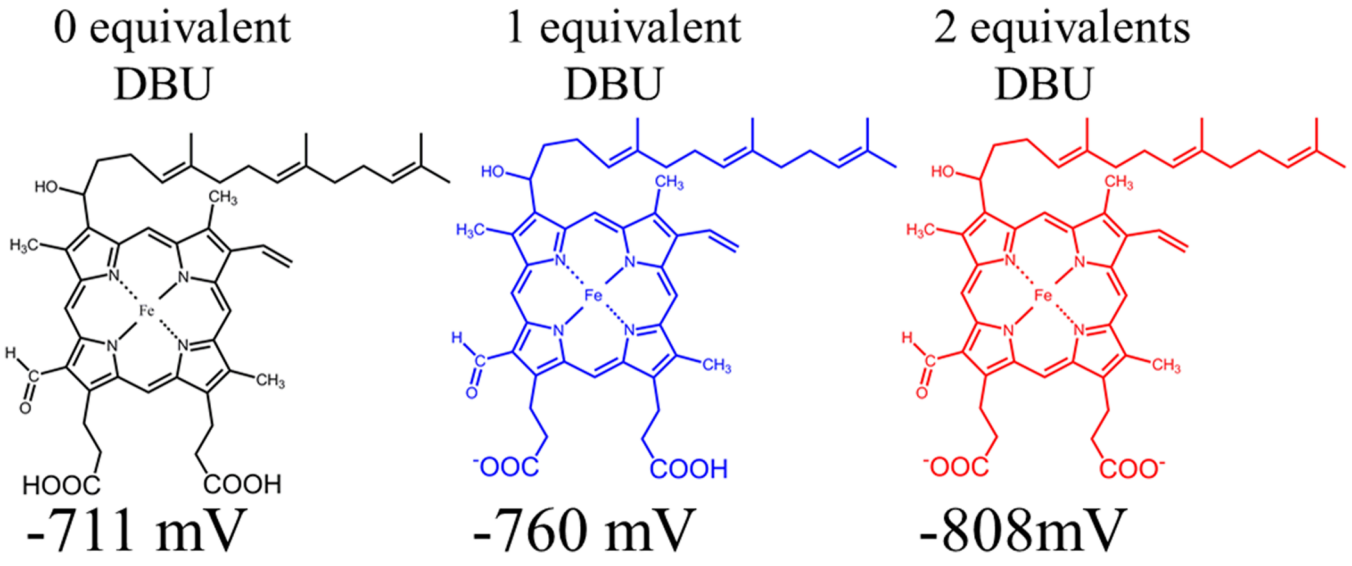
Scheme 1. Heme *b* Reduction Potentials in CH_2Cl_2



Scheme 2. Heme *a* Reduction Potentials in CH_2Cl_2



presence of a third middle peak in both heme *b* and heme *a*, Figure 3c,d, respectively. The difference in $E_{1/2}$ between the first peak and the middle peak (after addition of 1 equiv of added DBU) was −55 and −47 mV for heme *b* and heme *a*, respectively. The reductive waves span over 100 mV; hence the presence of an intermediate heme compound is difficult to detect unless a Gaussian fit is performed. Addition of 0, 1, and 2 equiv of DBU to heme *b* in CH_2Cl_2 generates the heme compounds shown in Scheme 1 with ferric-to-ferrous reduction potentials of −825, −877, and −935 mV (vs ferrocene), respectively. After addition of more than 2 equiv of DBU, the heme *b* reduction potential is maintained at −935 mV (vs ferrocene), Figure 2b.

The addition 0, 1, and 2 equiv of DBU to heme *a* in CH_2Cl_2 generates the heme compounds shown in Scheme 2 with ferric-to-ferrous reduction potentials of −711, −760, and −808 mV (vs ferrocene), respectively. The addition of more than 2 equiv of DBU to heme *a* does not shift the reduction potential below −808 mV (vs ferrocene), Figure 2b.

