

# Characterization of the High-Spin Heme $x$ in the Cytochrome $b_6f$ Complex of Oxygenic Photosynthesis<sup>†</sup>

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**ABSTRACT:** X-ray structures at 3.0–3.1 Å resolution of the cytochrome  $b_6f$  complex from the cyanobacterium *Mastigocladus laminosus* [Kurisu, G., Zhang, H., Smith, J. L., and Cramer, W. A. (2003) *Science* 302, 1009–1014] and the green alga *Chlamydomonas reinhardtii* [Stroebel, D., Choquet, Y., Popot, J.-L., and Picot, D. (2003) *Nature* 426, 413–418] showed the presence of a unique heme, heme  $x$ , that is covalently linked by a single thioether bond to a Cys residue (Cys35) on the electrochemically negative ( $n$ ) side of the cytochrome  $b_6$  polypeptide. Heme  $x$  faces the intermonomer quinone exchange cavity. The only axial ligand associated with this heme is a H<sub>2</sub>O or OH<sup>−</sup> that is H-bonded to the propionate of the stromal side heme  $b_n$ , showing that it is pentacoordinate. The spectral properties of this heme were hardly defined at the time of the structure determination. The pyridine hemochromagen redox difference spectrum for heme  $x$  covalently bound to the cytochrome  $b$  polypeptide isolated from SDS–PAGE displays a low-amplitude broad spectrum with a peak at 553 nm, similar to that of other hemes with a single thioether linkage. The binding of CO and a hydrophobic cyanide analogue, butyl isocyanide, to dithionite-reduced  $b_6f$  complex perturbs and significantly shifts the redox difference visible spectrum. Together with EPR spectra displaying  $g$  values of the oxidized complex of 6.7 and 7.4, heme  $x$  is defined as a ferric high-spin heme in a rhombic environment. In addition to a possible function in photosystem I-linked cyclic electron transport, the five-coordinate state implies that there is at least one more function of heme  $x$  that is related to axial binding of a physiological ligand.

The X-ray structure of the cytochrome  $b_6f$  complex from the thermophilic cyanobacterium *Mastigocladus laminosus* ( $I$ ) and the green alga *Chlamydomonas reinhardtii* ( $2$ ) documented the presence of a heme, which was almost unrecognized in the previous literature, that is adjacent and approximately orthogonal to the  $b$ -type heme ( $b_n$ ) on the electrochemically negative ( $n$ ) side of the complex (Figure 1).<sup>1</sup> The unprecedented absence of any amino acid side chain in its axial coordination, and its covalent coordination to the protein by one thioether linkage, led to the designation of this heme as heme  $x$  ( $I$ ) or, alternatively,  $c_1$  ( $2$ ). The former notation will be used in this report. Heme  $x$  was found in the site occupied by the ubiquinone molecule bound on the

$n$ -side of the cytochrome  $bc_1$  complex in the mitochondrial respiratory chain. The single covalent thioether bond involves a conserved cysteine residue (Cys35) on the  $n$ -side (stromal side) of helix A of the cytochrome  $b_6$  subunit of the  $b_6f$  complex. Cys35 is not part of a signature heme binding sequence (-Cys-X-Y-Cys-His-) that is diagnostic for  $c$ -type hemes. Electron density in the  $b_n$ -proximal axial ligand position is consistent with ligation of heme  $x$  by a water or OH<sup>−</sup> molecule that is hydrogen bonded to the carboxylate of the propionate side chain at position 7 of heme  $b_n$  and the backbone of the neighboring Gly38. However, no electron density has been resolved in the other axial position of heme  $x$  that contacts the intermonomer quinone exchange cavity. The absence of an amino acid axial ligand for heme  $x$ , together with its covalent attachment by only one thioether bond, confers on it properties of a unique heme prosthetic group.

Unlike the other  $c$ - and  $b$ -type cytochromes in the energy-transducing  $bc_1$  and  $b_6f$  complexes, which were unequivocally detected by chemical and light minus dark difference spectrophotometry, heme  $x$  had not previously been detected prior to the structure analysis by most analyses using such methods, and therefore had not been recognized and is not included in almost all descriptions of the photosynthetic electron transport chain. An exception is a set of analyses on the green alga *Chlorella sorokinia* using flash kinetic spectroscopy (3, 4). In these studies, the presence of an

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<sup>1</sup> Abbreviations: BIC, butyl isocyanide;  $E_{m7}$ , midpoint oxidation–reduction potential at pH 7; EPR, electron paramagnetic resonance; FeCN, ferricyanide;  $n$ - and  $p$ -side, membrane side with negative and positive electrochemical potential, respectively; NQNO, 2- $n$ -nonyl-4-hydroxyquinoline  $N$ -oxide; PQ, plastoquinone; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide electrophoresis; UDM,  $\beta$ -D-undecyl maltoside.

