A Map of Dielectric Heterogeneity in a Membrane Protein: the Hetero-Oligomeric Cytochrome $b_6f$ Complex

S. Saif Hasan,†,‖ Stanislav D. Zakharov,†,§‖ Adrien Chauvet,‡ Valentyn Stadnytskyi,‡ Sergei Savikhin,*‡§ and William A. Cramer*‡†

†Department of Biological Sciences and ‡Department of Physics, Purdue University, West Lafayette, Indiana 47907, United States §Institute of Basic Problems of Biology, Russian Academy of Science, Pushchino, Moscow Region 142290, Russian Federation

ABSTRACT: The cytochrome $b_6f$ complex, a member of the cytochrome $bc$ family that mediates energy transduction in photosynthetic and respiratory membranes, is a hetero-oligomeric complex that utilizes two pairs of $b$-hemes in a symmetric dimer to accomplish trans-membrane electron transfer, quinone oxidation—reduction, and generation of a proton electrochemical potential. Analysis of electron storage in this pathway, utilizing simultaneous measurement of heme reduction, and of circular dichroism (CD) spectra, to assay heme—heme interactions, implies a heterogeneous distribution of the dielectric constants that mediate electrostatic interactions between the four hemes in the complex. Crystallographic information was used to determine the identity of the interacting hemes. The Soret band CD signal is dominated by excitonic interaction between the intramonomer $b$-hemes, $b_n$ and $b_p$, on the electrochemically negative and positive sides of the complex. Kinetic data imply that the most probable pathway for transfer of the two electrons needed for quinone oxidation—reduction utilizes this intramonomer heme pair, contradicting the expectation based on heme redox potentials and thermodynamics, that the two higher potential hemes $b_n$ on different monomers would be preferentially reduced. Energetically preferred intramonomer electron storage of electrons on the intramonomer $b$-hemes is found to require heterogeneity of interheme dielectric constants. Relative to the medium separating the two higher potential hemes $b_p$, a relatively large dielectric constant must exist between the intramonomer $b$-hemes, allowing a smaller electrostatic repulsion between the reduced hemes. Heterogeneity of dielectric constants is an additional structure—function parameter of membrane protein complexes.

1. INTRODUCTION

Photosynthetic and respiratory electron transfer chains utilize a series of membrane bound multisubunit protein complexes.1–4 Recent advances in membrane protein structural biology have described static structure-related aspects of such large membrane protein assemblies. An exponential increase in the number of membrane protein structures solved to high resolution (http://blanco.biomol.uci.edu/mpstruc/),5 including hetero-oligomeric electron transfer complexes, has enhanced the understanding of the protein architecture and the intraprotein environment of the associated prosthetic groups. An important feature of the multisubunit membrane protein complexes involved in electron transfer is the presence of ordered, crystallographically resolvable lipids and lipid binding sites,6–8 and the presence of cavities lined by hydrophobic residues within the trans-membrane domain proposed to provide conduits for binding and transport of redox substrates, such as quinones.6,8–13 The redox prosthetic groups of membrane protein electron transfer complexes are mostly embedded in the trans-membrane domain. Domains of these complexes that contain an extended lipid matrix are assumed to have a low dielectric constant, approximately 2.5.14–16

Investigation of the effects of dielectric heterogeneity in membrane protein function requires a system with unique measurable signals across a medium with different dielectric constants. The cytochrome $b_6f$ complex is a dimeric lipoprotein integral membrane complex that catalyzes plastoquinone reduction—protonation at the electrochemically negative (n) side of the thylakoid membrane and plastoquinol deprotonation—oxidation on the electrochemically positive (p) side.11,17 The trans-membrane core of the dimeric cytochrome $b_6f$ complex is associated with four $b$-hemes with separable redox potentials between two of lower potential ($b_n$ or $b_p$) and two of higher potential ($b_n$ or $b_p$).18,20 The heme $b_n/b_p$ pair is embedded in a protein environment, within the monomeric unit of the $b_6f$ complex, while the two monomers are separated by a large cavity (30 Å high, 15 Å deep, 25 Å wide on the n-side), which is known to contain an appreciable lipid content.21 The presence of an intramembrane, asymmetrically located lipid-filled cavity in such a membrane protein complex is expected to contribute significantly to the energetics of electron transfer.