Since the uncertainties of the reduction potential measurements were ±10 mV, we cannot distinguish whether the $\Delta E_{1/2}$ due the first deprotonation is different from the $\Delta E_{1/2}$ due to the second deprotonation (relative to the first ionization). Essentially, these results show that the effects of consecutive ionizations of the propionates on the heme reduction potentials are purely electrostatic. Our measured $\Delta E_{1/2}$ of −55 mV per propionate in heme *b* is 2-fold greater than the change in the reduction potential measured by Warren and Mayer in their study of PPIX-monomethyl ester (bis)*N*-methylimidazole complexes in acetonitrile solvent.[18] The difference in the values of the reduction potential is due to their use of a more polar solvent (ε_r = 36) in the presence of an electrolyte. In contrast, our measured $\Delta E_{1/2}$ of about −55 mV per propionate is up to 2-fold lower than the heme reduction potential produced by the ionization of one heme propionate in sodium dodecyl sulfate and Triton X-100 micelles.[21] Our measured $\Delta E_{1/2}$ are instead comparable to the reduction potential changes induced by mutations of the surrounding amino acids that interact with the

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heme propionate-7 in yeast cytochrome *c*.[22] Differences in all these values can be attributed to differences in the dielectric constant of the media around the heme cofactor. Since our measurements of $\Delta E_{1/2}$ were performed in the absence of dissolved ions, we can conclude that in the absence of ion−dipole interactions, a measured $\Delta E_{1/2}$ value lower than −55 mV per propionate in heme *b* implies that the dielectric constant of the medium is larger than that of CH_2Cl_2, whereas a larger $\Delta E_{1/2}$ value means that the dielectric constant of the medium is lower than that of CH_2Cl_2. Measurements of the $\Delta E_{1/2}$ per heme propionate ionization in benzene should produce larger values because of the lower dielectric property of this solvent. In fact, mutagenic substitutions of the surrounding amino acids that interact with the heme propionate-7 in yeast cytochrome *c* for phenylalanine residues results in the largest $\Delta E_{1/2}$ per heme propionate (∼70 mV), presumably because of the lowering in the local ε_r.[22]

Cytochrome *c* Oxidase and Hydrogen Bonding to the Heme *a* Formyl Group. In heme proteins, heme propionates that are buried in the protein have significant heme redox-linked ΔpK_a ($pK_{aFe(II)} - pK_{aFe(III)}$) than those that are exposed to the solvent.[23] In this regard, the medium dielectric modulates this heme-redox-linked ionization. Electron transfer (ET) proteins such as cytochrome *c* oxidase (CcO) have buried hemes (hemes *a* and a_3), compared with myoglobin, whose heme is exposed to the solvent, see Figure 4. This study

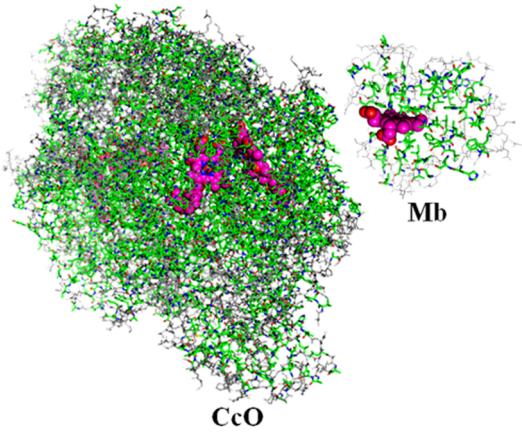

Figure 4. X-ray crystallographic structures of cytochrome *c* oxidase (PDB ID 1OCC)[29] and myoglobin (PDB ID 2V1I).[30] The hemes are in magenta, and the nonpolar residues are highlighted in green.

suggests that redox changes in hemes *a* and a_3 in CcO can lead to significant changes in the p*K*a of the heme propionates, which have been postulated as having a role in the proton pumping mechanism of CcO.[5,8,24−27] Another element, as important as the propionates in the proton pumping mechanism of CcO, is the involvement of the heme *a* formyl group. The formyl group is part of the π system of the porphyrin ring and changes in the interaction to this group result in changes in the optical spectra of heme *a*.[4,9,10] Based on the observation that heme *a* reduction in CcO strengthens the H-bond to the carbonyl of the formyl group, Babcock and co-workers proposed that this redox-linked change is significant enough to be considered as a major factor for proton pumping.[4,9] To evaluate the contribution of the H-bond to the formyl group on the heme *a* reduction potential, we measured the $E_{1/2}$ as a function of added trichloroethanol (TCE). The results are shown in Figure 5. The initial solution

