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Cyclic Voltammetry of Imidazolemicroperoxidase-8: Modeling the Control of the Redox Potential of the Cytochromes

A number of cytochromes have midpoint redox potentials that are strongly dependent upon pH; these include the bacterial cytochromes c_2[1,2] and both cytochrome *b*-562[3] and the cytochrome *d* terminal oxidase complex of *Escherichia coli*.[4] NMR and other spectroscopic evidence has shown, at least for the cytochromes c_2, that the redox potential is controlled by the state of ionization of a heme propionate or a noncoordinated imidazole of a His residue in close proximity to the iron porphyrin.[1] This provides an example of how proteins couple changes at one site ($\pm e^-$ at Fe) to changes at another site ($\pm H^+$ at an ionizable functional group), an effect that may be relevant, for example, to the proton-pumping activity of various components of the respiratory chain and physiological control of electron transport.[4-8]

We report here that the midpoint potential of the imidazole complex of the heme octapeptide from cytochrome *c*, microperoxidase-8 (MP8)[9-11] (Figure 1), determined by direct dc cyclic voltammetry at a glassy-carbon electrode, is strongly pH dependent and models these pH-linked redox effects in the cytochromes. The redox potential is controlled by at least five ionizations, three in the oxidized and two in the reduced heme peptide. We suggest that a heme propionate, the terminal amino group of the polypeptide, and the imino N atom of coordinated imidazole are the ionizing groups responsible.

The strong susceptibility of aquo-MP8 to dimerization in aqueous solution[9] makes a study of its electrochemical behavior problematic. A cyclic voltammetry investigation of the related heme undecapeptide, MP11,[12] yielded results that were apparently independent of MP11 concentration (0.1–1 mg/mL, i.e., 50–500 μM); with MP8, this would mean only 25%–9% monomer would be present in aqueous solution.[9] We therefore chose to perform our electrochemical experiments with MP8 in the presence of saturating concentrations of imidazole (0.15 M; the binding constant is 2.82×10^4 [10]). Successive dilutions of imidazole-MP8 were performed with buffered 0.15 M imidazole (0.1 M CAPS, pH = 10.2; μ = 1.0 M (KCl); 25 °C) to produce solutions with [MP8] = 1.2 mM to 80 nM, which were monitored at 527 nm in 0.1-, 1.0-, and 10.0-cm path-length cells. The complex obeys Beer's law over the entire concentration range (ϵ = 8540 ± 5 M^{-1} cm^{-1}). Observed effects are therefore strictly attributable to a monomeric complex in aqueous solution.

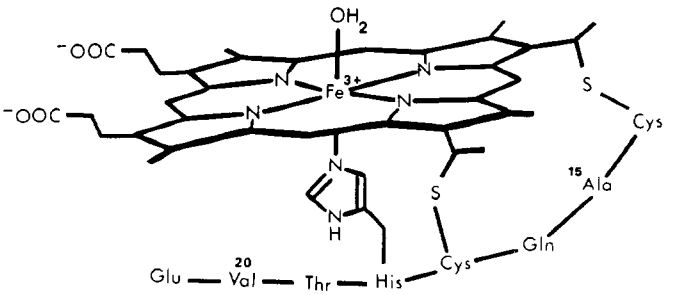

Figure 1. The heme octapeptide, microperoxidase-8 (MP8), obtained from proteolytic degradation of horse heart cytochrome *c*. The amino acid residue numbering is that of the parent protein.