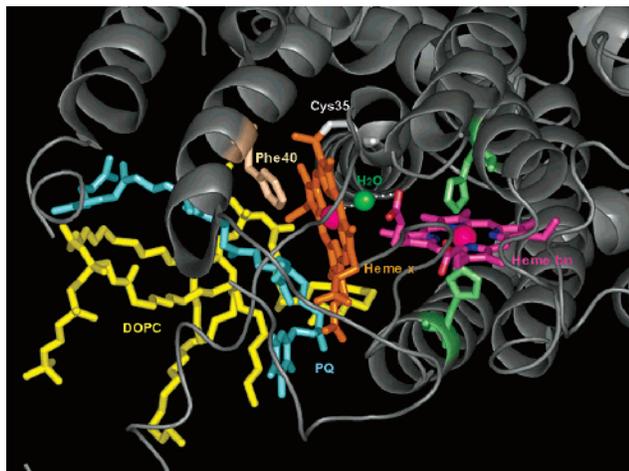


FIGURE 1: Heme  $x$  binding environment. View normal to the membrane plane from the stromal side: heme  $b_n$ , purple; His ligands for heme  $b_n$ , lime; heme  $x$ , orange; Cys35 thioether bound to heme  $x$ , white; Phe40 in subunit IV, wheat; PQ, cyan; lipid molecules (DOPC), yellow. The water or  $\text{OH}^-$  molecule that bridges heme  $b_n$  and  $x$  is displayed as a green ball; protein subunits are shown as gray ribbons.

additional electron transfer component in the  $b_6f$  complex, “G”, with properties resembling those of a high-spin cytochrome  $c'$ , was inferred from difference spectra induced by a light flash, and the perturbation of these spectra by CO. These properties have a significant correspondence to those associated with heme  $x$  in this study.

Now that the presence of the additional heme in the cytochrome  $b_6f$  complex has been unequivocally demonstrated by an X-ray structure from both a cyanobacterium (1) and green alga (2), it is important to determine the spectral properties of this unique heme to obtain information about its ligation and oxidation state, which have implications for its function. In this study, visible and EPR spectra have been obtained of heme  $x$  in the cytochrome  $b_6f$  complex prepared from the thermophilic cyanobacterium *M. laminosus* and spinach chloroplasts. The cytochrome  $b_6f$  complex from both sources was shown in previous studies to be pure by mass spectroscopic analysis (5) and formation of crystals that diffract to high resolution (1, 6).

## MATERIALS AND METHODS

**Purification of the Cytochrome  $b_6f$  Complex.** The cytochrome  $b_6f$  complex from spinach chloroplasts and a thermophilic cyanobacterium, *M. laminosus*, was purified according to the method of Zhang *et al.* (6–8).

**Absorbance Difference Spectra.** Optical chemical difference spectra were measured using a Cary 3 UV–visible spectrophotometer with a bandwidth of 2 nm. The spectral peaks and bandwidths are accurate to  $\pm 0.5$  nm.

**SDS–PAGE.** SDS–PAGE (15%) was carried out using a Laemmli system (9) with a 1 mm gel run under constant current (12 mA) conditions. Samples were prepared in 50 mM Tris-HCl (pH 8.0) containing 4 M urea, 2% SDS, 5% glycerol, and 2.5% mercaptoethanol and boiled for 2 min before being loaded on the gel.

**Isolation of Subunits from the Gel.** The top two pink bands in the SDS–PAGE gel (Figure 3A), corresponding to cytochromes  $f$  and  $b_6$ , respectively, were excised and soaked in a buffer containing 30 mM Tris-HCl (pH 7.5), 50 mM

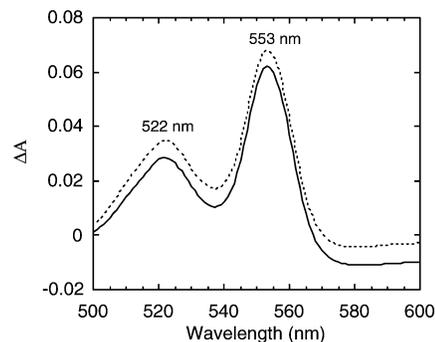


FIGURE 2: Pyridine hemochromagen difference (dithionite minus ferricyanide) spectrum of the complete cytochrome  $b_6f$  complex from *M. laminosus* (—) and spinach thylakoids (---).

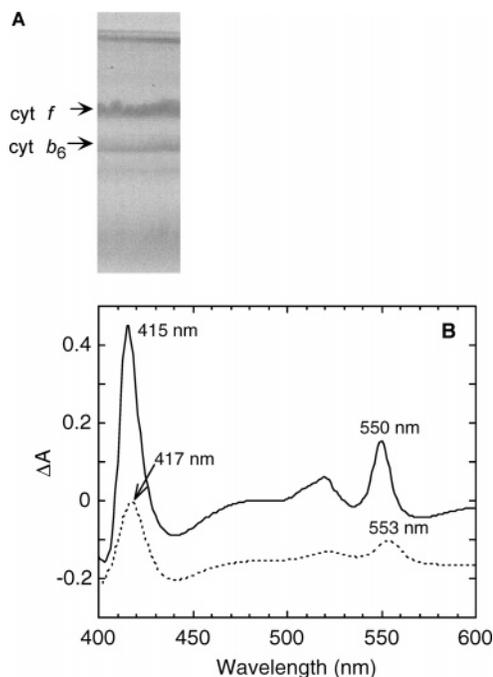


FIGURE 3: Pyridine hemochrome of extracted cytochrome  $f$  and cytochrome  $b_6$  from SDS–PAGE. (A) SDS–PAGE of the isolated cytochrome  $b_6f$  complex from *M. laminosus*. (B) Spectra of pyridine hemochromes of cytochrome  $f$  (—) and  $b_6$  (---) extracted from the gel.

