Consequences of the structure of the cytochrome $b_{6f}$ complex for its charge transfer pathways

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Abstract

At least two features of the crystal structures of the cytochrome $b_{6f}$ complex from the thermophilic cyanobacterium, *Mastigocladus laminosus* and a green alga, *Chlamydomonas reinhardtii*, have implications for the pathways and mechanism of charge (electron/proton) transfer in the complex: (i) The narrow 11×12 Å portal between the $p$-side of the quinone exchange cavity and $p$-side plastoquinone/quinol binding niche, through which all Q/QH$_2$ must pass, is smaller in the $b_{6f}$ than in the $bc_1$ complex because of its partial occlusion by the phytyl chain of the one bound chlorophyll a molecule in the $b_{6f}$ complex. Thus, the pathway for trans-membrane passage of the lipophilic quinone is even more labyrinthine in the $b_{6f}$ than in the $bc_1$ complex. (ii) A unique covalently bound heme, heme $c_n$, in close proximity to the $n$-side $b$ heme, is present in the $b_{6f}$ complex. The $b_{6f}$ structure implies that a Q cycle mechanism must be modified to include heme $c_n$ as an intermediate between heme $b_n$ and plastoquinone bound at a different site than in the $bc_1$ complex. In addition, it is likely that the heme $b_n$-$c_n$ couple participates in photosystem I-linked cyclic electron transport that requires ferredoxin and the ferredoxin: NADP$^+$ reductase. This pathway through the $n$-side of the $b_{6f}$ complex could overlap with the $n$-side of the $Q$ cycle pathway. Thus, either regulation is required at the level of the redox state of the hemes that would allow them to be shared by the two pathways, and/or the two different pathways are segregated in the membrane.

Keywords: Carbon fixation; Cytochrome $b_{6f}$, $bc_1$ complexes; Cyclic electron transport; Heme $c_n$; Q cycle

1. Introduction

Determination of the crystal structure of the cytochrome $b_{6f}$ complex at a resolution of 3.0 Å [1]–3.1 Å [2] completed the description of the protein architecture responsible for electron transport and proton translocation in oxygenic photosynthesis. With the exception of the $b_{6f}$ complex, for which structures have been obtained from both the thermophilic cyanobacterium, *Mastigocladus laminosus* (pdb id: stigmatellin and DBMIB complexes, 1VF5 and 2D2B, respectively), and the green alga *Chlamydomonas reinhardtii* (pdb id: stigmatellin complex, 1Q90), the structures of the other electron transport complexes that define the photosynthetic electron transport chain, the PSII [3] and PSI [4] reaction centers, are derived from thermophilic cyanobacteria. The high degree of similarity of the 3.1 Å and 3.0 Å structures from *M. laminosus* and *C. reinhardtii*, the similarity of mass spectra of the complex from *M. laminosus* and spinach [5], and a large array of spectrophotometric and sequence data (not shown), indicates that the $b_{6f}$ structure from the thermophilic cyanobacterium is representative of those in green algae and probably higher plants as well.

2. Structure of the complex

The structure of the dimeric $b_{6f}$ complex from *M. laminosus* contains 8 natural prosthetic groups per monomer (6 redox groups and 2 pigment molecules) and consists of eight...
polypeptide subunits, which can be divided into two groups of four: (a) Four “large” (MW = 17.5–32 kDa) core polypeptide subunits bind the redox prosthetic groups [four hemes, one [2Fe-2S] cluster, and a plastoquinone in *M. laminosus*], and participate in electron and proton transfer reactions. These subunits are cytochromes *f* and *b*₆, the [2Fe-2S] Rieske iron-sulfur protein (ISP), and subunit IV, which provides the binding environment for the two additional prosthetic groups in each monomer, a chlorophyll *a* and β-carotene. (b) Four “small” (MW = 3–4 kDa) subunits designated PetG, L, M, and N, each of which spans the complex and the membrane once, can be considered to be “hydrophobic sticks” forming a hydrophobic “picket fence”, with one-two C-terminal positively charged residues on the *n*-or stromal side of each of these subunits. The protein moiety of the eight subunit dimeric complex in *M. laminosus* has a molecular weight of 217 kDa [5]. Structures of protein components of the complex were previously obtained to a resolution of 1.9 Å for the C-terminal 250 residue extrinsic soluble domain of cytochrome *f* [6], and of 1.83 Å for the N-terminal 139 residue soluble domain of the ISP [7,8]. Cytochrome *b*₆ and subunit IV, which define the core of the complex (TM helices A-D and E-G, respectively), are highly homologous in their distribution of hydrophobic amino acids to the *N*-and C-terminal halves of cytochrome *b* in the *bc₁* complex [9].