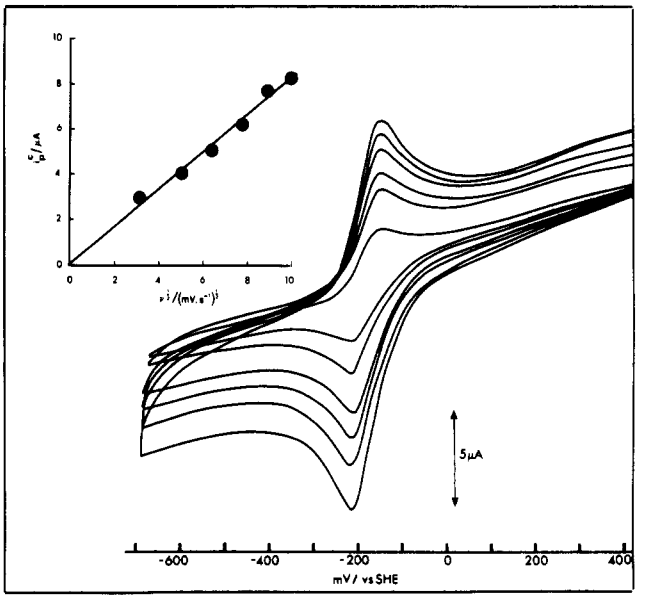

Figure 2. Cyclic voltammetry of MP8 (0.978 mM) is 0.15 M imidazole (μ = 1.0 M (KCl); 25 °C) on a glassy-carbon electrode, pH = 7.0, vs SHE. Sweep rates (mV s^{-1}): (a) 100; (b) 80; (c) 60; (d) 40; (e) 25; (f) 10. The inset shows a plot of peak cathodic currents as a function of (scan rate)$^{1/2}$. The slope gives the diffusion coefficient, D_0.[13]

MP8 was prepared as previously described.[9] Spectra were recorded on a Cary 2300 spectrophotometer fitted with thermostated cell holders. Cyclic voltammetry experiments were conducted on 3 mL of 0.978 mM MP8 in the presence of 0.15 M imidazole at 25 °C (μ = 1.0 M (KCl)) under N_2 by using a MINTEK Potential-GalvanoStat instrument (Council for Mineral Technology, Randburg, South Africa) operating in the potentiostat mode with a 3.0-mm-diameter glassy-carbon working electrode, a Ag/AgCl reference electrode, and a Pt-plate counter electrode. The electrode surface was manually polished on a polishing gauze with diamond powder slurry followed by sonication to produce a black mirrorlike surface. pH was adjusted by judicious addition of concentrated HCl or saturated KOH and measured with a Metrohm 605 pH meter and a microcombination glass electrode calibrated aganst standard buffers.

Voltammograms obtained at pH 7.0 are shown in Figure 2. The peak anodic to peak cathodic current ratio was 0.85 ± 0.06

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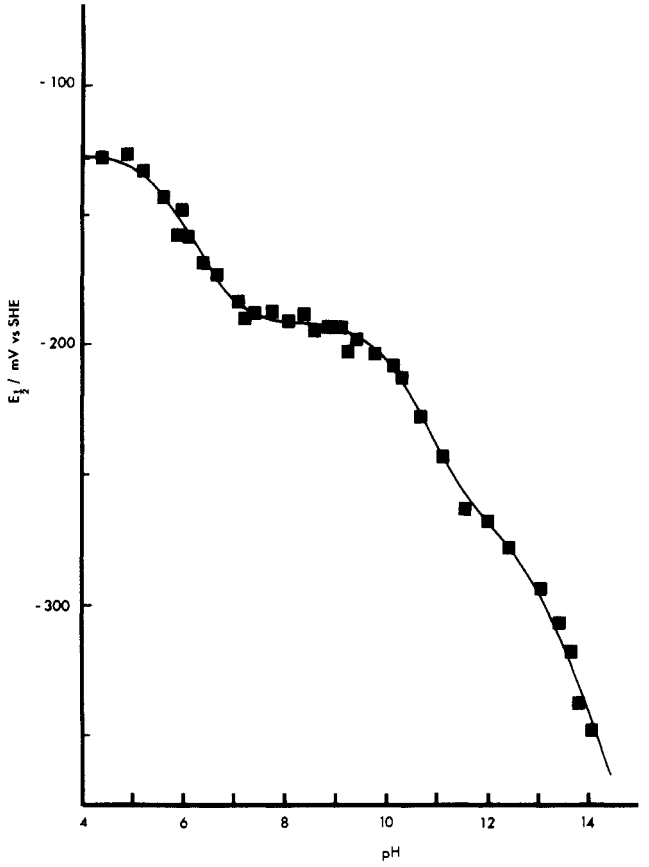