NaCl, and 0.05%  $\beta$ -D-undecyl maltoside overnight or until the gel strips became colorless. The light pinkish “soaking solution” was collected for determination of pyridine hemochrome spectra of covalently bound hemes.

**Determination of Pyridine Hemochrome Difference Spectra.** Pyridine hemochromogen spectra were determined on the basis of the method of Berry *et al.* (10) with minor modifications. The cytochrome  $b_6f$  complex (600  $\mu\text{L}$ , 1.5 mM) from *M. laminosus* or spinach chloroplasts was oxidized by addition of 1–2 grains of solid ferricyanide. Subsequently, 300  $\mu\text{L}$  of pyridine and 100  $\mu\text{L}$  of 1 N NaOH were added. After thorough mixing, the oxidized spectrum was recorded and used as a baseline for the difference spectrum. Solid dithionite ( $\sim 2$ –3 mg) was added, and the reduced minus oxidized difference spectrum was recorded.

**Butyl Isocyanide and CO Difference Spectra.** The cytochrome  $b_6f$  complex (1 mL, 1  $\mu\text{M}$ ) was reduced in a cuvette by an addition of an excess ( $\sim 2$ –3 mg) of solid dithionite. The cuvette was then sealed with Parafilm, and the sample was mixed by inverting the cuvette several times and

incubated for 10 min at room temperature. A series of baseline spectra were taken until there was no change in absorbance. Butyl isocyanide (BIC) (from Aldrich) was injected into the sealed cuvette to a final concentration of 10 mM, and a difference spectrum was recorded. For CO binding experiments, CO was delivered into the dithionite-reduced sample in a sealed cuvette for 2 min, and the difference spectrum was measured as described above.

**EPR Spectroscopy.** The cytochrome  $b_6f$  complex from *M. lamosus* (15  $\mu$ M) and spinach chloroplasts (17  $\mu$ M) was prepared in the presence of ferricyanide (125  $\mu$ M) or ascorbate (500  $\mu$ M), and dithionite-reduced samples by adding ~2–3 mg of dithionite. Azide or cyanide was added to ferricyanide-oxidized or dithionite-reduced samples to a final concentration of 2.5 mM.

Electron paramagnetic resonance spectra at 20 K were obtained using a Bruker ESP300E system (Bruker Instruments, Billerica, MA). The EPR parameters were as follows: temperature of 20 K, microwave frequency of 9.44 GHz, microwave power of 20 mW, modulation amplitude of 20 G, gain of  $1 \times 10^5$ , and conversion time of 20 ms.

EPR spectra of the spinach  $b_6f$  complex at 6 K were obtained using a Bruker 300 EPR spectrometer. The sample temperature was regulated using a GFS-300 transfer tube, an ESR-900 helium cryostat, and a model ITC4 temperature controller (Oxford Instruments, Oxford, U.K.). EPR parameters were as follows: temperature of 6 K, microwave frequency of 9.46 GHz, microwave power of 10 mW, modulation amplitude of 20 G, gain of  $2 \times 10^4$ , and conversion time of 40 ms. EPR spectra at 2.9 K were measured using a Bruker 380E spectrometer with a dielectric resonator immersed in liquid helium in a CFG-935 cryostat, with 1.56 kHz field modulation, and an amplitude of 10 G. The temperature was determined from the vapor pressure of the liquid helium.

## RESULTS

**Visible Absorbance Spectra of the Cytochrome  $b_6f$  Complex.** Reduced minus oxidized chemical difference  $\alpha$ -band spectra obtained at room temperature for the cytochrome  $b_6f$  complex (11) show essentially a single spectral band at 563–564 nm with a half-bandwidth of approximately 10–11 nm, in addition to a band at 553–554 nm associated with cytochrome  $f$ . Spectra of the complex in intact membranes show similar  $\alpha$ -band absorbance peaks for the two  $b$ -hemes,  $b_n$  and  $b_p$ , centered at 563–564 nm in thylakoids and cells (4, 12, 13). These spectra revealed no evidence for the presence of an additional spectral component that might be associated with heme  $x$ . Because of the spectral sharpening at low temperature, low-temperature difference spectroscopy is a more sensitive method for separating closely positioned spectral bands. Such spectra of the  $b_6f$  complex from spinach thylakoids, measured at 77 K, exhibited three blue-shifted spectral components in the  $\alpha$ -band region, at 557.5, 561.5, and 560.5 nm. The 560.5 nm band was assigned to the low-potential  $b_p$ , and the 557.5 and 561.5 nm peaks were ascribed to heme  $b_n$  based on their dependence on the ambient redox potential (14).

