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EPR Detection of an O_2 Surrogate Bound to Heme c_n of the Cytochrome b_6f Complex

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The membrane-embedded cytochrome (cyt) b_6f complex mediates electron transport between the photosystem II and photosystem I reaction center complexes in oxygenic photosynthesis.[1] The 220 kDa b_6f complex consists of eight polypeptide subunits in each monomer of a functional dimer.[2−5] Four relatively large subunits bind five redox cofactors of the complex (Fe_2S_2, hemes b_p, b_n, c_n, f). Cyt b_6f couples oxidation−reduction of lipophilic plastoquinol and reduction of high potential soluble plastocyanin or cyt c_6[6] to proton translocation across the membrane, thereby generating a transmembrane proton electrochemical potential gradient. The core structure resembles that of the cyt bc_1 complex of the mitochondrial respiratory[7] and photosynthetic bacterial electron transport chains.[8] However, the cyt b_6f complex contains three prosthetic groups per monomer not found in the cyt bc_1 complex: chlorophyll a, β-carotene, and heme c_n[2,3] that is unique in not utilizing any amino acid side chain as an axial ligand. The function of heme c_n is an open question and the focus of this paper. Heme c_n interacts electronically with heme b_n via an axial OH^- or H_2O bridged through a H-bond to a propionate oxygen of heme b_n (Figure 1).[9,10] The other axial site of heme c_n is unoccupied and open to the quinone-exchange cavity that separates the two b_6f monomers. c-Type hemes with an open coordination site are uncommon and usually associated with catalytic functions. Heme c_n can bind the quinone analogues NQNO[4,9−12] or TDS[4] at the open coordination site, suggesting that plastoquinone (PQ) can bind near heme c_n, be reduced by hemes b_n/c_n that have been reduced by the Q cycle or cyclic electron transport, and thereby serve as an n-side electron transfer intermediate electron donor from the b_6f complex to the PQ pool. Based on these observations, it was proposed that this heme pair facilitates a concerted two-electron reduction of the n-side PQ, thereby avoiding the generation of a plastosemiquinone intermediate and ensuing formation of reactive oxygen species.[9,13]

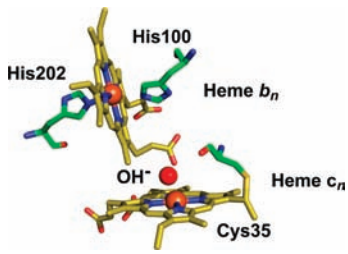

Figure 1. Hemes b_n and c_n of the cyt b_6f complex.

The present study examines the ligand-binding properties of heme c_n. Previous studies have shown that cyanide, butyl isocyanide, and azide do not bind to isolated cyt b_6f.[9,14] Three other common heme ligands (fluoride, imidazole, or nitric oxide) were added to the cyt b_6f complex in the present study and examined for binding to the complex. Addition of these small molecules in large excess to the isolated cyt b_6f complex did not result in a change to the EPR spectra. The binding constant of NO for ferric hemes is $K_{eq} = 10^3$ to 10^5 M^{-1}.[15,16] If the binding constant of NO to heme c_n is within this range, the loss of the native cyt b_6f signal due to NO binding would be readily detectable. Thus, the native ferric state of the heme c_n does not bind these molecules. All known five-coordinate ferric hemes bind cyanide, except when sterically hindered by close protein residues, which is not seen in crystal structures of wild type cyt b_6f.[2−5] Membrane proteins with active sites deep within the membrane (e.g., cyt c oxidase) also bind cyanide, where charged molecules might not penetrate. The crystal structure data indicate that access to heme c_n is not blocked by the side chains of the neighboring amino acid residues and that the heme c_n site is located near the surface of the protein and the membrane.[2−5] Furthermore, neutral forms of these putative inhibitor molecules also do not bind. At present, it is unclear what interferes with the coordination of these small molecules. Since the quinone analogues NQNO and TDS are known to bind near heme c_n, an obvious candidate would be PQ. PQ has not been detected at this location in the crystal structures of cyt b_6f.[2−5] However, the O_2 surrogate NO is shown here to serve as a ligand to reduced heme c_n as presented next.

Samples of cyt b_6f were anaerobically reduced with sodium dithionite, and their X-band EPR spectra were recorded (Figure 2A). As expected, these spectra showed loss of all signals except for the Fe_2S_2 cluster signal, which was significantly more intense and quantified to a concentration in agreement with the protein concentration determined optically from cyt f. Upon addition of NO to the anaerobically reduced complex, a new signal appeared (Figure 2B). Figure 2C shows this new signal after a subtraction of the overlapping Fe_2S_2 cluster signal. Figure 2D shows a Q-band spectrum of the same sample state after subtraction of the Fe_2S_2 signal. The simulations overlaid on the data (Figure 2C, D) are calculated with the same parameter set: a nearly axial $S = {}^1/_2$ system with $\mathbf{g} = (2.003, 2.011, 2.108)$ and ^{14}N nuclear hyperfine coupling tensor of $\mathbf{A} = (49, 57, 45)$ MHz. The multifrequency EPR data allows determination of the full hyperfine tensor of the species. The magnitude of the observed hyperfine splitting is typical of NO. Thus, this new signal originates from an $[FeNO]^7$ spin system, indicating binding of a single molecule of NO to heme c_n. The conversion of the ferrous heme c_n to the NO-bound form was quantitative, indicating that NO binds tightly to heme c_n.

