Role of Heme Propionate Groups in Cytochrome b_5 Electron Transfer

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Abstract: The involvement of the heme propionate groups in the mechanism of the cytochrome b_5 oxidation–reduction transition has been evaluated by preparation and functional characterization of a derivative of the protein (DME-b_5) which the native prosthetic group has been replaced by ferriprotoporphyrin IX dimethyl ester. Spectroelectrochemical studies of DME-b_5 show that esterification of the heme propionate groups increases the potential of the protein from 5 mV observed for the native protein to 69 mV vs. NHE [pH 7.0 (phosphate), μ = 0.1 M, 25 °C]. The dependence of $E°$ on pH is analogous to that seen for the native protein and indicates that these propionate groups are not responsible for this effect. The ionic strength dependence of $E°$ has been analyzed in terms of Debye–Hückel theory to produce an apparent net electrostatic charge on oxidized DME-b_5 of −8. The thermodynamic parameters for this equilibrium are $\Delta H° = -11$ (1) kcal/mol and $\Delta S° = -31$ (2) eu [pH 7.0 (phosphate), μ = 0.1 M]. Kinetics analysis of DME-b_5 reduction by $Fe(EDTA)^{2-}$ produces a second-order rate constant of 1.97 (2) × 10^3 M^{-1} s^{-1} [pH 7.0 (phosphate), μ = 0.1 M, 25 °C] and a pH dependence similar to that of the native protein. Activation parameters for this reaction are $\Delta H^* = 4.05$ (5) kcal/mol and $\Delta S^* = -30$ (2) eu. Analysis of the ionic strength dependence of DME-b_5 reduction by $Fe(EDTA)^{2-}$ in terms of the Marcus ionic strength equation yields an apparent net electrostatic charge on oxidized DME-b_5 of −6.9. These results have been used to calculate the apparent self-exchange rate constant demonstrated by DME-b_5 in this reaction to produce a value (k_{11}^{corr}) of 7.6 × 10^2 M^{-1} s^{-1}, a value 70-fold greater than that observed for the native protein. We conclude that the present analysis of DME-b_5 provides strong evidence for (1) the involvement of the partially exposed heme edge in heme protein electron transfer reactions in general, (2) short-range electrostatic effects in the $Fe(EDTA)^{2-}$ reduction of the native protein that are not adequately accommodated by available models for protein behavior, and (3) a major role of at least one heme propionate in determining the reduction potential of native cytochrome b_5.

Hepatic cytochrome b_5 (m_r 16000) occurs in both a microsomal[1] and mitochondrial[2] form. Although the functions of the mitochondrial form are not defined clearly at this time, the microsomal form participates in the catalytic cycle of cytochrome P-450[3] and in fatty acid desaturation.[4] A smaller (m_r 10000), soluble form of this cytochrome occurs in erythrocytes and is responsible for mediating electron transfer between methemoglobin reductase and methemoglobin.[5] A soluble derivative of hepatic cytochrome b_5 that can be isolated by proteolytic treatment of microsomes[1] has been shown recently to be virtually identical in amino acid sequence with the erythrocytic protein.[6] The three-dimensional structure of the protease-solubilized hepatic microsomal cytochrome has been determined to high resolution in both the reduced and oxidized states by Mathews and co-workers.[7]

Despite the importance of the metabolic functions in which cytochrome b_5 participates and the detailed structural characterization to which it has been subjected, relatively little effort has been directed toward understanding the mechanism by which cytochrome b_5 changes oxidation state. For this reason, we have previously undertaken kinetic[8] and spectroelectrochemical[9] studies of trypsin-solubilized hepatic microsomal cytochrome b_5. The results of this work combined with NMR[10] and crystallographic data[7] were consistent with a model[7c] for cytochrome b_5 function in which the negative charge of one of the heme propionate groups stabilizes the net positive charge on the iron in the oxidized form of the protein. In the reduced state, this coulometric interaction is abolished and the heme propionate is thought to bind a cation from solution. To evaluate directly the role of heme propionate groups in affecting the oxidation–reduction behavior of cytochrome b_5, we now report the first successful reconstitution of this protein with ferriprotoporphyrin IX dimethyl ester and present a kinetic and spectroelectrochemical characterization of this reconstituted derivative (DME-b_5).