A search for heme  $x$  in difference spectra at 20 K was carried out. Dithionite minus ascorbate-reduced spectra were measured at 20 K, and first and second derivatives of the

absorbance spectra were calculated to look for minor bands or “shoulders” in the spectra. Only two peaks were resolved, at 557 and 561 nm, in the absolute spectra and calculated second-derivative functions. Following Hurt and Hauska (14), these peaks were assigned, respectively, to a split spectrum of heme  $b_n$  and overlapping peaks of hemes  $b_n$  and  $b_p$  [ $n$  and  $p$  corresponding to  $h$  and  $l$ , respectively, in the notation of Hurt and Hauska (14)]. A search for the signature of heme  $x$  in the visible spectra of the isolated  $b_6f$  complex was then carried out by analysis of the pyridine hemochromagen.

**Spectra of Pyridine Hemochromes of the Cytochrome  $b_6f$  Complex.** There are apparently no previously published pyridine/hemochromagen spectra of the cytochrome  $b_6f$  complex. Room-temperature chemical difference (dithionite minus ferricyanide) spectra of pyridine hemochromes of the  $b_6f$  complex from *M. lamosus* (Figure 2, solid line) and spinach chloroplasts (Figure 2, dashed line) are virtually identical with an absorbance maximum at 553 nm and a half-bandwidth of 16 nm. These spectra contain overlapping contributions from one  $c$ -type heme (cytochrome  $f$ ), two  $b$ -type hemes ( $b_p$  and  $b_n$ ), and presumably heme  $x$ . The reported absorbance maximum of the cytochrome  $f$  pyridine hemochromagen is at 550 nm (15) and that of cytochrome  $b$  in the  $bc_1$  complex at 556 nm (10) in more narrow spectra having half-bandwidths of 10.6 and 14.5 nm, respectively. The large extent of spectral overlap between the four heme components in the  $b_6f$  complex, and the compression of their spectra, make it impossible to accurately resolve the spectra of the individual hemes in room-temperature pyridine hemochromagen difference spectra. A search for heme  $x$  in the isolated cytochrome  $b_6$  polypeptide was then carried out.

**Spectra of Pyridine Hemochromes of the Isolated Cytochrome  $f$  and  $b_6$  Polypeptides.** SDS–PAGE was used to separate the four large subunits in the cytochrome  $b_6f$  complex. Covalently bound peroxidase-staining heme is retained on the gel in two positions, the cytochrome  $f$  polypeptide to which the heme is covalently bound and also at the position of the cytochrome  $b_6$  polypeptide (16, 17). The noncovalently bound  $b$ -type hemes were removed from the latter by the denaturing conditions in SDS–PAGE and are not seen on the gel (Figure 3A). Pyridine hemochromagen difference spectra were separately determined for the cytochrome  $f$  and  $b_6$  polypeptides extracted from the gel. The extracted cytochrome  $f$  band shows a typical  $c$ -type pyridine hemochrome spectral peak at 550 nm with a half-bandwidth of 10.5 nm (Figure 3B, solid line) which, as expected, is identical to the spectrum of the cytochrome  $f$  pyridine hemochromagen from higher plants (15). However, the pyridine hemochrome spectrum of the heme chromophore attached to the cytochrome  $b_6$  polypeptide shows a broad low-amplitude peak at 553 nm with a half-bandwidth of ~14.5 nm (Figure 3B, dashed line). The latter spectral peak is different from the reported value of hemochrome  $b$  that has an absorbance maximum at 556 nm (10), but it resembles that of other single-thioether bond  $c$ -type cytochromes from *Euglene gracilis* and *Crithidia oncopelti* (18). The spectral amplitude of the heme  $x$  pyridine hemochromagen is approximately 30% of that of cytochrome  $f$ , indicating a smaller extinction coefficient. The actual  $\alpha$ -band peak in the native state of the high-spin heme  $x$ , which is not determined here, depends on the identity of the proximal heme ligand, whether it is  $H_2O$  or hydroxyl ion, the two possibilities that are

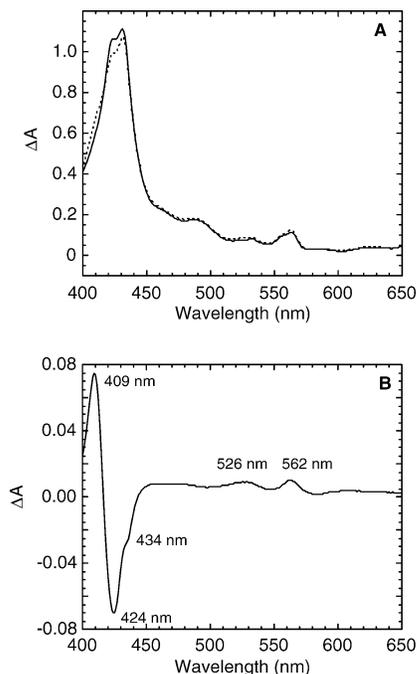


FIGURE 4: Visible spectra of CO derivatives of ferrous heme  $x$  in the cytochrome  $b_6f$  complex. (A) Dithionite-reduced spectra in the presence (---) and absence (—) of CO. (B) CO-reduced minus reduced difference spectrum.

allowed from the X-ray structures of the cytochrome  $b_6f$  complex (1, 2).