### 2.1. An additional subunit in the *b*₆*f* complex from higher plant chloroplasts; the ferredoxin:NADP⁺ reductase

The same eight subunits found in the *b*₆*f* complex from *M. laminosus* and *C. reinhardtii* are also found, with very similar masses determined by electrospray mass spectroscopy, in the complex isolated from spinach (*spinacea oleracea*) thylakoid membranes [5]. In addition, a ninth subunit was found to be present in the complex from spinach which, at 35.3 kDa, is the largest in the set, and was identified to be ferredoxin:NADP⁺ reductase (FNR). The occupancy of FNR in the complex, where it was first identified in biochemical studies [10,11], was estimated to be 70–90% [11]. FNR has also been identified in *b*₆*f* complex isolated from pea (H. Zhang and G. Kurisu, unpublished).

The dimeric complex is organized as a twenty-six transmembrane TM helix bundle, thirteen per monomer (view parallel to membrane plane, Fig. 1), with the following distribution of TMH: cytochrome *b*₆ (four), subunit IV (three), and cytochrome *f*, ISP, and the PetG, L, M, and N subunits (one each). As in the *bc₁* complex [12–16], the 25–27 residue TMH of the ISP is domain-swapped from the soluble domain in one monomer to the hydrophobic TM domain of the other (Fig. 1), a property that contributes to the stabilization of the dimer.

### 2.2. The quinone exchange cavity; function of the dimer

The two monomers enclose a large (30 Å) along the symmetry axis of the dimer, 25 Å× 15 Å in the membrane plane (Fig. 1) cavity, the “quinone exchange cavity.” This cavity, first observed in the mitochondrial cytochrome *bc₁* complex [12], exchanges quinone-quinol with the bulk phase lipid of the membrane. The lipophilic nature of the cavity was defined by the observation that two of the ten lipid molecules per monomer added to the complex for crystallization [17] reside in the cavity [1], and also presumably contribute to its stability. The identification of the cavity as serving a function of Q/QH₂ exchange was inferred from: (i) the quinone analogue inhibitor, TDS, in both *M. laminosus* and *C. reinhardtii*, bound in an 11 Å×12 Å portal on the p-side of the cavity [1,2]; (ii) electron density near the n-side of the cavity in *M. laminosus* most readily identified as plastoquinone near the heme *c₅* [1] (see section II.3). The other prosthetic groups observed to protrude or insert into the Q/QH₂ exchange cavity are: (i) the novel heme *c₆*, (ii) the phytol chain of the bound chlorophyll *a* [18,19] that extends through the 11 Å×12 Å portal that connects the “exchange cavity” and the *p*-side quinone binding niche near the ISP cluster [12–16]. In both *b*₆*f* and *bc₁* complexes, the portal defines the only route for QH₂/Q entry/exit to the quinone binding niche near the ISP reaction site at the *p*-side interface of the complex [1,2,20–22]. The penetration of the 20 carbon phytol chain through the portal results in partial occlusion of the portal, which would make the pathway of Q/QH₂ transfer across the complex even more labyrinthine in the *b*₆*f* than in the *bc₁* complex.