Experimental Section

The tryptic fragment of bovine liver microsomal cytochrome b_5 was prepared as described previously.[8] Apoprotein was prepared by the method of Teale.[11] Specifically, cytochrome b_5 (100 mg, 12.5 mg/mL) was dialyzed into water (4 °C) and placed in a conical, glass-stoppered test tube on ice, and the pH was lowered to 1.5 with cold HCl (1 N). Heme was extracted by adding an equal volume of ice-cold 2-butanone (Burdick and Jackson) and inverting the stoppered tube gently several times. The dark brown ketone layer was drawn off with a pipet, and the extraction was repeated if necessary to produce a colorless solution of apoprotein. This preparation was dialyzed (4 °C) against 1 L of 0.6 mM $NaHCO_3$ containing 1 mM EDTA and then against 1 L of 0.6 mM $NaHCO_3$ alone. The apoprotein precipitated at this point as it reached its isoelectric pH but readily redissolved during further dialysis against 1-L changes of 20 mM sodium phosphate (pH 7.2) followed by 0.6 mM $NaHCO_3$. The apocytochrome concentration was generally 0.5 mM (ϵ_{280} = 10500 M^{-1} cm^{-1} [12]).

As ferriprotoporphyrin IX dimethyl ester (Porphyrin Products, Logan UT) is insoluble in aqueous media, reconstitution was achieved by dissolving the heme in freshly distilled, dry Me_2SO (2 mg/mL) and adding it (10% excess) dropwise to the apoprotein on ice with gentle stirring. After incubation of this mixture for 24 h at 4 °C, an additional 0.1 equiv of heme was added, and the solution was allowed to stand in the cold for another 24 h. The solution was then concentrated to 1.5 mL by ultrafiltration (Amicon YM-5 membrane) and clarified by centrifugation at 16000*g* (4 °C). The supernatant fluid was eluted over a column of

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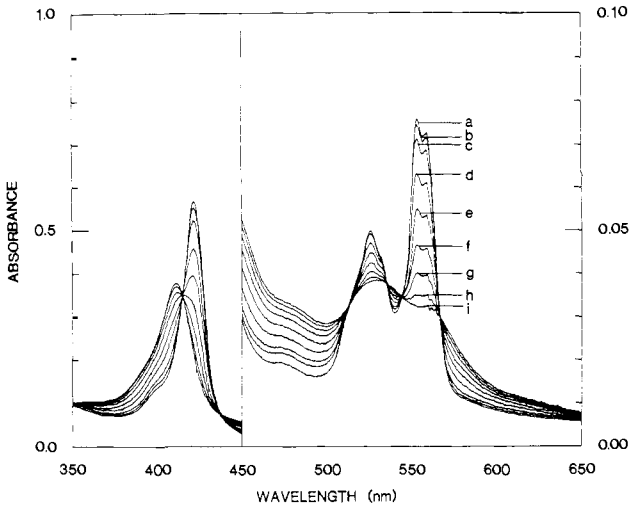

Figure 1. Representative family of thin-layer spectra of DME-b_5 at various values of applied potential, E_{app} (mV vs. NHE): DME-b_5 (120 μM), $Ru(NH_3)_6Cl_3$ (12 μM), 20 °C, pH 7.0 (phosphate), μ = 0.1 M: (a) −205.6, (b) −5.4, (c) 24.2, (d) 54.6, (e) 74.7, (f) 95.0, (g) 114.9, (h) 144.3, (i) 344.1.

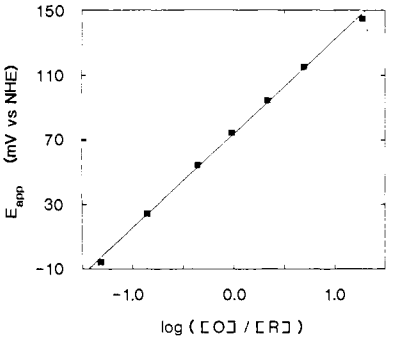

Figure 2. Nernst plot calculated from the spectra shown in Figure 1 based on ΔA_{544}. The midpoint reduction potential determined from this analysis is 77.3 (1) mV and the slope is 58.4 (2) mV.

Sephadex G-75 Super-Fine (2.5 × 90 cm, 4 °C) that was equilibrated with 20 mM sodium phosphate (pH 7.2). Excess heme eluted as a green band followed by the cherry-red reconstituted protein. Fractions with an A_{412}/A_{280} ratio ≥6 were pooled and concentrated by ultrafiltration. The recovery of protein was 80–85%.