Supporting Information

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transfer reactions, and perhaps to contribute to a heterogeneous distribution of dielectric constants within the complex.

The structure of the hetero-oligomeric lipoprotein cytochrome $b_{6:}f$ complex, which couples proton translocation and electron transport in oxygenic photosynthesis, has been obtained from two filamentous cyanobacteria, *Mastigocladus laminosus* (Figure 1A) and *Nostoc* sp. PCC 7120 (Figure 1A) and *Nostoc* sp. PCC 7120,11–13,21,22 and the green alga, *Chlamydomonas reinhardtii*.24 The hydrophobic core of the $b_{6:}f$ complex is similar21 to the core of the related cytochrome $b_{c}$ complex in the mitochondrial respiratory chain and purple photosynthetic bacteria.6,9,25–27 In this core, two trans-membrane $b$-hemes, $b_{p}$ and $b_{p}$, respectively, on the n- and p-sides of the complex, cross-link the "B" and "D" TMH of the cytochrome $b$ subunit through bis-histidine ligation in each monomer of a symmetric dimer,29 a structure motif also present in the cytochrome $b_{c}$ complex.9,25,28 The $b_{6:}f$ complex accepts electrons from plastoquinol, transfers one electron to the plastocyanin or cytochrome $c_{6}$ of higher oxidation–reduction potential on the p-side of the complex, and can transfer the other to a lower potential trans-membrane pathway involving the two $b$-hemes $b_{p}$ and $b_{p}$ directed to a plastoquinone bound axially to the heme $c_{6}$.11,13 The electron transfer reactions are coupled to net proton transfer, whose pathways have been described11 into the aqueous phase on the p-side of the membrane, thereby contributing to the trans-membrane electrochemical proton gradient utilized for ATP synthesis. A summary of the net electron and proton transfer pathways is shown (Figure S1, Supporting Information) along with edge–edge and center–center distances between the trans-membrane $b$-hemes in the $b_{6:}f$ complex (Figure 1B) and heme center (Fe)–center (Fe) distances (Figure 1B, Table 1).

The cytochrome $b_{6:}f$ complex, its structure determined from crystallographic analysis shown in ribbon format (Figure 1A), contains five redox prosthetic groups: the extra-membrane heme of cytochrome $f_{i}$ the two trans-membrane $b$-hemes; heme $c_{6}$ which is electronically coupled to heme $b_{6:}f$ and the [2Fe–2S] cluster in the iron–sulfur protein subunit at the p-side membrane interface. The edge–edge distances between the trans-membrane $b$-hemes, which are a major determinant of the rate of intraprotein electron transfer,31–35 are, respectively, 8.1 and 12.8 Å (Figure 1B), between the intramonomer $b$-hemes, $b_{p}$ and $b_{p}$, and the intermonomer hemes, $b_{p}$ as seen in the crystal structure of the *Nostoc* PCC 7120 cytochrome $b_{6:}f$ complex (PDB ID 4H44).11 Heme center–center distances, one determinant of the strength of interheme exciton interactions, are shown in Figure 1B and summarized (Table 1).

In addition to conferring structure stability, an obligatory function in electron transfer of the dimeric structure of the cytochrome $b_{6:}f$ complex has not been described, and little is known about the physical interactions that govern the pathways of trans-membrane electron transfer between bound quinone molecules on the two sides of the membrane via the two $b$-type hemes. The electron transfer pathways in cytochrome $b_{c}$ complexes have been studied extensively in the respiratory and photosynthetic bacterial cytochrome $b_{c}$ complexes,36–44 for which crystal structure information has been summarized.24,45,46 It was inferred from the absence of a sigmoidal dependence of flash-induced cytochrome $b$ reduction in the presence of the inhibitor myxothiazol,38 and the shorter distance between the intramonomer hemes $b_{p}$ and $b_{p}$ compared to the distances between the intermonomer $b$-hemes,42 that intramonomer electron transfer from heme $b_{p}$ to heme $b_{p}$ is more likely than intermonomer transfer between the two hemes $b_{p}$. A different viewpoint is that interheme transfer