This is the first clear evidence for access of O_2-like molecules to the open coordination site of heme c_n. Previous reports have observed small changes in the absorption spectra of cyt b_6f upon addition of CO, but conclusions regarding the effect on cyt b_6f were ambiguous.[14] The g- and A-values of cyt b_6f-NO and several related five- or six-coordinate NO complexes are given in Table S1 for

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comparison. The *g*- and *A*-values values for heme c_n-NO of cyt b_6f are in the range typical of five-coordinate heme−NO complexes.

Heme c_n has no axial protein ligation, and the hemes in the heme b_n/c_n pair are bridged by OH^- or H_2O.[2−4] The protonation state of this axial ligand is relevant to the function of the heme pair in determination of the reduction potential,[17] and possibly as part of the proton pump mechanism of the complex.[3] Five-coordinate Fe(III) porphyrin complexes have been characterized for which the axial ligand is either OH^-[18] or H_2O.[19] The hydroxide complexes are high spin ($S = {}^5/_2$), whereas the water complexes are intermediate spin ($S = {}^3/_2$) species. The EPR data indicate that native heme c_n is high spin.[9] On the basis of comparison with characterized porphyrin complexes, we conclude that the axial ligand for oxidized heme c_n is OH^-, as indicated in Figure 1.

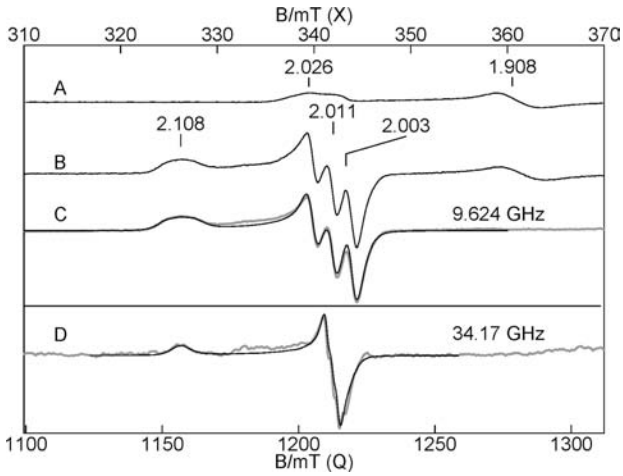

Figure 2. (A−C) X- and (D) Q-band EPR spectra of (A) anaerobically reduced cyt b_6f complex from spinach, (B) after addition of nitric oxide, (C, D, gray) after subtraction of reduced state without NO. Simulations (C, D, black) of the $S = {}^1/_2$ heme c_n-NO species using the parameters given in the text, additionally, σ_g = (0.0054, 0.0016, 0.0060). Experimental conditions: temperature 8 K (X), 34 K (Q); power, 0.002 mW (X), 0.2 mW (Q).

Implications for Function. The discovery in the present study that heme c_n can bind NO implies that heme c_n may also function as an oxidase. NO is a useful O_2 surrogate, as O_2 binding to ferrous hemes cannot be readily probed with EPR spectroscopy, and optical detection of O_2 binding to the b_6f complex is complicated by the presence of the three other hemes and a chlorophyll whose absorbance may mask that of the heme c_n.

The existence of a heme in the photosynthetic electron transport chain with properties of an oxidase is meaningful for a large number of experimental phenomena in oxygenic photosynthesis in which a low rate of oxygen uptake, or a water to water pathway, has been documented in the intermediate electron transport chain.[20−25] In addition, pathways of dark oxidation from NAD(P)H to plastoquinone have been documented.[21,26,27] Given the large number of pathways for which a plastoquinol terminal oxidase has been proposed, but the actual oxidase not identified, it is proposed that one of the functions of heme c_n, the only prosthetic group in the electron transport chain with oxidase-like properties, is the putative oxidase. It is recognized that this hypothesis has difficulties: (a) Studies on chlororespiration in *C. reinhardtii* with a cyt b_6f mutant implied the lack of involvement of the b_6f complex in this system;[22] (b) In recent studies on the green alga, *Ostreococcus*,[24] and the cyanobacterium, *Synechococcus*,[25] the lack of complete inhibition of the light-induced oxygen uptake by the quinone analogue inhibitor, DBMIB, led to the same conclusion. However, the DBMIB inhibition site, on the p- or lumenal side of the membrane, would be bypassed if PQH_2 was directly oxidized by heme c_n. The crystal structure data for the b_6f complex show that there are no structural barriers to this reaction.[2−5] The crystal structures with bound quinone-analogue inhibitors imply that heme c_n is the *n*-side site for reduction of PQ. Of course, the structure data do not distinguish the direction of the redox reaction at heme c_n. The similarity of the midpoint potentials of PQ/PQH_2 and heme c_n[11] would allow reversible redox reactions. It is then hypothesized that heme c_n can serve as an electron donor to PQ or acceptor at a low rate from PQH_2, depending on whether the heme b_n/c_n couple is in a redox state that is mostly reduced by the Q-cycle or the cyclic pathway[4] or is oxidized in the dark or the post illumination state.

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Supporting Information Available: Materials, methods, and EPR parameters of heme−NO complexes. This material is available free of charge via the Internet at http://pubs.acs.org.

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