Electrochemical measurements were made with an optically transparent thin-layer electrode system with $Ru(NH_3)_6Cl_3$ as mediator as previously described.[9] Kinetics analysis of cytochrome reduction by $Fe(EDTA)^{2-}$ used the stopped-flow apparatus, data reduction techniques, solution preparations, and anaerobic operations employed in our work with the native protein.[8]

Results

The electronic absorption spectra of reduced and oxidized DME-b_5 are virtually identical with those of the native protein. The principal changes are a small shift in the Soret maximum of the oxidized protein from 412.5 to 412 nm and a more pronounced splitting of the α-band in the reduced form of the protein. This latter feature is illustrated in the family of thin-layer spectra from a representative electrochemical experiment shown in Figure 1. Isosbestic points observed in such measurements occurred at 350, 414, 436, 513, 535, 543.5, and 566 nm. As with the native protein,[9] DME-b_5 was electrochemically reversible in the thin-layer cell and could be cycled between reduced and oxidized states repeatedly. The Nernst plot derived from the data in Figure 1 is shown in Figure 2 and demonstrates that the heme-substituted protein is well-behaved under these conditions. The reduction potential of DME-b_5 under the normal reference conditions [pH 7.0 (phosphate), μ = 0.1 M, 25 °C] was found to be 68.8 (8) mV vs. NHE.

The effect of pH on the midpoint reduction potential of DME-b_5 is shown in Figure 3. Following the approach developed by

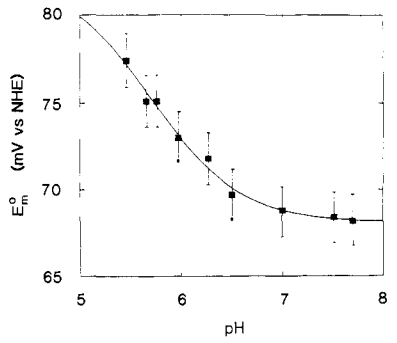

Figure 3. pH dependence of the DME-b_5 midpoint reduction potential [μ = 0.1 M (phosphate), 25 °C]. The solid line is the theoretical fit of the data to eq 1 described in the text.

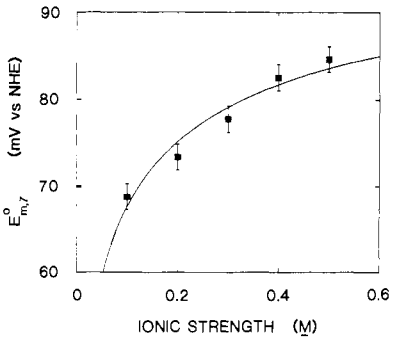

Figure 4. Variation of the DME-b_5 midpoint reduction potential with ionic strength [pH 7.0 (phosphate), 25 °C]. The solid line is the theoretical fit of the data to eq 2 described in the text.

Clark,[9,13] these results may be analyzed in terms of a redox-linked functional group that undergoes a change in pK_a as the protein changes oxidation state. The dependence of the reduction potential on pH in this case is described by eq 1. The solid line in Figure

$$E_m^\circ = E + \frac{RT}{2.303nF} \ln \frac{K_{red} + [H^+]}{K_{ox} + [H^+]} \quad (1)$$

3 is the nonlinear least-squares fit of the data to this relationship. From this analysis we calculated pK_{ox} = 5.60, pK_{red} = 5.84, and E = 81 mV vs. NHE [μ = 0.1 M (phosphate), 25 °C].

The variation of the DME-b_5 reduction potential with ionic strength is shown in Figure 4. These data may be analyzed in terms of a Debye–Hückel-type expression:[14]

$$E_{obsd}^\circ = E^\circ - \frac{RT}{F} A(q_{ox}^2 - q_{red}^2) f(\mu) \quad (2)$$

where E_{obsd}° is the reduction potential observed at a given ionic strength, E° is the standard reduction potential (μ = 0), A is the Debye–Hückel constant (0.5115), and q_{ox} and q_{red} are the net electrostatic charges of the oxidized and reduced protein, respectively ($q_{ox} = q_{red} + 1$). Several forms of the function $f(\mu)$ have been suggested,[15] but in our previous study,[9] we found that $f(\mu) = \mu^{1/2}/(1 + \beta R\mu^{1/2})$, where R is the radius (Å) of the protein, provides the most reasonable numerical results for data of this type. The solid line in Figure 4 is the nonlinear least-squares fit of the data to eq 2 in which this form of $f(\mu)$ has been used with a value of R = 17 Å estimated from the protein crystal structure.[7] This analysis yields a value for q_{ox} of −8 (1). A similar result (q_{ox}