### 2.3. Heme *x* or *c₅* or *c₅*

The structures of the cytochrome *b*₆*f* complex showed the presence of a unique heme facing the inter-monomer quinone exchange cavity on the *n*-side of the complex that is covalently linked by a single thioether bond to a Cys

![Fig. 1. Structure of the cytochrome *b*₆*f* complex showing the eight-subunit dimeric organization. The color code is as follows: cytochrome *b*₆ (green), subunit IV (blue); cytochrome *f* (pink); ISP (yellow); PetG, -L, -M and -N (orange). The iron-sulfur cluster is shown as orange and purple spheres. Other prosthetic groups are shown as sticks: heme *f* (pink); heme *b* (purple); heme *c₅* (pink); PQ (blue); chlorophyll (green); β-carotene (orange); TDS (orange).](image-url)
2.5. Effects of inhibitors; visible and EPR spectral properties

The $E_{m7}$ of heme $c_n$ is shifted by approximately $-225$ mV in the presence of the quinone analogue inhibitor, NQNO [28]. Heme $c_n$ is characterized by an absorbance band in redox difference spectra [28], or in CO-or butyl isocyanide-difference spectra [27], in the Soret region at approximately 425 nm. The pyridine hemochromagen redox difference spectrum for heme $c_n$ covalently bound to the cytochrome $b$ polypeptide isolated from SDS-PAGE displays a broad spectrum of low amplitude in the $\alpha$-band region with a peak at 553 nm, similar to that of other hemes with a single thioether linkage [31]. EPR spectra displaying ‘g’ values of the oxidized complex at 6.7 and 7.4 define heme $c_n$ as a high spin ferric heme in a rhombic environment [27]. In the crystal structures of the $b_6f$ complex from both $M. laminosus$ and $C. reinhardtii$, the binding site of heme $c_n$ (Fig. 2) occupies most of the binding site of the $n$-side quinone ($Q_n$) and its analogue inhibitor, antimycin A, seen in the structure of the $bc_1$ complex [12].

2.6. Inferred function of heme $c_n$: evolutionary origin

The absence of this heme in the $bc_1$ complex implies that a likely function in the $b_6f$ complex is photosystem I-linked cyclic electron transport (section III.1). In addition, the gene sequence motifs coding for both hemes $b$ and $c_n$ are seen not only in cyanobacteria, but also in the non-photosynthetic firmicutes that include gram-positive bacteria such as $B. subtilis$ (Table 1). The presence of the conserved residues Cys35 that serves as a ligand for hemes $c_n$ and His86, 100, 187, and 202, which serve as ligands for hemes $b_p$ and $b_c$, can be noted. The presence of a complex resembling the cytochrome $b_6f$ complex [32] and of a cofeavantly (cysteine-) bound c-type heme [33] has been shown in $B. subtilis$, implying a metabolic role for these hemes in primitive non-photosynthetic micro-organisms.

3. Electron-proton transfer

It has been inferred that electron transport and coupled proton translocation through the $b_6f$ complex are described, at least under conditions of low $\Delta\tilde{\mu}_{H+}$ [34], by a “Q-cycle” model [35–37] essentially identical to that operating in the cytochrome $bc_1$ complex from the respiratory chain and purple photosynthetic bacteria [38–40]. Two experimental complications in establishing an unequivocal description of the electron transfer pathway in the $b_6f$ complex are: (i) the similarity ($\Delta\Lambda_{\text{max}} \approx 0.5$ nm) of the peaks of the difference spectra of the two $b$ hemes [24], which prevents direct measurement of inter-heme electron transfer; (ii) the absence of a quinone analogue inhibitor that binds tightly to a $Q_n$ site as does antimycin A in the $bc_1$ complex. The effect of NQNO on the $b_6f$ complex mimics antimycin A in its ability to block oxidation of heme $b_n$ [41,42], although this does not result in a parallel inhibition of linear electron transport [43].
<table>
<thead>
<tr>
<th>Sequence alignment of the 4-helix cytochrome b polypeptide in the cytochrome b(C) complexes from higher plant chloroplasts, green algae, cyanobacteria and eubacteria</th>
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<tr>
<td><strong>Spinach</strong></td>
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<td><strong>Chlamydomonas reinhardtii</strong></td>
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<td><strong>Synechocystis elongatus</strong></td>
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<td><strong>Synechocystis sp._PCC_6803</strong></td>
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<td><strong>Synechocystis sp._PCC_7002</strong></td>
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<td><strong>Nostoc sp.</strong></td>
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<td><strong>M._laminosus</strong></td>
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<td><strong>Helobacterium gestii</strong></td>
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<td><strong>Bacillus subtilis</strong></td>
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<td><strong>Bacillus cereus</strong></td>
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Conserved Cys residues are highlighted in cyan. The two pairs of histidine ligands to the cytochrome b hemes are highlighted in red. (For interpretation of the references to colour in this table legend, the reader is referred to the web version of this article.)
The reactions of a Q-cycle mechanism that would describe the pathway of oxidation of plastoquinol through bifurcated high and low potential electron transfer pathways, are summarized (formulae 1–6):