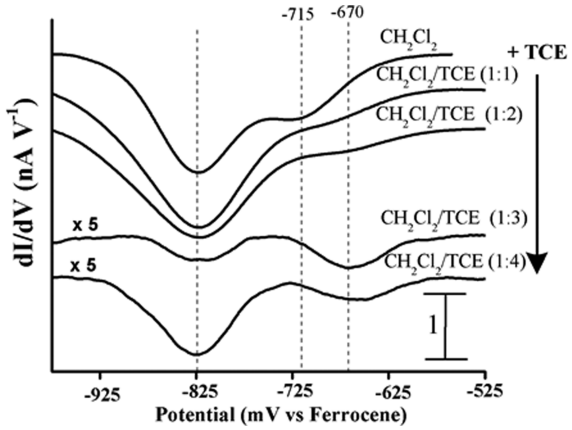

Figure 5. Low-current voltammograms (first-order derivatives) of heme *b* (left peak) and heme *a* in different CH_2Cl_2−TCE solvent mixtures without electrolyte. The scan rate was 20 mV s^{-1}.

contains heme *a* and heme *b* in CH_2Cl_2 with no electrolyte. In this case, we used heme *b* as a control because it does not have a carbonyl that forms part of the heme π system. Addition of TCE (1:1 mixture) shifted the $E_{1/2}$ of heme *a* but not the $E_{1/2}$ of heme *b*. After addition of TCE to a 1:2 (CH_2Cl_2/TCE) mixture, the measured reduction potentials of heme *a* reached a final value of −670 mV (vs ferrocene). Thus, H-bonding between TCE and the formyl on heme *a* produces a reduction potential change of +45 mV. Based on the measured C═O stretching vibrations in the ferric and ferrous forms of heme *a* in a CH_2Cl_2−phenol mixture, the calculated redox change due to the H-bond on the formyl group is equivalent to about 0.8 kcal mol^{-1} (+35 mV).[4,9] Since the H-bond strengths to the formyl are approximately the same in phenol as in TCE, the Gibbs free energy for heme *a* reduction in TCE is within the expected range predicted by Babcock and Callahan for heme *a* in the CH_2Cl_2−phenol mixture.[4] Given the weak H-bond between TCE and the heme *a* formyl, relative to the H-bond between arginine (R38) and the formyl in bovine CcO,[4] we surmise that the redox change is significantly larger in the enzyme than our measured $\Delta E_{1/2}$ in TCE solvent. This study suggests that H-bonding to the heme *a* formyl can contribute to the heme reduction potential as much as the heme propionate ionization. Furthermore, we presume that addition of a strong acid to heme *a* should produce a stronger H-bond to the heme *a* formyl. In this study, we were not able to use TFA as a strong H-bond donor because at high concentrations it can react with the iron heme *a* model compounds.[4] Thus, we have been careful in the use of TFA, only adding substoichiometric amounts for heme propionate protonation. Babcock and Callahan instead used copper porphyrin *a* compounds to study the spectroscopic effects of added TFA. Based on this study, TFA had the largest effect on the C═O stretching vibration of the formyl, and they predicted that H-bond to the heme *a* formyl in the enzyme can cause a heme *a* reduction potential change as large as +110 mV.