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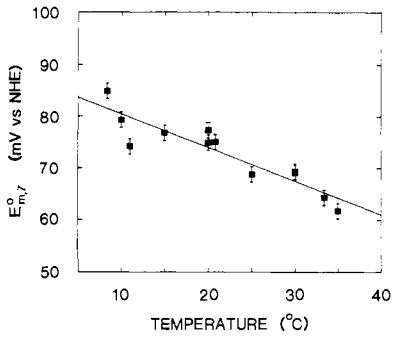

Figure 5. Temperature dependence of the DME-b_5 midpoint reduction potential [pH 7.0 (phosphate), μ = 0.1 M]. The solid line is a weighted linear least-squares fit to the data.

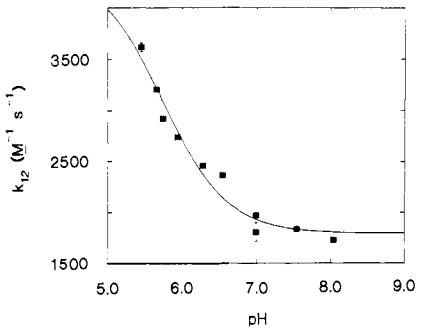

Figure 6. Dependence of the second-order rate constant for the reduction of DME-b_5 by Fe(EDTA)$^{2-}$ on pH [μ = 0.1 M (phosphate), 25 °C]. The solid line is the theoretical fit of the data to eq 3 described in the text.

= −9 (1)) is obtained if an estimate of R (15.5 Å) is derived[9] from the molecular weight of the protein.

The thermodynamics of the DME-b_5 oxidation–reduction equilibrium have been analyzed as described by Taniguchi et al.[16] (Figure 5). From this analysis, $\Delta S°$ = −31 (2) eu and $\Delta H°$ = −11 (1) kcal/mol. $\Delta S°$ is the difference in partial molal entropies between the oxidized and reduced halves of a one-electron oxidation–reduction couple adjusted to the $S°_{H^+}$ = 0 convention.

The kinetics of DME-b_5 reduction by Fe(EDTA)$^{2-}$ were studied under pseudo-first-order conditions by monitoring the change in absorbance at the Soret maximum (423 nm). First-order plots were linear for at least 90% of the reaction in all cases. In contrast to the results with native cytochrome b_5, Fe(EDTA)$^{2-}$ was able to reduce the heme-substituted protein completely under all conditions. This finding presumably arises from the much higher reduction potential of DME-b_5. Under standard conditions [pH 7.0 (phosphate), μ = 0.1, 25 °C], the second-order rate constant for this reaction is 1.97 (2) × 10^3 M^{-1} s^{-1}.

The pH dependence of DME-b_5 reduction by Fe(EDTA)$^{2-}$ is shown in Figure 6. As before,[8] these data may be interpreted as resulting from reduction of protonated (k_a) and unprotonated (k_b) forms of the protein and analyzed in terms of the relationship described in eq 3. Here, K_a is the equilibrium constant for the

$$k_{12} = \frac{k_a[H^+] + k_bK_a}{[H^+] + K_a} \quad (3)$$

deprotonation–protonation reaction and k_{12} is the second-order rate constant observed at a given pH. The nonlinear least-squares fit of the present data to this relationship is illustrated in Figure 6 and is consistent with k_a = 4.4 × 10^3 M^{-1} s^{-1}, k_b = 1.8 × 10^3 M^{-1} s^{-1}, and pK_a = 5.74.

