Acknowledgment. This work was supported by grants from the National Institute of General Medical Sciences (GM 19035) and the Yale University NSF Regional Instrumentaion Center (NSF CDP 7916210).

A Ferrous, High-Spin Heme *a* Model for Cytochrome a_3 in the Dioxygen Reducing Site of Cytochrome Oxidase

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Received July 20, 1981

Cytochrome oxidase catalyzes the four electron reduction of dioxygen to water in its role as the terminal oxidase in mitochondrial electron transport.[1] The catalysis is remarkably efficient: the enzyme operates at an overvoltage which is only 300–400 mV above the reversible potential of the water/dioxygen couple.[2] The structural and mechanistic basis for this efficiency appears to lie in the binuclear nature of the dioxygen reducing site. A copper atom, denoted Cu_{a_3}, and a heme *a* bound iron atom, cytochrome a_3, comprise this site and in the oxidized enzyme are strongly exchange coupled;[3] in the reduced enzyme they apparently act in concert to reduce dioxygen to a bound peroxy intermediate which is subsequently reduced to water.[4] Some evidence exists which indicates that the dioxygen reducing site is apolar[5] but that it communicates with bulk solution by a hydrophilic channel.[6] Sporadic attempts to prepare heme *a* model compounds for deoxycytochrome a_3^{2+} have been reported;[7] however, these have been limited to aqueous solution and have been only partially successful. For the experiments reported here we developed techniques for producing both high- and low-spin ferrous heme *a* in aprotic solvents. A comparison of the optical and resonance Raman properties of these models, and of the same species in water, with key spectroscopic properties of the enzyme shows that the iron of deoxycytochrome a_3 is high spin and that the dioxygen reducing site is hydrophobic.[8]

Reduction of heme *a* in aprotic solvents was carried out in an inert atmosphere by titrating solutions of the oxidized chromophore in the solvent of interest with a near-stoichiometric amount of sodium dithionite/[2.2.2]-cryptand solution, a procedure derived from that used by Mincey and Traylor.[9] Anaerobicity was achieved either by purging the solutions with oxygen-free argon gas or by using successive freeze–pump–thaw cycles. The extent of heme *a* reduction was monitored optically during the titration; excess dithionite was found to alter the spectrum of heme *a*, possibly by inducing adduct formation at the formyl group on the porphyrin periphery. Other reducing systems (e.g., hydrazine hydrate, hydrogen/Pd, $NaBH_4$) attack this group and were not examined as potential reductants in any detail. The technique of overlaying the organic solvent/heme *a* solution with aqueous dithionite was also found unsuitable owing both to the amphiphilic nature of heme *a* and to the slight solubility of H_2O in the solvents of interest. For five-coordinate high-spin models, 2-methylimidazole was employed as the sole axial ligand.[10] Low-spin models had bis(*N*-methylimidazole) ligation in the axial sites. Raman spectroscopy with Soret laser excitation was carried out as described previously.[5a]

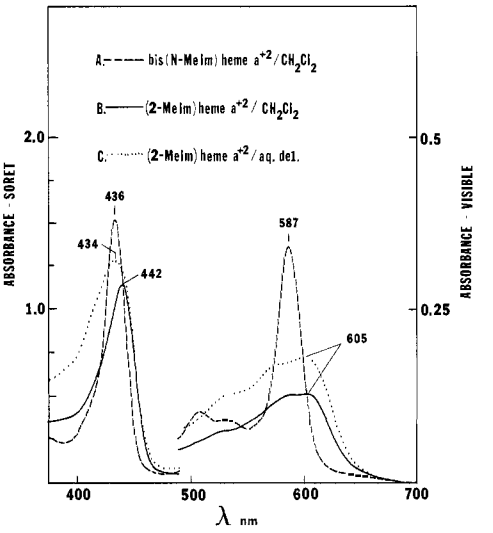

Figure 1. Optical spectra of reduced heme *a* model compounds (cell path length is 0.5 cm). (A) Low-spin reduced heme *a*; ~27 μM in heme, 0.3 M in *N*-methylimidazole; aprotic solvent is CH_2Cl_2; (B) high-spin reduced heme *a*; approximately 22 μM in heme, 2.5 mM in 2-methylimidazole; aprotic solvent is CH_2Cl_2; (C) high-spin reduced heme *a*; approximately 33 μM in heme, 1.2 mM in 2-methylimidazole; aqueous solvent is 0.07 M cetyltrimethylammonium bromide and 0.1 M potassium phosphate buffered to pH 7.4.

Figure 1 presents optical spectra of low-spin heme a^{2+} in CH_2Cl_2 and of high-spin a^{2+} in both CH_2Cl_2 and aqueous detergent solution. The high-spin model in aprotic solution has optical properties clearly resembling the 443-nm Soret maximum and the weak 600-nm visible absorption determined for cytochrome a_3^{2+}.[11] When the ferrous high-spin species is prepared in water, there is a shift to 434 nm in the Soret region with little change in the visible spectrum. Low-spin heme *a* shows a significant increase in visible absorption, and the Soret maximum occurs at 436 nm. Interestingly, there is very little solvent dependency in the absorption properties of the low-spin species.[12] Evidence presented elsewhere indicates that low-spin bis(imidazole) heme *a* is a reasonable first-order model for the other heme *a* chromophore in cytochrome oxidase, cytochrome *a*.[7b,13]