*Derivatives of Heme  $x$  with Strong Field Ligands.* A search in the visible spectra for the high-spin nature of heme  $x$  was carried out using small molecule strong field axial ligands that are expected to perturb the spectra of high-spin hemes. The addition of azide and cyanide had no effect on the visible spectra, perhaps because of their limited access to the heme in a hydrophobic environment. However, the addition of CO and butyl isocyanide caused a large perturbation and shift of the peaks of the visible absorbance spectrum. The CO difference spectrum of dithionite-reduced complexes from *M. lamosus* and spinach chloroplasts displayed a pronounced peak and trough in the Soret region around 409 and 424 nm, respectively, with a shoulder at 434 nm. Although it is not possible to precisely equate ligand-induced band shifts of isolated  $b_6f$  complex to the peaks of the light minus dark difference spectra previously measured in *C. sorokinia*, the major trough at 424 nm is similar to that seen in the difference spectrum attributed to component “G” observed by Lavergne (3) and Joliot and Joliot (4). This suggests that the redox component in the  $b_6f$  complex that binds CO is the component labeled G in those studies. An identification of the trough in the Soret band difference spectra (Figures 4B and 5B derived from Figures 4A and 5A) with the absorbance peak of heme  $x$  can be made because the band shifts (11–15 nm) in the difference spectra are similar to the width of the Soret band difference spectra. Two other broad peaks centered at 562 and 526 nm, along with a broad spectral trough centered at 583 nm, were also observed in both complexes (Figure 4B).

The difference spectrum in the presence, relative to the absence, of the BIC- $b_6f$  complex exhibited two broad absorbance maxima centered at 554 and 520 nm, a peak and a trough at 416 and 427 nm, respectively, in the Soret region,

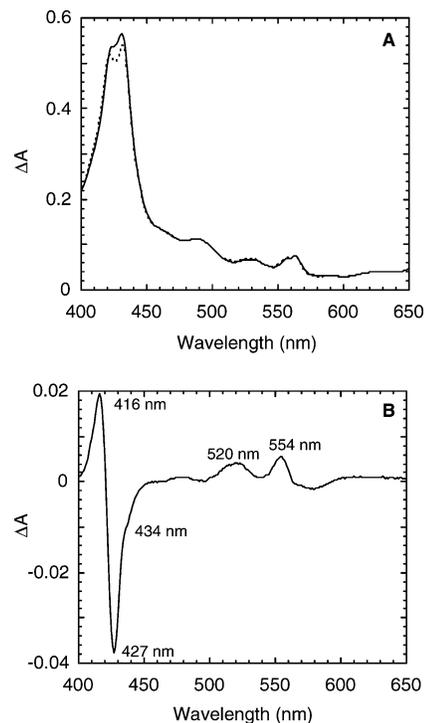


FIGURE 5: Visible spectra of BIC derivatives of ferrous heme  $x$  in the cytochrome  $b_6f$  complex. (A) Dithionite-reduced spectra in the presence (---) and absence (—) of BIC. (B) The BIC-reduced minus reduced difference spectrum, measured as described in Materials and Methods.

and a shoulder at 434 nm (Figure 5B). This trough at 427 nm also resembles that seen in the studies on component G (4). Moreover, the shoulder on the trough at 434 nm is common to both the CO and BIC difference spectra (Figures 4B and 5B) and was seen in spectra of a cytochrome  $c'$  (19). A broad spectral trough centered at 579 nm was also present in the difference spectrum. Butyl isocyanide has been used to perturb the spectra of high-spin myoglobin and hemoglobin (20), and the flavocytochrome  $b$  in neutrophil membranes (21). Spectral perturbation caused by butyl isocyanide was not observed on the purified yeast  $bc_1$  complex under the same experimental conditions (data not shown). Together with the absence in the CO and BIC difference spectra of a trough at the Soret and  $\alpha$ -band region (432 and 563 nm, respectively) of cyt  $b_6$ , it is inferred that the ligands do not bind significantly to the hemes of cytochrome  $b_6$ .

If an extinction coefficient of  $26 \text{ mM}^{-1} \text{ heme}^{-1} \text{ cm}^{-1}$  for cytochrome  $b_6$  is used for the spectral band at 563 nm, and if all of the heme  $x$  binds butyl isocyanide, then the extinction coefficient for the BIC-heme  $x$  complex at 554 nm would be  $10.6 \text{ mM}^{-1} \text{ heme}^{-1} \text{ cm}^{-1}$  with half-bandwidths of 14 and 22 nm for  $\alpha$ - and  $\beta$ -bands, respectively. As discussed above (first paragraph of the Results), heme  $x$  in the native state without any strong field ligand is almost invisible in the cytochrome  $\alpha$ -band region in the background of the three other heme proteins in the complex. A small amplitude in the  $\alpha$ - $\beta$  region is a common feature of high-spin heme proteins (19, 22).