The variation in the rate of this reaction with ionic strength is illustrated in Figure 7. As expected for a reaction between two negatively charged species, the second-order rate constant increases with ionic strength. These results may be evaluated in

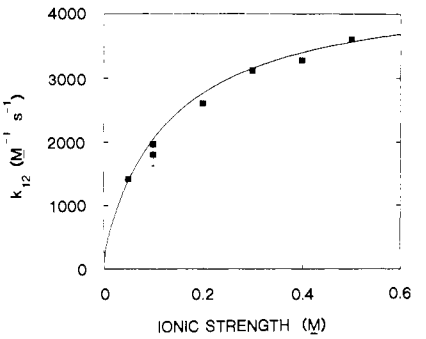

Figure 7. Variation of the second-order rate constant for the reduction of DME-b_5 by Fe(EDTA)$^{2-}$ with ionic strength [pH 7.0 (phosphate), 25 °C]. The solid line is the theoretical fit of the data to eq 4 described in the text.

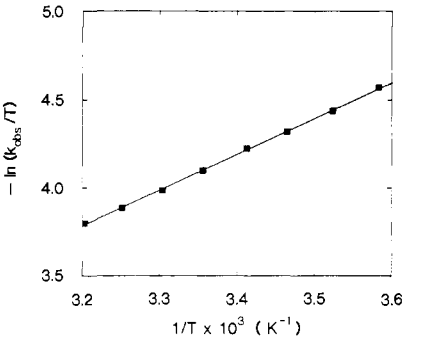

Figure 8. Eyring plot of the rate data for the reduction of DME-b_5 by Fe(EDTA)$^{2-}$ [pH 7.0 (phosphate), μ = 0.1 M]. [Fe(EDTA)$^{2-}$] = 2.5 × 10^{-3} M.

a more quantitative manner by fitting the data to the Wherland–Gray adaptation[17] of the Marcus ionic strength relationship:[18]

$$\ln k = \ln k_\infty - 3.576 \frac{e^{-\kappa R_2}}{1 + \kappa R_1} + \frac{e^{-\kappa R_1}}{1 + \kappa R_2} \frac{Z_1Z_2}{R_1 + R_2} \quad (4)$$

In the present case, $\kappa = 0.329\mu^{1/2}$, R_1 is the radius of DME-b_5 (17 Å), R_2 is the radius of Fe(EDTA)$^{2-}$ (4 Å), and Z_2 is the electrostatic charge on Fe(EDTA)$^{2-}$ (2−). The nonlinear least-squares fit of our data to this equation (solid line, Figure 7) provides an estimate of the apparent net electrostatic charge on DME-b_5 in this reaction of −8.0 (3). If the smaller protein radius is assumed (vide supra), the apparent net charge becomes −6.9 (2).

The activation parameters for reduction of the cytochrome were determined from an Eyring plot (Figure 8) of the rate constants observed at different temperatures. From this analysis, $\Delta H^‡$ = 4.05 (5) kcal/mol and $\Delta S^‡$ = −29.9 (2) eu.

Discussion

The most profound effect of heme propionate esterification on the electrochemical behavior of cytochrome b_5 is an increase in the reduction potential from 5.1 (6) to 68.8 (8) mV vs. NHE (pH 7.0 (phosphate), μ = 0.1 M, 25 °C). In terms of the Argos–Mathews model,[7c] this result can be attributed to a relative decrease in the stability of ferricytochrome b_5 of ~1.5 kcal/mol that results from the loss of the coulometric stabilization of the net positive charge on the iron atom in the oxidized protein. Analysis of the pH and ionic strength dependences of the DME-b_5 reduction potential produces numerical results very similar to those of the native protein. This finding establishes that these medium effects do not involve participation by the heme propionate groups as previously suggested.[9] The enthalpic component of the temper-

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ature dependence for DME-b_5 (−11 kcal/mol) is identical with that observed for native cytochrome b_5. This value is approximately 4 kcal/mol less negative than that reported for cytochrome c,[16] a difference that we orginally attributed[9] to the coulombic stabilization postulated for ferricytochrome b_5. It is now apparent that other effects, possibly related to a greater redox-linked conformational change in cytochrome c,[7,19] are responsible for this difference between the two types of cytochromes. Significantly, the entropic component of the temperature dependence is 6 eu less negative than that of the native protein, making it identical with the value reported for cytochrome c (−30.5 eu[16]). This observation can be explained by the inability of reduced DME-b_5 to bind a cation at the heme propionate as presumably occurs in the native protein.[7c]

As expected from the higher reduction potential of DME-b_5, the rate at which it is reduced by $Fe(EDTA)^{2-}$ is significantly greater than that for the native protein. Again, the pH dependence is virtually identical for the two forms of the protein, establishing that the heme propionates are not involved in this effect. Although several origins of the pH effect are conceivable, the information available is not sufficient to permit development of a viable model at this time.