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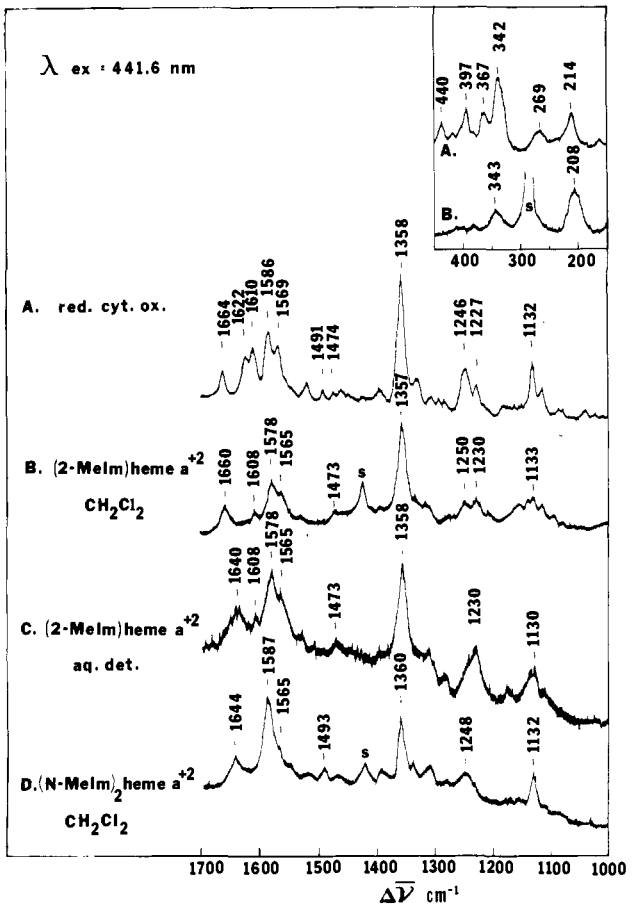

Figure 2. Resonance Raman spectra taken with 441.6-nm excitation, 15-mW incident power. Scan rate 50 cm^{-1} m^{-1}; time constant 1 s; spectral resolution 7 cm^{-1}. CH_2Cl_2 solvent peak marked "S". (A) Reduced cytochrome oxidase; (B) high-spin 2-methylimidazole heme a^{2+}/CH_2Cl_2; (C) high-spin 2-methylimidazole heme a^{2+}/aqueous CTAB; (D) low-spin bis(*N*-methylimidazole) heme a^{2+}/CH_2Cl_2. Heme concentrations: 60–120 μM. Inset: low-frequency region of traces A and B, reduced cytochrome oxidase and 2-methylimidazole heme a^{2+} dissolved in CH_2Cl_2, respectively.

The Raman spectra of the heme *a* model compounds, along with the spectrum of reduced oxidase, are shown in Figure 2. Soret laser excitation was used in the experiments, Franck-Condon scattering dominates, all modes in the spectra of Figure 2 have depolarization ratios $<^3/_4$.[14] Although the Raman spectrum of the reduced enzyme is composed of contributions from both cytochrome a^{2+} and a_3^{2+}, we showed in previous work[15] that some of the vibrations were due solely to cytochrome a_3^{2+}. These include the formyl vibration at 1664 cm^{-1}, the ring mode at 1230 cm^{-1}, and the low-frequency 214-cm^{-1} vibration. An investigation of the model spectra shows that for the high-spin ferrous heme *a* species in CH_2Cl_2 (Figure 2b) the formyl vibration occurs at 1660 cm^{-1} and reproduces the behavior of a_3^{2+} well. In aqueous solution (Figure 2c) the high-spin heme *a* formyl vibration shifts to 1640 cm^{-1} which we interpret to reflect hydrogen bonding. In low-spin derivatives the carbonyl occurs at 1644 cm^{-1} in aprotic media (Figure 2d) and shifts to still lower frequency in protic solvents.[16] High-spin ferrous heme *a* in aprotic solvents also reproduces the a_3^{2+} 1230-cm^{-1} mode. However, the frequency of this mode, in contrast to the formyl vibration, appears to be independent of solvent. In the low-frequency region (inset, Figure 2), five-coordinate high-spin ferrous heme *a* in CH_2Cl_2 shows a 208-cm^{-1} vibration which corresponds well with the a_3^{2+} 214-cm^{-1} mode. This vibration is missing in the low-spin derivatives and weak or absent in high-spin species in protic solvents (not shown). By analogy with work on hemoglobin,[17] we had assigned the protein 214-cm^{-1} band to the a_3^{2+} Fe–N_ϵ(histidine) vibration;[5a] the high-spin model compound provides additional support for this assignment. Moreover, a comparison of the a_3^{2+} 214-cm^{-1} frequency with recent work by Stein et al.[18] indicates that in the protein the histidine N_δ is protonated and diminishes considerably the likelihood that this species serves as the bridging ligand between Cu_{a_3} and Fe_{a_3} in the dioxygen reducing site.[19]

The data on ferrous heme *a* model compounds solidify the assignment of cytochrome a_3^{2+} as a five-coordinate, high-spin ferrous heme *a* species with an unaltered formyl in a non-hydrogen-bonding environment.[5a,20] That high-spin heme a^{2+} in CH_2Cl_2, but not in aqueous detergent solution, has optical properties in close agreement with a_3^{2+}, together with the IR data reported by Alben et al.,[5b] indicates that hydrophobic character in the dioxygen reducing site is not restricted to the immediate environment of the formyl group but rather is a property of a major portion of the pocket.

Acknowledgment. The portion of this research which was carried out at Michigan State University was supported by NIH Grant GM25480. We thank Patricia Callahan for helpful discussions.

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Correlation of Nonadditive Kinetic Effects with MINDO/3 Derived Molecular Geometries[1]

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Received May 20, 1981

Few studies have been reported which attempt to correlate chemical reactivity with molecular geometrical parameters.[2-6] A significant and valuable effort over the past 20 years has focused on the derivation of linear free energy relationships (LFER).[7] Although at least 11 different steric substitution parameters have

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