### Table 1. Relative Contribution of $b$-Heme Pairs to the CD Signal in the Dimeric Cytochrome $b_{6:}f$ Complex

<table>
<thead>
<tr>
<th>heme pair $b$</th>
<th>center–center distance (Å)</th>
<th>interplanar angle (deg)</th>
<th>relative contribution to CD spectrum (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$b_{p}$–$b_{p}$</td>
<td>20.6</td>
<td>52.0</td>
<td>+50.8%</td>
</tr>
<tr>
<td>$b_{p}$–$b_{p}$</td>
<td>22.0</td>
<td>17.2</td>
<td>−3.5%</td>
</tr>
<tr>
<td>$b_{p}$–$b_{p}$</td>
<td>33.8</td>
<td>48.4</td>
<td>+3.2%</td>
</tr>
<tr>
<td>$b_{p}$–$b_{p}$</td>
<td>34.9</td>
<td>18.9</td>
<td>−4.5%</td>
</tr>
</tbody>
</table>

$^a$Center-to-center distances, interplanar angles, and calculation of relative CD amplitudes determined from the crystal structure (PDB ID 4H44). $^b$Additional numerical subscripts in heme notation used to distinguish the hemes in the two monomers of the dimeric complex.
between hemes $b_{p}$ in the two monomers can occur with comparable likelihood,\textsuperscript{36,41,43,47} described as “half sites reactivity”,\textsuperscript{37,44} and proposed\textsuperscript{48} in the context of the first crystal structures of the mitochondrial $bc_{1}$ complex. Efficient communication between the two monomers at the level of the $b$-hemes was demonstrated through mutagenesis of the $bc_{1}$ complex in the photosynthetic bacteria, \textit{Rhodobacter capsulatus}\textsuperscript{59,41,43} and \textit{Rh. sphaeroides}.\textsuperscript{49} The issue in the latter studies is the pathway preferred for transfer of a single electron. The present study concerns the most probable pathway for transfer of the pair of electrons required for the complete two-electron reduction of the quinone bound on the n-side of the cytochrome $bc$ complex.

While low temperature spectra distinguish hemes $b_{p}$ and $b_{w}$, the pathway of electrons through the $b_{hf}$ dimer cannot be determined, as the spectra of the two hemes $b_{p}$ (or $b_{n}$) in the dimer cannot be distinguished in kinetic studies at room temperature.\textsuperscript{50} However, the asymmetric arrangement of the $b$-hemes within the $b_{hf}$ dimer results in unique interactions between various $b$-heme pairs. The existence of physical interactions between the $b$-hemes involved in trans-membrane electron transfer in mitochondrial and chloroplast membranes has previously been inferred from excitonic circular dichroism (CD) spectra of dithionite-reduced isolated cytochrome $bc_{1}$\textsuperscript{51} and $b_{hf}$\textsuperscript{52} complexes. In the Soret band region, the CD spectra of dithionite-reduced cytochrome $bc_{1}$ and $b_{hf}$ complex are split, due to excitonic heme–heme interactions, into two lobes of opposite sign around a node near the Soret band absorbance peak (432 nm). The amplitude of the split CD spectrum is a function of the geometry (interheme angle and distance) of the interacting hemes. High resolution crystal structure information\textsuperscript{14} can be utilized to identify the particular $b$-hemes that are reduced in the dimeric $b_{hf}$ complex.