\[
PQH_2 + [2Fe - 2S (ox)] + PQ - + [2Fe - 2S (red)] + 2H^+ [p-side aqueous phase] \tag{1}
\]

\[
FeS (red) + Cyt f (ox) \rightarrow FeS (ox) + Cyt f (red) [high potential chain] \tag{2}
\]

\[
POH_2 + +heme b_p (ox) \rightarrow PQ + heme b_p (ax) \tag{3}
\]

\[
heme b_p (red) + heme f (ox) \rightarrow b_p (ax) + h_n (red) [trans-membrane e' transfer] \tag{4}
\]

\[
heme b_p (red) + PQ \rightarrow heme b_p (ox) + PQ - [n-side e' transfer] \tag{5}
\]

\[
heme b_n (red) + PQ \rightarrow +2H^+ \rightarrow h_n (ox) + PQH_2 [n-side e' transfer,H^+ uptake] \tag{6}
\]

### 3.1. Cyclic electron transport

A **caveat** to an exact application of the Q-cycle to the \(bhf\) complex of oxygenic photosynthesis is that the latter complex may also participate in a cyclic electron transport pathway in which it acts as an electron acceptor from ferredoxin or ferredoxin-FNR that is reduced by the photosystem I reaction center (Fig. 3). Thus, the \(n\)-side of the \(bhf\) complex would participate in electron transfer reactions with a second electron donor-acceptor system. The ATP provided by such a cyclic electron transport pathway, coupled to formation of the \(\Delta\mu_{ii}\), is a metabolic requirement to establish a proper balance of NADPH and ATP for the fixation of \(CO_2\) [44]. The magnitude of the contribution of this cyclic pathway to the \(bhf\) complex of oxygenic photosynthesis is not precisely known, and the regulatory mechanisms that may apply under different light intensities and conditions of plant stress are not well understood. However, the requirement of the cyclic pathway and the extent of its contribution can be estimated from information on the utilization of the \(\Delta\mu_{ii}\) by the \(H^+\)-ATPase, as follows: (i) The maximum \(H^+/2e^-\) ratio = 6 for a two electron reduction of NADP+, and \(H^+/4e^-\) ratio = 12 for reduction of two NADP+, via linear electron transport from water, assuming that a “Q cycle” is completely obligatory and continuously engaged [37]. In this mechanism, 2/3 of the protons that form the \(\Delta\mu_{ii}\) result from electron transfer through the \(bhf\) complex. The remainder of the proton deposition on the \(p\)-side of the membrane results from water oxidation by PSII. (ii) Carbon fixation by the Calvin-Bassham C3 cycle requires three ATP and two NADPH per \(CO_2\) fixed. The number of \(H^+\) needed to synthesize the required 3 ATP would then be 9–12, depending on the \(H^+/\)

ATP stoichiometry, 3–4. In this case, a fully engaged Q cycle would be sufficient to drive carbon fixation. (iii) From the perspective of the \(H^+\) requirement for rotation of the ATPase, 3 ATP are synthesized in one complete rotation of the \(CF_1\) [45], the \(F_0\) sector of the ATPase contains 14 c-subunits [46], and thus 14 \(H^+\) are required for synthesis of the 3 ATP. In this case, \(H^+\) production by the linear electron transport pathway, even with a completely engaged Q cycle, falls short of what is necessary to generate sufficient ATP for carbon fixation. In fact, ATP production by the linear pathway is known to be less than fully efficient [47,48], implying that it is necessary to produce ATP by the PSI-linked cyclic pathway.