Figure 3. pH dependence of the redox potential of imidazole-MP8. Other experimental conditions are as in the caption to Figure 2. The curve is a fit of eq 1 of the text and shows that $E_{1/2}$ is controlled by at least five ionizations (all ± 0.1): $pK_1^{III} = 5.6$; $pK_1^{II} = 6.8$; $pK_2^{III} = 10.1$; $pK_2^{II} = 11.5$; $pK_3^{III} = 12.9$.

with a peak separation of 75 ± 5 mV at a sweep rate of 100 mV s^{-1}, which decreased to a minimum value of 60 ± 5 mV at a sweep rate of 40 mV s^{-1}. This indicates[13] a reversible one-electron-transfer reaction. The diffusion coefficient was found to be 4.5 ± 0.6 × 10^{-5} cm^2 s^{-1} (inset to Figure 2); hence the rate constant for heterogeneous electron transfer was calculated[14] to be (4 ± 2) × 10^{-2} cm s^{-1}. The midpoint potential at pH 7.0, −185 ± 5 mV vs SHE, is in reasonable agreement with that obtained by reductive titration for imidazole-MP11 (−200 mV) and for imidazole-MP8 (−210 mV) at 30 °C.[15] The value is also close to that for aquo-MP11 (−160 mV),[12] confirming an early report[15] that aquo and imidazole complexes of the microperoxidases have very similar redox potentials. The diffusion coefficient and the rate constant for electron transfer are significantly larger than reported for aquo-MP11 (2 × 10^{-6} cm^2 s^{-1}; 3 × 10^{-3} cm s^{-1} [12]), but the values are not strictly comparable because of the differences in axial ligand and in the size of the polypeptide and the possible involvement of dimeric species (see below) in the MP11 work.

As indicated in Figure 3, the midpoint redox potential is pH dependent in the pH range 4–14 (Figure 3);[16] this process is independent of the nature of the working electrode since cyclic voltammetry at a few pH values between pH = 7 and pH = 13 on a Pt working electrode and between pH = 5 and pH = 7 on a gold electrode yielded very similar results.[17] A minimum requirement for the system is five pK_a values, three (pK^{III}) in the oxidized and two (pK^{II}) in the reduced heme peptide; this leads[19] to eq 1, which, when fitted to the data by standard nonlinear

$$E_{1/2} = E' + 59.13 \times \log\left[\frac{[H^+]^3 + K_1^{II}[H^+]^2 + K_1^{II}K_2^{II}[H^+]}{[H^+]^3 + K_1^{III}[H^+]^2 + K_1^{III}K_2^{III}[H^+] + K_1^{III}K_2^{III}K_3^{III}}\right] \quad (1)$$

least-squares methods, gave $E' = -127 \pm 3$mV (vs SHE), $pK_1^{III} = 5.6 \pm 0.1$, $pK_1^{II} = 6.8 \pm 0.1$, $pK_2^{III} = 10.1 \pm 0.1$, $pK_2^{II} = 11.5 \pm 0.1$, and $pK_3^{III} = 12.9 \pm 0.1$.

Our results indicate that uptake of an electron at the metal (which reduces the net charge on the metal center from +1 to 0) causes a group with $pK_1^{III} = 5.6$ to increase to $pK_1^{III} = 6.8$. The only candidates for this ionizing group are the heme propionates, the side chain of Glu-21, and the C-terminal carboxylate (Figure 1). In the free amino acid, the last two pK_as are 4.31 and 2.13, respectively,[20] whereas the pK_as of heme propionates are usually in the range 5–6.[21] This suggests that the ionizing group is a heme propionate and that the difference in its pK_a in the ferric and ferrous complex is due to a decrease in electrostatic interaction with the metal center on reduction. A $\Delta(pK_a)$ of 1.2 is in agreement with the $\Delta(pK_a)$ of ~1 reported for the cytochromes.[1] $pK_3^{III} = 12.9$ is probably due to the ionization of coordinated imidazole, a process that can be monitored spectrophotometrically with $pK_a = 13.10 \pm 0.09$. Formation of an imidazolate will increase electron density at the metal[22,23] (the α, β, and Soret bands increase from 555, 527, and 404.1 nm to 565, 535, and 410.1 nm, respectively) and causes the midpoint potential to shift to more negative values. The only likely candidate for the group ionizing with $pK_2^{III} = 10.1$ and $pK_2^{II} = 11.5$ is the terminal amino group on Cys-14 ($pK_a = 10.34$ in the free amino acid[20]).