In the present study, the time course of $b$-heme reduction in the $b_{hf}$ complex, and of the increase in amplitude of the CD spectra associated with excitonic heme interactions, has for the first time been measured simultaneously. The amplitude of the absorbance spectra assays the fraction of the total $b$-heme population in the cytochrome complex that is reduced, while the split CD spectrum indicates the particular $b$-heme pair that is reduced. A difference between the midpoint redox potentials of the intramonomer hemes $b_{p}$ and $b_{w}$ of purified $b_{hf}$ complex has been described.\textsuperscript{19,20,53,54} Two of the studies,\textsuperscript{19,20} utilizing a crystallizable complex from the green alga, \textit{Chlorella vulgaris},\textsuperscript{48} that is characterized by physiological electron transfer rates of approximately 200 electrons/cytochrome $f$, skewed determination a midpoint potential difference between the two hemes $b_{p}$ and $b_{w}$ of 75–100 mV. \textit{In-situ} titrations of thylakoid membranes indicated that the midpoint redox potential difference could be as large as 50 mV.\textsuperscript{55,56} Given access to and equilibration between both monomers, thermodynamics dictates that the first electrons to be transferred to the dimer would equilibrate to the higher potential $b$-heme in each of the two monomers. In the present study, however, the combined spectrophotometric and circular dichroic analysis of these electron transfer events implies that this expectation is not correct. The preferred two-electron-reduced state of the complex is the $b$-heme pair in the monomeric unit of the complex, which consists of the “high” and “low” potential hemes ($b_{p}$ and $b_{w}$) defined in the redox titrations.\textsuperscript{19,20} Consideration of the energetics implies that preferential pairwise reduction of the two $b$-hemes in the monomer would be favored only if the dielectric constant of the protein medium between the intramonomer $b$-heme pair, $b_{p}$ and $b_{w}$, which have different redox potentials and which span the membrane from the electrochemically positive and negative sides, is greater than the dielectric constant $\varepsilon = 2.5$ which operates in the intermonomer space between the two higher potential (greater electron affinity) hemes $b_{p}$ located in the different monomers of the complex. Thereby, the protein medium between these $b$-hemes is more polarizable than that between the two hemes $b_{w}$ whose reduction would be considered to be thermodynamically more favorable strictly on the basis of their redox potentials. Preferential reduction of the intramonomer $b$-heme pair would collect electrons into one monomer of the dimeric cytochrome $b_{hf}$ complex, resulting in a more efficient reduction of the n-side bound quinone, and less efficient formation of superoxide mediated by plastosemiquinone.\textsuperscript{57}

2. MATERIALS AND METHODS

2.1. Purification of Cytochrome $b_{hf}$ Complex. The cytochrome $b_{hf}$ complex was isolated from leaves of \textit{Spinacea oleracea} as previously described.\textsuperscript{58} The dimeric complex was separated from the monomer by size exclusion chromatography and sucrose density gradient centrifugation.\textsuperscript{58} Densitometry on Blue Native-PAGE to determine the relative content of monomer and dimer fractions of the $b_{hf}$ complex was measured with a Fluor Chem E densitometer. All assays were performed in 30 mM Tris–HCl (pH 7.5), 50 mM NaCl, 0.2 mM EDTA, and 0.04% UDM (n-undecyl-β-D-maltopyranoside).

2.2. Electron Transfer Activity. The specific electron transfer activity of the purified cytochrome $b_{hf}$ complex was measured as described previously.\textsuperscript{59}

2.3. Measurement of Absorbance Changes; Cytochrome Difference Spectra. Absorbance spectra were measured with a Cary 4000 spectrophotometer (Varian/Agilent) in single-beam mode. For redox difference spectra, the $b_{hf}$ complex was oxidized by potassium ferricyanide (50 μM). Na-ascorbate (0.25 mM) was added to reduce the relatively high potential ($E_{m} \approx +0.36$ V) cytochrome $f$. Reduction of the two lower potential hemes $b_{p}$ and $b_{w}$ associated with the cytochrome $b_{hf}$ subunit of the complex was achieved by an aliquot of 1 M dithionite, freshly prepared using a degassed solution of 0.1 M Tris, pH 8.0, yielding a final concentration in the cuvette of 3−4 mM ($E_{m} \approx −0.5$ V).\textsuperscript{56} Sufficient buffer was added to prevent a decrease in pH during the course of the measurements. A decrease in the medium pH resulted in a significantly increased rate of $b$-heme reduction (>10-fold at pH < 6.0). The state associated with complete heme reduction is stable for 30 min, indicating maintenance of anaerobic conditions. Concentrations of cytochromes $f$ and $b_{w}$ were calculated from the amplitude of the redox difference absorbance spectrum (ascorbate-reduced minus ferricyanide-oxidized) at 554 or 563 nm (dithionite-reduced minus ascorbate-reduced), respectively, using extinction coefficients of 25 and 23−25 mM$^{-1}$ cm$^{-1}$ determined for difference spectra of cytochromes $f$\textsuperscript{61} and $b_{w}$.

