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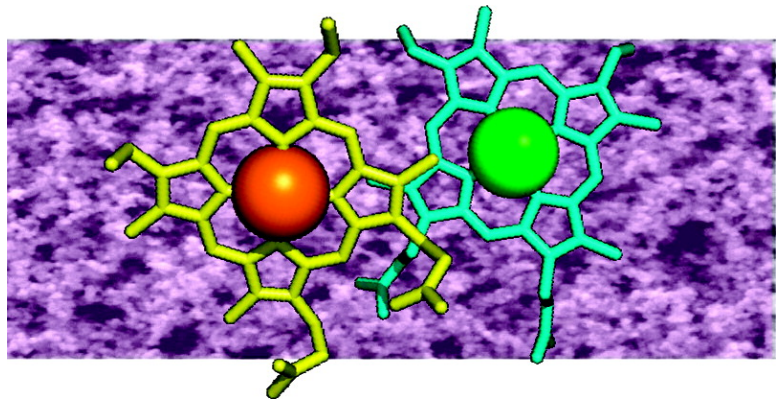

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Spectroelectrochemical Characterization of a Pentaheme Cytochrome in Solution and as Electrocatalytically Active Films on Nanocrystalline Metal-Oxide Electrodes

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Redox enzymes are numerous and ubiquitous. To date the majority of these enzymes have been found to catalyze two-electron transformations of their substrates. However, a pentaheme containing cytochrome, NrfA, from *Escherichia coli* catalyzes the six-electron reduction of nitrite, in addition to the five-electron reduction of nitric oxide, to achieve transformations that would otherwise require four, or three, distinct enzymes respectively.[1–5] The search for an understanding of the NrfA reaction mechanism(s) is usefully inspired by several structures.[1,6,7] In addition, protein film voltammetry (PFV) has provided insight into rate-limiting events during the NrfA-catalyzed reduction of various substrates.[2–4] However, unusually for such a well-studied enzyme, the thermodynamic descriptions of its redox activity are incomplete.[1,8] Here we rectify this situation through simultaneous electrodic manipulation and spectroscopic characterization of NrfA both in solution and as electrocatalytically active films on mesoporous SnO_2 electrodes.

In oxidized NrfA the active site heme 1 is high-spin ferric with lysine and water/hydroxide as axial ligands while hemes 2 to 5 are low-spin ferric with his−his ligation, Figure 1.[1] All of these hemes give rise to intense electronic absorption bands in the Soret region (350−420 nm) but ambient temperature magnetic CD (MCD) spectra at these wavelengths are dominated by broad bands from the low-spin hemes that are bisignate, that is, have positive and negative features, Figure 2. Reduction of the sample by mediated electrodic potentiometry[9,10] shows the MCD Soret bands decrease in intensity below ca. 50 mV versus SHE and in parallel with the appearance of a sharper bisignate band centered on 550 nm, the characteristic α-band of low-spin ferrous heme, Figure 2. A plot of α-band intensity versus equilibration potential is well described by the sum of four $n = 1$ Nernstian contributions with equal intensities. The average of two experiments gave midpoint potentials (E_ms) of −20, −153, −206, and −292 mV (all ±20 mV) for the low-spin hemes.

At the concentration of NrfA used in the MCD titers, weak features at 600−650 nm which are generally used as "marker" bands for high-spin ferric heme cannot be detected. However, a broad bisignate MCD band centered on 436 nm and characteristic of high-spin ferrous heme appears on sample reduction, Figure 2. The intensity of this signal is well described by a single $n = 1$ Nernstian component with $E_m = -108$ mV and that we assign to heme 1. Oxidative titration of NrfA returned the MCD spectrum to that displayed by the as isolated enzyme and gave E_ms for the high- and low-spin hemes in good agreement with those from the reductive titer.

The five E_ms defined here usefully inspire a re-evaluation of the NrfA titer monitored by EPR spectroscopy at X-band in the perpendicular mode where, since only three types of resonance were observed, some ambiguities remained.[1]Tying E_ms to EPR signatures is also valuable because low-spin heme resonances can be related to axial ligand conformation which distinguishes hemes 2 and 3 from hemes 4 and 5.[1] The $E_m \approx -20$ mV has a counterpart in reduction ($E_m \approx -37$ mV) of the rhombic trio of EPR resonances assigned to heme 2. The $E_m \approx -292$ mV correlates with titration ($E_m \approx -320$ mV) of a large g_{max} resonance that should now be assigned to reduction of one, rather than both, of hemes 4 or 5. Reduction of heme 1 with $E_m \approx -108$ mV is consistent with the titration ($E_m \approx -107$ mV) of resonances from the spin-coupled ferric pair formed by hemes 1 and 3. Loss of resonances from the spin-coupled ferric pair was not accompanied by the appearance of new resonances from ferric hemes, so coordinated reduction of hemes 1 and 3 was a possibility. However, this is not supported by the MCD titer. It remains to be established whether this difference arises from distinct electronic properties of NrfA at ambient and liquid-helium temperatures or a coupling of high-spin ferrous heme 1 and low-spin ferric heme 3.