The analysis of the ionic strength dependence of this rate, however, is considerably different from that of the native protein. In the latter case, the Wherland–Gray analysis yielded[8] an apparent net electrostatic charge for ferricytochrome b_5 of −15.6 which compared with a value of −7.5 estimated from the amino acid sequence of the protein. This difference in charges was attributed to the well-known limitations of the Debye–Hückel relationship at the ionic strengths used in our studies and to the remarkably asymmetric distribution of aspartyl and glutamyl residues around the partially exposed heme edge of the protein. The apparent electrostatic charge now estimated from the same analysis of DME-b_5 kinetic behavior (−7 or −8) is in much closer agreement with the value estimated from the sequence (−5.5). Despite the theoretical limitations of this type of analysis, Feinberg and Ryan have demonstrated[20] that the charge estimated from the fit to the Wherland–Gray equation generally demonstrates much closer agreement with the charge estimated from the sequence than we had found previously.[7] For this reason, we conclude that the present results argue strongly in favor of short-range electrostatic effects dominating in the reaction of $Fe(EDTA)^{2-}$ with native cytochrome b_5 to produce an anomalously large and negative apparent net electrostatic charge in the analysis of the ionic strength dependence data. These short-range electrostatic interactions arise from the presence of the heme propionates at the heme edge, the site at which electron transfer is thought to occur with this reductant. Elimination of these short-range interactions by esterification of the heme propionate groups significantly reduces the discrepancy in the electrostatics analysis.

The difference between the rates of reduction of cytochrome b_5 and DME-b_5 by $Fe(EDTA)^{2-}$ is produced by thermodynamic effects (the increased reduction potential of DME-b_5) and electrostatic effects. The one quantitative means of accounting for both contributions is through application of Marcus theory. From the electrochemical and kinetics data now available, we can calculate[17] the apparent self-exchange rate constants [$(k_{11}^{corr}$ (pH 7, 25 °C, μ = 0.1 M)] that the two proteins demonstrate in their reactions with $Fe(EDTA)^{2-}$. If we assume values for the protein charges that are derived from the ionic strength dependence data, k_{11}^{corr} for the native protein is 11 M^{-1} s^{-1} while the same value for DME-b_5 is 7.6×10^2 M^{-1} s^{-1}. Comparison of these rates with the corresponding value reported[17a] for cytochrome c (6.2 M^{-1} s^{-1}) and consideration of the relative degree of heme exposure in the two types of cytochrome as calculated by Stellwagen[21] (6% of heme surface exposed in cytochrome c and 23% in cytochrome b_5) indicate that the low rate constant calculated for the native protein results from a difference between the mechanism that is employed in its reduction by $Fe(EDTA)^{2-}$ and that employed by cytochrome c. This difference in rate is produced by the marked difference in the orientation of the heme groups in the two proteins. In cytochrome c the heme propionate groups are directed more toward the back of the heme binding pocket while in cytochrome b_5 they are on the edge of the heme that is exposed to external solvent. Esterification of the heme propionate groups that are directed toward the solvent in cytochrome b_5 largely eliminates this mechanistic difference by removing short-range electrostatic interactions to produce a value for k_{11}^{corr} that is more consistent with the greater degree of heme group exposure in this protein. These results provide some of the most compelling experimental evidence available for the involvement of the heme edge in heme protein electron transfer reactions.

In conclusion, we note that both heme propionate groups are likely to play important but distinctly different roles in cytochrome b_5 electron transfer reactions in vivo. As demonstrated in the present work, one of these groups is partially responsible for determining the reduction potential of the iron center. The other heme propionate appears likely to be involved in recognition of and interaction with heme proteins that are physiological redox partners of cytochrome b_5.[22] The availability of DME-b_5 and knowledge of its functional properties should facilitate experimental assessment of this latter possibility in subsequent studies.

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Registry No. $Fe(EDTA)^{2-}$, 15651-72-6; cytochrome b_5, 9035-39-6.

Supplementary Material Available: Listing of reduction potentials, Nernst slopes, and observed first-order rate constants (4 pages). Ordering information is given on any current masthead page.

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