2.4. Low Temperature (77 K) Determination of $b$-Heme Spectra. Upon addition of dithionite to $b_{hf}$ complex suspended in a detergent solution containing 50% glycerol, small aliquots (0.8 mL) were frozen in liquid nitrogen. The absorbance was monitored using a UV–vis Cary 300 Bio (Varian) spectrometer with a measuring beam half-bandwidth of 0.2 nm.

2.5. Simultaneous Measurement of Circular Dichroism and Absorbance Spectra. Soret band CD spectra and
absorbance spectra of the $b_{6f}$ complex were measured simultaneously in a cuvette with 1 cm optical path length and magnetic stirring, with a half-bandwidth of 1 nm, using a “Chirascan” (Applied Photophysics, Ltd., U.K.) spectropolarimeter equipped with thermal control and magnetic stirring of samples. Dithionite as reductant was added as described above in section 2.3, and consecutive spectra were collected at intervals of approximately 16 s.

2.6. Structure-Based Calculation of Circular Dichroism Spectra Derived from the Set of Heme–Heme Excitonic Interactions. Simulations of CD spectra for all four possible pairs of reduced $b$-hemes of the dimeric cytochrome $b_{6f}$ complex were performed using a formalism previously described.51 The dipole–dipole coupling potential, $V_{12}$ (in cm$^{-1}$), between the optical transitions of two spectroscopically identical chromophores can be expressed as eq 1:

$$
V_{12} = \frac{5040}{R_{12}^3} (\mu_1 \cdot \mu_2 - 3(\mu_1 \cdot \hat{r})(\mu_2 \cdot \hat{r}))
$$

(1)

where $R_{12}$ is the center–center distance (in Å) between the two hemes, $\hat{r}$ is a unit vector in the direction of $R_{12}$, and $\mu_1$ and $\mu_2$ are the transition dipole moments of the two interacting hemes expressed in Debye units, 3.34 $\times$ 10$^{-30}$ C·m. The numerical coefficient 5040 in eq 1 results from the use of Debye units and expression of the coupling potential, $V$, in cm$^{-1}$.51 The interaction between the two hemes causes a separation of the absorbance band into two excitonically split bands with maxima at energies $\nu_1$ and $\nu_2$, as described by eq 2:

$$
\nu_2 = \nu_0 \pm V_{12}
$$

(2)

where $\nu_0$ is the transition energy of a noninteracting heme. Following the notation of Palmer et al.,51 the rotational strength, in Debye units, of the two transitions is described by eq 3:

$$
R_{12} = \frac{170}{\lambda} R_{12} [\hat{r} \cdot (\mu_1 \times \mu_2)]
$$

(3)

where $\lambda$ is the optical transition wavelength, in Å. The optical CD signal as a function of wavelength was calculated by adding two Gaussian bands that mimic the shape of the $b$-heme absorbance. One of amplitude $R_1$ is centered at $\nu_1$, and the other of amplitude $R_2$ is centered at $\nu_2$. Equations 1–3 have been used to derive expressions for CD signal strength expressed in terms of the interplanar angles between the $b$-hemes.51 The angular information required for eqs 1–3 for calculation of the interaction energy and the CD signal was derived from the crystal structure of the $b_{6f}$ complex (PDB ID 4H44). The $Q_y$ and $Q_x$ transitions of the reduced $b$-hemes are known to point along the directions between nitrogens, $N_{A-N_C}$ and $N_{B-N_D}$ (Table S1, Supporting Information), respectively, and the distances between the pairs of $b$-hemes were calculated as Fe–Fe distances derived from the crystallographic structure (PDB ID 4H44) (Figure 1B; Table 1). The transition moment of each transition was set to be 4.6 D.51 Since the heme $Q_y$ and $Q_x$ transitions are degenerate, the CD spectra of all four interacting transitions between two $b$-hemes ($Q_y-Q_y$, $Q_y-Q_x$, $Q_x-Q_y$, $Q_x-Q_x$) were calculated separately and then combined. As the goal of these simulations was estimation of the relative contribution of each $b$-heme pair to the CD spectrum, the absorbance spectrum of each $b$-heme was modeled as a Gaussian band with a half-width of approximately 10 nm.