■ CONCLUSIONS

Our study can be summarized by Scheme 3. Heme propionate ionizations can cause a reduction potential change of about 50 mV per propionate in CH_2Cl_2. Such heme-redox-linked ΔpK_a is present in heme *b* as in heme *a*. Additionally, the presence of the formyl group in heme *a*, not present in heme *b*, adds extra modulation of the heme reduction potential of at least ∼50 mV

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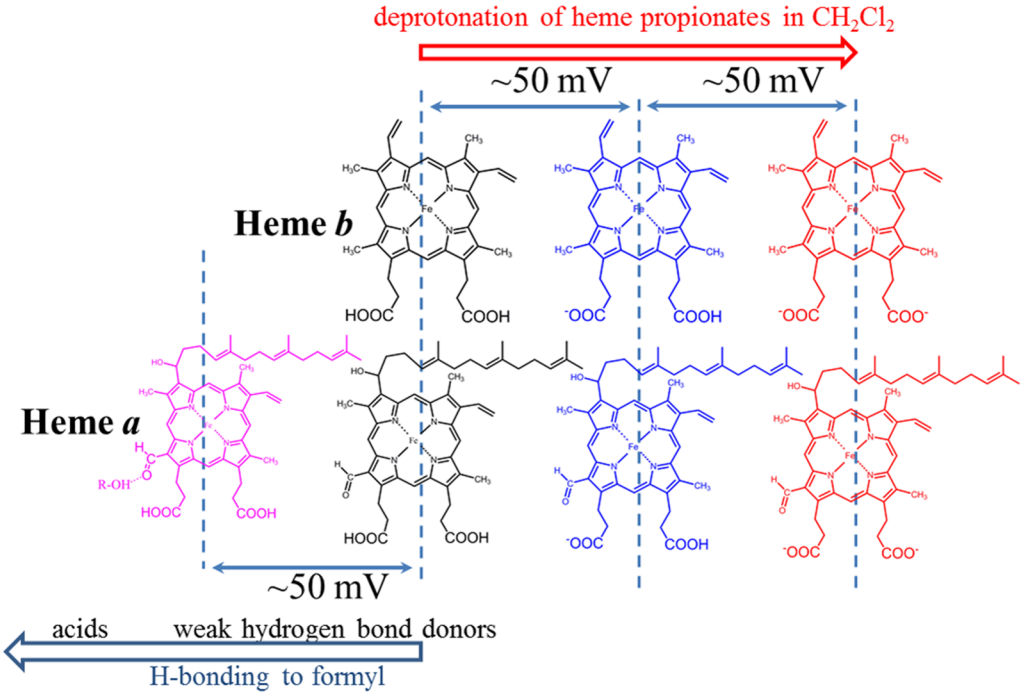
Scheme 3. Heme *b* and Heme *a* Reduction Potentials in CH_2Cl_2



with weak H-bond donors. Addition of stronger acids should increase this effect. Considering that the ionization of both heme *a* propionates causes an overall reduction potential change of least a 97 mV, our study suggests that heme *a* can be used to transduce over 200 mV (4.6 kcal mol^{-1}) of redox energy for proton pumping in CcO upon reduction of the enzyme .

EXPERIMENTAL SECTION

Reagents. Methylimidazole, anhydrous benzene, anhydrous dichloromethane, acetonitrile, dimethyl sulfoxide (DMSO), acetone, tichloroethanol (TCE), tetrabutylammonium hexafluorophosphate (TBAHFP), trifluoroacetic acid (TFA), 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), and ferrocene (denoted herein as ferrocene when used as the reference potential), all over 99% purity, were purchased from Sigma-Aldrich. Hemin (heme *b*), iron(III) 2,4-diacetyl deuteroporphyrin IX dimethyl ester (DAH), and iron(III) protoporphyrin IX dimethyl ester (PPIX-DME) were purchased from Frontier Scientific Inc. (Logan, Utah).

Heme *a*. Purified heme *a* was obtained by extraction from used cytochrome *c* oxidase (CcO) samples. The CcO samples were a kind gift from Dr. Denis Proshlyakov at Michigan State University. Purification was performed by using the procedure outlined by Takemori and King.[28] The extracted heme *a* was dissolved in acetonitrile and then lyophilized. The heme *a* powder was then dissolved in the appropriate solvent as needed.

Tetrahexylammonium Perchlorate (THAP). The reagent was purchased from GSF Chemicals Inc. (Powell, Ohio). Tetrahexylammonium perchlorate was then recrystallized in ethanol.