Because PFV has provided much kinetic analysis of NrfA, with heme redox events proposed to drive boosts and attenuations of activity on traversing the electrochemical potential domain,[2–4] we now turn to consider how the properties of adsorbed NrfA relate to those displayed in solution.

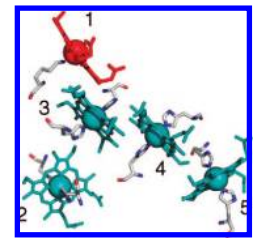

Figure 1. The low-spin (blue) and high-spin (red) hemes of *E. coli* NrfA and their amino acid ligands.

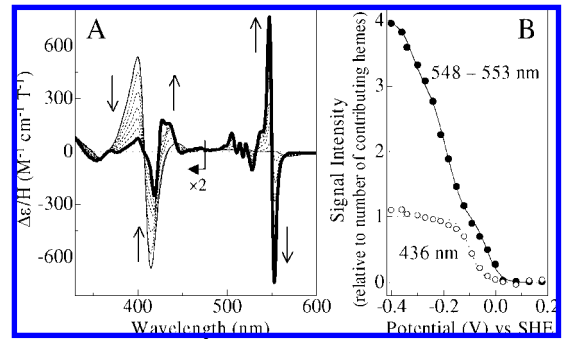

Figure 2. (A) Ambient temperature MCD spectra of *E. coli* NrfA equilibrated at +180 (thin solid line), 0, −90, −150, −210, −240, −300, and −400 (heavy solid line) mV. NrfA (30 μM) in 50 mM Hepes, 100 mM NaCl, pH 7. Magnetic field, 6 T. Open headed arrows indicate spectral change on reduction. (B) Variation of MCD signal intensity (circles) with potential for the wavelengths indicated. Solid line is a fit to four $n = 1$ Nernstian components with E_ms = −35, −164, −217, and −326 mV. Broken line is a fit to one $n = 1$ Nernstian component with $E_m = -99$ mV.

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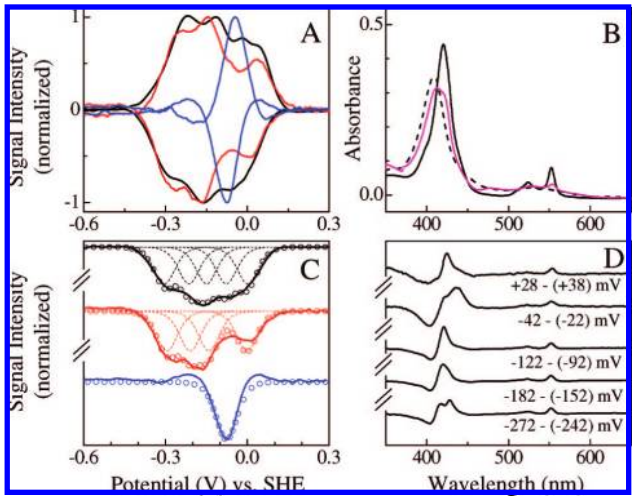

Figure 3. Spectroelectrochemical characterization of *E. coli* NrfA adsorbed on mesoporous SnO_2. (A) Faradaic current (black), dA_{552nm}/dE (red) and dA_{442nm}/dE (blue); scan rate = 5 mV s^{-1}. (B) Electronic absorbance spectra in 0 (black) and 1 mM nitrite (purple) at +366 mV (broken line) and −634 mV (solid lines). (C) Data from reductive sweep (solid lines), fits (circles), and $n = 1$ Nernstian contributions (broken lines) for Faradaic current (black), dA_{552nm}/dE (red), and dA_{442nm}/dE (blue). (D) Electronic absorbance difference spectra for the potentials indicated. Buffer-electrolyte: 2 mM $CaCl_2$, 50 mM Hepes, pH 7, 24 °C.