*EPR Spectra.* EPR spectra of the cytochrome  $b_6f$  complex from *M. lamosus* and spinach chloroplasts were measured at 20 K (Figure 6A) and 6 K (Figure 6B). The principle  $g$  values at 7.3–7.4, 6.6–6.7, 6.0, 4.7, 4.3, and 3.5 can be seen at 20 K in the ferricyanide-oxidized (Figure 6A, traces

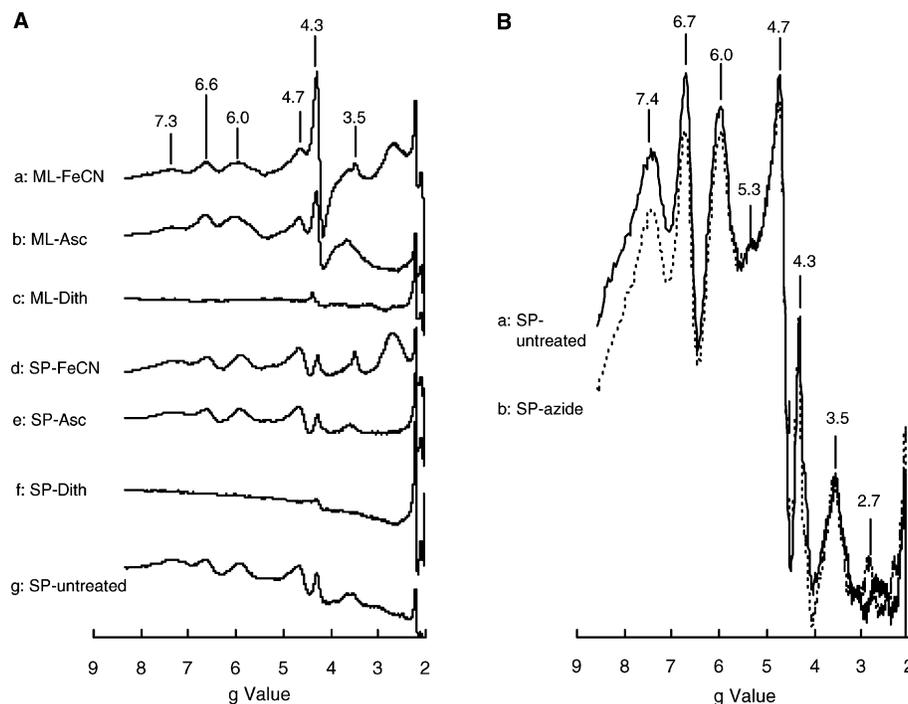


FIGURE 6: EPR spectra of (A) the cytochrome  $b_6f$  complex from *M. lamosus* and spinach thylakoid membranes at 20 K. Spectra from the *M. lamosus* complex in the presence of (a) ferricyanide (FeCN, 125  $\mu$ M), (b) ascorbate (Asc, 400  $\mu$ M), and (c) dithionite ( $\sim$ 1 mM). Spectra of the spinach complex in the presence of (d) ferricyanide, (e) ascorbate, and (f) dithionite and (g) the untreated spinach complex. (B) Cytochrome  $b_6f$  complex from spinach chloroplasts (a) untreated and (b) with azide, measured at 6 K.

a and d), ascorbate-reduced (Figure 6A, traces b and e), and the untreated complex at 6 K (Figure 6B, trace a). The presence of the low field lines in the ascorbate-reduced samples implies that these lines originate from a redox component with an  $E_m$  of less than 50–100 mV, the operating potential of ascorbate. The larger amplitudes and smaller bandwidths of the 6 K spectra, shown most dramatically for the high-spin  $g = 7.4$  signal, document qualitatively a large temperature dependence for the underlying transitions. The major spectral difference between the complex from *M. lamosus* (Figure 6A, traces a–c) and spinach (Figure 6A, traces d–g) at 20 K is the amplitude of the  $g = 4.3$  signal, which arises from the first excited Kramer's doublet (23) that disappears at low temperatures (Figure 7).

The  $g \sim 3.5$  signal in the ferricyanide-oxidized and the ascorbate-reduced complex mainly arises from the low-spin ferric hemes of cytochrome  $f$  and cytochrome  $b_6$  (Figure 6A, traces a, b, d, and e). These  $g$  values and redox properties are consistent with those previously reported in EPR spectra of the cytochrome  $b_6f$  complex (24, 25). The 6 K spectra confirm that there is no reactive site for azide in the complex (Figure 6B), consistent with the absence of an effect on the visible difference spectra as discussed above in the context of Figure 4.