The decrease in the pK_a of imidazole from 14.4[24] when free to ~13 when coordinated to MP8 has many precedents in the hemoproteins; for example, it is 10.34 when imidazole is coordinated to sperm whale metMb[25] and 10.45 in *Chironomus plumosus* metHb.[26] The effect is undoubtedly a consequence of the polarizing effect of the metal ion. Formation of an imidazolate causes the redox potential to increase, and partial deprotonation by hydrogen bonding of the proximal His[26,27] may be one of the key factors that decreases the redox potential of horseradish peroxidase ($E_{1/2} = -270$ mV[28]) relative to myoglobin ($E_{1/2} = +50$ mV[29]), consistent with the function of the former as a peroxidase that features high-oxidation state intermediates in the catalytic

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(16) Below this pH, the cathodic wave due to the complex is replaced by one at more positive potential due to free MP8. This process can be monitored spectrophotometrically ($pK_a = 4.47 \pm 0.04$) as low-spin imidazole-MP8 is converted into a high-spin complex. At pH 2.5, the Soret maximum is at 394 nm, which indicates[10] the loss of both axial ligands. Aquo-MP8 reversibly loses His-18 with $pK_a = 4.43 \pm 0.09$.[10]
(17) Polished glassy carbon has surface carboxylate groups that undergo ionization with an apparent $pK_a = 5.6$.[18] This might be expected to influence the observed pK_a attributed to the iron porphyrin. Although there is a decrease in Faradaic response at pH ≤ ~6 as found for cytochrome *c* and $Fe(CN)_6^{3-}$ among others,[18] we obtained very similar results on a gold electrode. The surface ionization of glassy carbon thus appears to have no significant influence on the *position* of the midpoint potential.
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cycle and of the latter as a dioxygen storage protein that requires the ferrous state.

The present results show that reversible electron transfer to imidazole-MP8 is controlled by three electrostatic gates. Protonation of the heme propionate and deprotonation of the N-terminal amino group decrease the midpoint potential of the Fe(III)/Fe(II) couple because of changes in Coulombic interaction between these groups and the metal ion. This models equivalent electrostatic perturbations of the redox potential of various cytochromes where ionizing groups have been identified as a heme propionate ($\Delta(pK_a) = \sim 1$) or a noncoordinated His ($\Delta(pK_a) = \sim 0.4$[1]) and means that electron uptake at one center is coupled to proton uptake at another as decreased charge on the metal ion raises the pK_a of these ionizing groups. MP-8 has previously proved a useful protein-free model for the peroxidase enzymes,[30,31] for studying reactions of protein-free heme-bound dioxygen,[32] and for delineating features of hemoprotein topology that control access to the heme.[33] The effects described here now provide a plausible mechanism for control of redox potentials and proton pumping by the cytochromes. Work with other MP8 derivatives and other small heme peptides that will further explore this hypothesis is planned and will be reported elsewhere.

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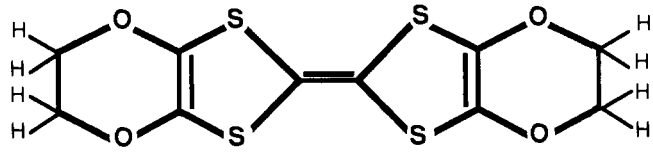
The First Ambient Pressure Organic Superconductor Containing Oxygen in the Donor Molecule, β_m-(BEDO-TTF)$_3$Cu$_2$(NCS)$_3$, T_c = 1.06 K