The role of the \(bhf\) complex in this cyclic pathway has been extensively debated. On one hand, a comparison of the effect of antimycin A on flash-induced reduction of cytochrome \(b\) heme and ferredoxin-mediated ATP synthesis led to the conclusion that the \(bhf\) complex is not involved in the cyclic pathway [49–51]. In retrospect, the effects of antimycin A on cytochrome \(b\) reduction have been ambiguous [49,50], and it is now known that the binding site of antimycin A in the \(bc_1\) complex is occluded by heme \(c_n\), NADH dehydrogenase (NDH) can provide an alternative pathway for cyclic electron transport [52,53]. However, relative to the \(bhf\) complex, the amount of ndh that can be isolated from any oxygenic photosynthetic membrane system is small. Recent studies indicate kinetic competence of a ferredoxin/FNR cyclic pathway through the \(bhf\) complex that operates most efficiently in dark-adapted leaves or when photosystem II is inhibited, and which resides in the structurally separated non-appressed region of the thylakoid membrane [54–56].

A role of heme \(c_n\) in an \(n\)-side ferredoxin-and FNR-dependent pathway is implied by: (i) the ferredoxin [30,57] and FNR [11] dependence of heme \(b\) reduction by NADPH; (ii) a \(\Delta E_m\) between hemes \(b_n\) and \(c_n\) [28] that implies that the
sequence of electron transfer is $b_n \rightarrow c_n$; (iii) a pH dependence of the $E_{m}$ of heme $c_n$ [28] that implies a proton uptake pathway [2]; (iv) the 4 Å separation of hemes $b_n$ and $c_n$ that results in rapid oxidation of heme $b_n$ and a small amplitude of its reduction in flash experiments; (v) the negative shift caused by NQNO in the $E_{m}$ of heme $c_n$ [28], which provides an explanation of its inhibition of heme $b_n$ oxidation [41].

Thus, the $n$-side of the $b_{6}f_{6}$ complex could be utilized in a PSI-linked ferredoxin (Fd) cyclic pathway in which heme $b_n$ and protonatable heme $c_n$ are considered as a coupled di-heme complex (section 2.4):

$$2 Fd \text{(red)} + FNR \text{(ox)} + 2 H^{+} \rightarrow 2 Fd \text{(ox)}$$

$$+ FNRH_{2} \text{(red)}$$

$$FNRH_{2} \text{(red)} + 2 \text{heme } b_n \text{(ox)} \cdot c_n \text{(ox)} \rightarrow FNR \text{(ox)}$$

$$+ 2 \text{heme } b_n \text{(red)} \cdot c_n \text{(ox)} \cdot H^{+}$$

$$\rightarrow (\text{inhibited by NQNO}) 2 \text{heme } b_n \text{(ox)} \cdot c_n \text{(red)} \cdot H^{+}$$

$$2 \text{heme } b_n \text{(ox)} \cdot c_n \text{(red)} + PQH_{2}$$

$$\rightarrow 2 \text{heme } b_n \text{(ox)} \cdot c_n \text{(ox)} + PQH_{2}$$

The reduced plastoquinol, PQH$_2$, traverses the quinone exchange cavity of the $b_{6}f_{6}$ complex (Fig. 1), enters the p-side/QH$_2$ niche through the portal in the roof of the cavity described above (section II.2), where it serves as an electron and proton donor to the [2Fe–2S] cluster.

The use of hemes $b_n$ and $c_n$ on the $n$-side of the $b_{6}f_{6}$ complex for both cyclic electron transport and the Q cycle raises a problem of interference and cross-talk between the two pathways. That is, reduction of heme $b_n$ by the ferredoxin-dependent pathway would prevent its reduction by heme $b_p$. Alternatively, it has been proposed that the cyclic pathway in higher plant and algal chloroplasts is structurally separated and concentrated in $b_{6}f_{6}$ complex from which FNR is absent, which is located in the non-appressed membrane fraction [54–56] defined by Romanowska and Albertsson [58], and whose occupancy in the latter membrane fraction may be controlled by state transitions [59].

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References