Electrochemistry. Cyclic voltammetry (CV) was carried out by using an Epsilon electrochemical workstation from Bioanalytical System Inc. (West Lafayette, IN). The electrochemical cell consisted of three electrodes: a glassy carbon working electrode, a platinum wire as the auxiliary electrode, and a silver wire as the pseudo-reference electrode. The working electrode was a 1.6 mm diameter glassy carbon disk electrode (Bioanalytical System Inc.). Low-current voltammograms were obtained by using a 10 μm diameter glassy carbon ultramicroelectrode from Bioanalytical System Inc.

Low-Current Voltammograms of DBU Titrations of Heme *b* in CH_2Cl_2 with No Electrolyte. For the DBU titrations, an aliquot of a 5 mM heme *b* stock solution in CH_2Cl_2 with 1 M *N*-methylimidazole was dissolved in 5 mL of CH_2Cl_2 with 1 M *N*-methylimidazole and ~1 mM ferrocene to a final cocentration of ~0.3 mM. The solution was maintaned under anaerobic conditions by using an argon gas flow throught the experiment. The measurements were performed by using the 10 μm diameter ultramicroelectrode. An initial voltammogram was obtained prior to the DBU titration. This was followed by adding substoichiometric ammounts of a 10 mM TFA solution, which increased the reduction potential of heme *b*. TFA was added until no further changes were observed in the heme reduction potential (0.1 to 0.2 equiv per heme *b*). We were careful with the TFA additions because excess concentrations can cause changes to the heme spin state.[4] The heme reduction potential typically increased to about 30 mV. The titration consisted in additions of 20 μL of 10 mM DBU, dissolved in the same solvent conditions, to the 5 mL heme solution. Low-current voltammograms were obtained after each addition of DBU. Between DBU additions, the UME was rinsed with acetone to avoid fouling of the electrode surface during the measurements. After the DBU titration, TFA was added to verify that the deprotonation was reversible.

Low-Current Voltammograms of DBU Titrations of Heme *a* in CH_2Cl_2 with No Electrolyte. For the DBU titrations, an aliquot of a 1 mM heme *a* stock solution in CH_2Cl_2 with 1 M *N*-methylimidazole was dissolved in 0.2 mL of CH_2Cl_2 with 1 M *N*-methylimidazole and ~1 mM ferrocene to a final concentration of ~0.15 mM. The solution was contained in a 1 mL v-vial and maintaned under anaerobic conditions by using an argon gas flow throught the experiment. The measurements were performed by using the 10 μm diameter UME. An initial voltammogram was obtained prior to the DBU titration. This was followed by adding substoichiometric ammounts of a 10 mM TFA solution, typically 1 μL additions, which increased the reduction potential of heme *a*. TFA was added until no further changes were observed in the heme reduction potential (0.1 to 0.2 equiv per heme *a*). Similar to heme *b*, the heme reduction potential typically increased to about 30 mV. The titration consisted in additions of 1 μL of 10 mM DBU, dissolved in the same solvent conditions. Low current voltammograms were obtained after each addition of DBU. Between DBU additions, the UME was rinsed with acetone to avoid fouling of the electrode surface during the measurements. After the DBU titration, stoichiometric amount of TFA was added, which resulted in the increase of the heme *a* reduction potential. Also, a UV–vis

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absorption spectrum of the heme *a* solution after addition of TFA did not show evidence of ionized propionates.

Low-Current Voltammograms of Heme *a* in TCE. The initial solution before TCE addition was prepared as described above, except that heme *b* was also added to the solution. TCE with 1 M *N*-methylimidazole was added in increments of 0.2 mL to a final CH_2Cl_2/TCE ratio of 1:4.

Voltammograms and Data Handling. The first-order derivative (dI/dV) of the low-current cyclic voltammograms and Gaussian fits were performed by using Origin Microcal Software (Northampton, MA).

■ ASSOCIATED CONTENT

Supporting Information

Heme UV−vis absorption spectra and additional voltammograms. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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