To date, gold and graphite electrodes have been employed for PFV of NrfA.[2–4,11] These strategies have failed to resolve signals that report on redox events in the absence of substrate, a situation that is most likely to arise from low electroactive coverage of NrfA. By contrast, clear peaks are observed from cyclic voltammetry of mesoporous, nanocrystalline SnO_2 electrodes that have been soaked with NrfA[12,13] and rinsed thoroughly with buffer−electrolyte (Figure 3A). Integration shows that the oxidative and reductive peaks, not seen in the absence of enzyme, have equal areas. Electronic absorption spectroscopy, permitted by the optical transparency of SnO_2, shows that the peaks reflect complete reduction and oxidation of adsorbed NrfA (Figure 3B). Combining the voltammetric and spectroscopic information allows the electroactive enzyme to be quantitated. For example, the voltammetric peaks from an electrode of geometric area 0.7 cm^2 soaked in 22.4 μM NrfA for 18 h arise from 3.03 ± 0.20 nmol electrons that equates to ca. 0.6 nmol electroactive enzyme given the five hemes of NrfA. Since the effective surface area of the mesoporous nanocrystalline SnO_2 electrodes is ca. 300× their geometric area[13] this equates to ca. 3 pmol NrfA cm^{-2} which is close to that predicted for a monolayer.

To assess the catalytic activity of NrfA films voltammetry was performed in 1 mM nitrite. The voltammograms were transformed with the peaks replaced by a catalytic reduction wave and electronic absorption spectra similar to that of oxidized enzyme were collected across the entire potential window examined, e.g., Figure 3B. Thus, the majority of adsorbed NrfA is active toward nitrite reduction with catalysis limited by the rate of enzyme reduction.

The voltammetric peaks seen in the absence of nitrite describe the total redox activity of the NrfA film. However, the contributions from low- and high-spin hemes can be resolved by virtue of their distinct electronic absorbances. Features typical of low-spin heme reduction with maxima between 418 and 429, 523, and 554 nm are present in difference spectra generated for closely spaced equilibration potentials (Figure 3D). An additional Soret maximum at 435.5 nm typical of high-spin heme reduction dominates difference spectra collected between +20 and −60 mV. Thus, redox transitions primarily associated with the low- and high-spin hemes are resolved by monitoring absorbance at 552 and 442 nm, respectively, during cyclic voltammetry. Overlaying the first derivative of each absorbance with respect to applied potential (dA/dE)[13] demonstrates their distinct contributions to the total redox response (Figure 3A). The dA_{442nm}/dE and dA_{552nm}/dE plots are well described by one and the sum of four equal intensity, $n = 1$ Nernstian contributions, respectively (Figure 3C). Averaging the responses from eight films gave an E_m of −56 mV for high-spin heme 1 and of +22, −117, −189, and −275 mV for the low-spin hemes (all ±15 mV). For any given film, the voltammetric peaks are well described by the sum of five $n = 1$ Nernstian components of equal intensity and E_ms within 20 mV of those determined optically. Since the voltammetry should arise from five equal intensity contributions this supports the assumption of equal extinction coefficients used to extract the low-spin heme E_ms.

The results presented here show NrfA displays similar electronic properties at ambient temperatures in solution and as an adsorbed film. The order of E_ms, with that for low-spin heme 2 > high-spin heme 1 > low-spin hemes 3 to 5, can be compared to the modulations of activity resolved by catalytic PFV at similar temperatures and nitrite concentrations <15 μM that are likely to be physiologically relevant. The onset of activity is centered on ca. −100 mV in a feature with the steepness of an $n = 2$ process and attenuated in an $n = 1$ process centered on ca. −320 mV. Since the studies presented here provide no evidence for an $n = 2$ event in the thermodynamic properties of NrfA this must arise as a consequence of substrate interactions with the enzyme and as proposed for the *Desulfovibrio desulfuricans* NrfHA complex.[14] By contrast, the attenuation process correlates with the thermodynamic description for reduction of the lowest potential heme, and we anticipate that the identity of this heme will be resolved when the methods described here are applied to site-specific variants of NrfA.

In conclusion, spectroelectrochemical studies of *E. coli* NrfA have provided unprecedented resolution of its thermodynamic properties, and it is likely that the methodologies used here will facilitate studies of other oxidoreductases for which catalytic PFV is well defined, but comparable thermodynamic descriptions remain elusive.

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Supporting Information Available: Cyclic voltammetry of NrfA coated SnO_2 electrode in nitrite, experimental and fitting procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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