In this communication, we report the discovery of superconductivity in an organic charge-transfer salt containing an oxygen-bearing organic-donor molecule. A review of the literature[1] on organic conductors and superconductors reveals that all such superconductors are based on Se- or S-containing organic electron-donor molecules. In fact, an increasingly large number of *ambient-pressure* organic *superconductors* occur as the atomic number of the chalcogen atoms in the electron- donor molecule decreases from Se to S. So far, one ambient-pressure superconductor has been discovered in an all-Se-containing system, (TMTSF)$_2$ClO$_4$ ($T_c \cong 1.1$ K),[2] and at least nine such superconductors are found in S-containing systems based on BEDT-TTF.[3] The latter system has yielded the highest transition temperature obtained thus far in an organic superconductor, for κ-(BEDT-TTF)$_2$Cu(NCS)$_2$, $T_c \cong 10.4$ K.[4] If this trend continues one might expect to find a number of O-based organic superconductors, as appropriate organic electron-donor molecules are synthesized. As suggested by Wudl and co-workers,[6a] there may be a remote connection between oxygen containing organic superconductors and Cu–O high-T_c superconductors.[5] In addition, according to BCS theory[18] (isotope effect), a lighter atom (oxygen) than S or Se in otherwise identical organic-donor molecules could cause T_c to rise in the lighter atom salt. Expanding on this possibility, we point out that the discovery of superconductivity in an oxygen-containing organic system could be important for systematically expanding the range of possible organic superconductors and for potentially establishing a link between organic superconductors and the recently discovered high-temperature oxide superconductors.[5] In fact, organic superconductors have many features in common with the high-T_c oxide superconductors; viz., both are layered compounds, both have short anisotropic superconducting coherence lengths (type-II superconductors), and both have superconducting ground-states competing with magnetic instabilities. In this communication, we report the first organic superconductor based on the oxygen-bearing electron-donor molecule BEDO-TTF[6,7] [BEDO-TTF is bis(ethylenedioxy)tetrathiafulvalene, **1**],



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and a copper-containing inorganic anion. We describe herein the synthesis of this new material, its crystal structure, its characteristic ESR properties, inductive measurements of its superconducting transition, and its band electronic structure.

Synthesis and ESR Study. The salt, which we denote as β_m-(BEDO-TTF)$_3$Cu$_2$(NCS)$_3$, was prepared by electrocrystallization of the organic donor BEDO-TTF[6a] (7.0 mg, 0.022 m mol), CuSCN (27.5 mg, 0.23 m mol), KSCN (22.1 mg, 0.23 m mol), and 18-crown-6 (~60 mg, 0.23 m mol) in 1,1,2-trichloroethane with 10% (vol) absolute ethanol at room temperature. Crystal growth was carried out in a drybox purged with nitrogen gas. The current density applied was 0.07 μA/cm^2, and small black crystals were harvested after 25 days. Preliminary room-temperature ESR data indicate an anisotropic single line absorption in which the peak-to-peak line width varies from 17.5 to 27 G. No other line width was observed in several samples, indicating that the title compound is the only product formed under the above preparative conditions. An analysis of the copper content was carried out by use of an inductively coupled plasma/atomic emission spectrometer (ICP/AES). A similar, but well-defined, compound, κ-(BEDT-TTF)$_2$Cu(NCS)$_2$, was also analyzed for comparison; % Cu, found (calcd) = 6.53 ± 0.33 (6.69). The result for β_m-(BEDO-TTF)$_3$Cu$_2$(NCS)$_3$; % Cu, found (calcd) = 9.90 ± 0.50 (10.06). Low-temperature single-crystal ESR studies revealed a monotonic decrease in line width from 12.6 G at 150 K to 1.5 G at 4.2 K, and the ESR spin susceptibility was approximately constant from 150 to 30 K, below which temperature a Curie-like impurity tail was observed. The Curie-like tail could be caused by chemical contamination from a magnetic impurity or by the formation of paramagnetic centers due to twinning or packing defects. The constant spin susceptibility (30–150 K) is consistent with the Pauli paramagnetism expected of a metallic specimen.

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