The major EPR signals seen at  $g$  values of 7.3–7.4, 6.6–6.7, 6.0, and 4.7 (Figure 6A, traces a, b, d, e, and g; Figure 6B) fall into two groups based on temperature dependence and power saturation. A small presumably broadened signal is seen at  $g = 5.3$  in the spectrum measured at 6 K (Figure 6B). The  $g = 7.4$ , 6.7, and 4.7 signals are saturates at a moderate microwave power at a low ( $\sim$ 3 K) temperature, while the  $g = 6.0$  signal does not. At 2.9 K, the amplitudes of all high-spin signals except the  $g = 6.0$  signal are lost at a microwave power of 0.01 mW, but the  $g = 6.0$  signal is

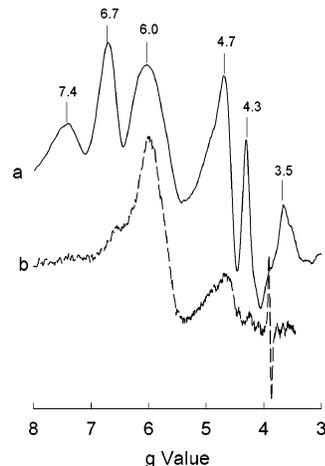


FIGURE 7: High- $g$ -value EPR signals from the  $b_6f$  complex at 14 K (top trace) and 2.9 K (bottom trace). The sharp line near  $g = 3.9$  in the bottom trace is from a Cr(III) impurity in the dielectric resonator. The microwave power was 0.01 mW for both spectra; modulations for traces a and b are 30 G at 100 kHz and 10 G at 1.56 kHz, respectively. The two spectra have been scaled so that they have comparable amplitudes.

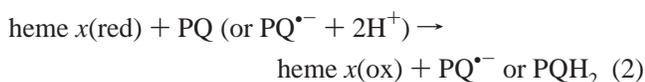
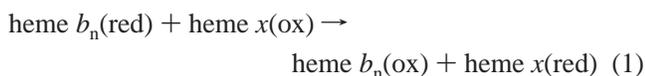
much less affected (Figure 7). The different temperature dependence of the  $g = 6.0$  band implies that it does not have the same origin as the other high-spin signals. The  $g = 6.0$  component has previously been associated with heme signals from the denatured  $b_6f$  complex (24). In the case presented here, denatured cytochrome  $b_6$  was not detected in the X-ray structure. It is possible that a small amount of the complex is denatured during sample freezing, and this component is excluded from the crystals. The spectra in Figure 6 imply the existence of two pairs of rhombic signals,  $g = 7.4$  and 4.7 and  $g = 6.7$  and 5.3, which correspond, respectively, to values of the rhombic distortion parameter,

$E/D$  (23), of 0.058 and 0.029. It is hypothesized that the species with the more symmetrical, lower-rhombicity signal ( $g = 6.7$  and  $5.3$ ) corresponds to the structure observed in the crystal structure. The broadening of the  $g = 5.3$  signal is attributed to dipolar interactions (26) with a magnitude of 1–2 GHz from the nearby (9–10 Å Fe–Fe distance) heme  $b_n$ . Considering the high-spin  $g$  values in the spectra, there are four distinct species indicated by the spectra: the two rhombic pairs listed above, a  $g = 6.0$  axial species, and a highly rhombic species giving rise to the  $g = 4.3$  peak. The latter two might arise from denaturation. The second ( $g = 7.4$  and  $4.7$ ) rhombic pair observed with the soluble complex could arise from a conformational change in or near heme  $x$ , which decreases the dipolar broadening and changes the zero-field splittings.

## DISCUSSION

**Accessibility of Heme  $x$  to Exogenous Ligands.** The cavity side heme surface of heme  $x$  is surrounded by aromatic or other hydrophobic side chains. Phe40 and Leu37 in helix E of subunit IV, together with a bound plastoquinone and two lipid molecules, form a hydrophobic shield over the heme  $x$  surface, and the accessibility of exogenous ligands to the sixth coordination site of the Fe of heme  $x$  is limited. Thus, addition of cyanide and azide caused no change in the absorbance spectra, and they are assumed to be unreactive with heme  $x$ . This is a property of many high-spin cytochromes (19). In contrast, the small neutral molecule, CO, and a hydrophobic cyanide, butyl isocyanide, bind to the reduced heme  $x$  and perturb its visible spectrum. The visible spectral changes associated with the binding of BIC and CO are similar to those observed for the binding of EIC and CO to a cytochrome  $c'$  from the photosynthetic bacterium *Rhodospirillum rubrum* (27). The spectral shifts caused by addition of CO or BIC are indicative of a high-spin–low-spin transition.

**Redox Potential of Heme  $x$ .** Spectral changes caused by the addition of butyl isocyanide and CO were observed only in the dithionite-reduced complex, not in ascorbate-reduced or ferricyanide-oxidized samples. It is known that CO can bind to only the reduced heme proteins (19). Therefore, it was inferred that the midpoint potential of the CO- and BIC-reactive component is less than that of the operating potential of ascorbate (approximately 50–100 mV). With regard to a more precise determination of the  $E_m$ , the available information is somewhat disparate. From the equilibrium between G and heme  $b_n$  in *C. sorokinia*, it was inferred that the midpoint potential of G is 20 mV more positive than that of heme  $b_n$  [ $E_m(\text{G, heme } x) > \text{heme } b_n$ ], so  $E_m(\text{heme } x) \sim -30$  mV (4). This  $E_m$  is consistent with the presence of oxidized heme  $x$  as defined by the spectroscopic analysis in this study. An  $E_m$  of approximately  $-30$  mV, together with the position of the bound plastoquinone observed within 3.1 Å of heme  $x$  in the *M. laminosus* structure (1), suggests an  $n$ -side electron transfer pathway involving heme  $x$ :



This pathway and the position of PQ in the structure suggest that the mechanism of action of the quinone analogue inhibitor, NQNO, which is known to increase the amplitude of the flash-induced cytochrome  $b$  reduction (28), is to displace the PQ molecule in the intermonomer cavity.

On the other hand, consideration of other data in the literature in the context of the scheme described in reactions 1 and 2 implies a redox potential of heme  $x$  more reducing than that suggested in ref 4. In particular, it has previously been observed that at a positive potential, where the PQ pool is fully oxidized, flash-induced turnover of PS II results in a large amplitude, and quite stable reduction of cyt  $b_n$ , even in the absence of  $Q_n$  site inhibitors (29, 30). This would not be expected if heme  $x$  served as the efficient electron acceptor of heme  $b_n$ . Therefore, in contrast to the prediction of the reaction direction of eq 1, the working redox potential from functional assays would appear to yield the relation  $E_m(\text{heme } x) < E_m(\text{heme } b_n)$ , i.e.,  $E_m(\text{heme } x) < -50$  mV, so that heme  $b_n$  could not readily reduce heme  $x$ . During the operation of the Q-cycle, electron transfer from cyt  $b_n$  to PQ might thus involve a transient, energetically uphill reduction of heme  $x$ .

All of the above estimates of the  $E_m$  of heme  $x$  are consistent with its existence in the oxidized ferric state under physiological ambient conditions, as are the pronounced  $g \geq 6$  EPR signals that are a diagnostic signature for an oxidized ferric state of heme  $x$ . The inference that heme  $x$  in the ambient environment of the isolated  $b_6f$  complex is in a high-spin ferric state is not in agreement with the conclusion, derived from resonance Raman spectra, that heme  $x$  is reduced in the  $b_6f$  complex from *Ch. reinhardtii* (31). A reduced heme  $x$  *in vivo* would preclude the redox changes in *C. sorokinia* that were attributed to component G (3, 4). It is unlikely that the oxidation state of heme  $x$  differs as a consequence of structural differences between the complex in *Ch. reinhardtii* and *M. laminosus*, because the structure of the  $b_6f$  complex from the two sources is virtually identical in all major aspects (1, 2). As noted in the resonance Raman study (31), photoreduction of the heme by the laser light used to excite the signals in the Raman spectrum may be an alternate explanation of the presence of a reduced heme  $x$  under the conditions of those experiments (31).

**Function of Heme  $x$ : Significance of Its High-Spin Five-Coordinate State.** It has previously been proposed that the function of heme  $x$ , which is not found in the cytochrome  $bc_1$  complex, is related to the pathway of cyclic electron transport (1, 2). Although the  $b_6f$  and  $bc_1$  complexes have numerous identities and homologies in structure–function relationships, it has been suggested that the requirement of the cyclic pathway connected to the low-potential electron carriers, ferredoxin and FNR, may be a pathway that is unique to the  $b_6f$  complex (1, 2, 12, 32, 33), and suggests a role for heme  $x$  in this pathway. If heme  $x$  functions in cyclic electron transfer, acting as an acceptor of one electron from ferredoxin, or from FNR (7), and as a donor of one electron to plastoquinone or plastosemiquinone (eqs 1 and 2 above), the operation of the Q-cycle (34) would require the oxidative turnover of only one quinol on the  $p$ -side of the membrane to accomplish the two-electron reduction of a quinone on the  $n$ -side.

However, caution must be observed in assigning a role for heme  $x$  in cyclic electron flow, since the observed rate

of cytochrome *b* reduction by NADPH-ferredoxin shows a kinetic limitation (12, 35, 36), which implies regulation and control of this pathway that is not understood at present (37). Moreover, work on C3 plants suggests that electron transport is apparently inhibited by mutation of a Fd:PQ oxidoreductase and a NADPH:PQ oxidoreductase (38). This implies that the major physiological pathway for reduction of PQ from stromal reductants might not include the cyt *b<sub>6</sub>f* complex (39).

The assignment of a function of heme *x* in a cyclic electron transport pathway, even if ultimately proven correct, would not answer all of the questions about its properties. The question of the functional significance of the five-coordinate high-spin state of heme *x* remains to be answered. The high-spin state implies that, in addition to its electron transfer function, heme *x* may have another function that is related to binding of a physiological ligand.

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## NOTE ADDED IN PROOF

An  $E_m = +75$  mV for heme *x* was recently reported (Rappaport, F., Pierre, Y., Picot, D., and Lavergne, J., Proc. 13th Int. Cong. Photosyn., Montreal, Sept., 2004, Abstr. 7394), based on redox titrations of the *b<sub>6</sub>f* complex from *Ch. reinhardtii*, with the possibility noted that a more negative  $E_m$  could arise from electrostatic interactions with heme *bn*.

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