3. RESULTS

Purified predominantly dimeric cytochrome $b_{6f}$ complex, prepared as described, was obtained by hydrophobic chromatography and separated from inactive monomer by size exclusion chromatography (Materials and Methods). The dimeric character of the isolated complex was confirmed by clear native gel electrophoresis analysis, its subunit composition defined by SDS-PAGE analysis (Figure S2A, Supporting Information), and its electron transfer rate from donor decyl- plastoquinol to the plastocyanin/ferricyanide determined (150–200 electrons-cyt$^{-1}$·s$^{-1}$). Monomeric $b_{6f}$ complex prepared as described in the Materials and Methods section, and displayed by native gel electrophoresis in Figure S2B of the Supporting Information, was inactive.

3.1. Dithionite Reduction of Hemes $b_6$ and $b_{6f}$ Room Temperature and 77 K. The $b$-hemes in the dimeric $b_{6f}$ complex are completely reduced in a pH-dependent reaction by dithionite (3–4 mM) in 10–12 min (room temperature, 23 °C) at pH 7.5 (Figure 2A and B). The rate of heme $b$ reduction by dithionite at pH 8.0 was approximately 100-fold slower compared with that at pH 5. The fully reduced state was stable for at least 30 min. Absorbance spectra measured at 77 K resolve the presence of heme $f$, $b_{6f}$, and $b_{52}$ (Figure S3, Supporting Information), with the dithionite-minus-ascorbate difference spectrum defining the spectra of hemes $b_6$ and $b_{52}$. Dithionite addition was performed under anaerobic conditions. On the basis of the area under the domains of the low temperature (77 K) difference spectra in the $\alpha$-band region that arise from hemes $b_6$ and $f$ (Figure S3, Supporting Information), the measured molar ratio of heme $b$ to heme $f$ was 2.0:1.

3.2. Simultaneous Measurement of Circular Dichroism (CD) and Absorbance Spectra. Soret band CD spectroscopy, utilized as described elsewhere52 and in section 2.6 in the Materials and Methods section, has been previously used to describe exciton interactions between $b$-hemes of respiratory cytochrome $bc_{1}$,53,62,63 and in studies on the C. reinhardtii photosynthetic $b_{6f}$ complex,52 although the identity of the interacting hemes was not determined. The basis for the method rests on the net transition dipole moment of two or more individual transition moments in a molecular array resulting from superposition of the individual moments. The net polarization of circularly polarized light results from preferential absorption of right- and left-hand polarized light by the array.64

The time course of $b$-heme reduction measured by the Soret band absorbance change at room temperature (Figure 2A and B) and of heme–heme exciton interaction, assayed by CD spectra (Figure 2C) of active dimer, are experimentally indistinguishable (Figure 2D). As in the previous CD studies on the $bc_{1}$51 and $b_{6f}$52 complexes, the amplitude of the short wavelength (428 nm) positive lobe of the split CD spectrum is approximately 20% larger than the longer wavelength negative lobe centered at 436 nm52 (Figure 2C). In the $b_{6f}$ complex, this asymmetry could be a consequence of a CD signal arising from the reduction of the unique heme $c_6$, identified in refs 12, 24, and 29, whose Soret band maximum in the reduced state is approximately 424 nm.19 However, neither heme $c_6$ nor any features unique to $b_{6f}$ compared to $bc_{1}$ are likely to be the source of this asymmetry, as the asymmetry is similar in the CD spectra of the $bc_{1}$ complex51 in which heme $c_6$ is absent. The normalized CD spectra of monomer and dimer are similar (Figures 3, Figure S4, Supporting Information), implying that...
the major excitonic interaction responsible for the split CD spectrum arises from the two intramonomer hemes, \( b_p \) and \( b_n \). This conclusion is in excellent agreement with the CD spectra calculated from the information available from crystal structures on interheme distances and dihedral angles (Materials and Methods, section 2.6), shown in Figure 3. The major source of the split CD signal (Figure 2C, Figure S4, Supporting Information) is the interaction between the intramonomer \( b \)-heme pairs on the electrochemically positive (p) and negative (n) sides of the membrane, \( p_1-n_1 \) and \( p_2-n_2 \) (red trace). Significantly smaller CD signals are expected from all other \( b \)-heme pairs (Figure 3, Table 1). Table 1 summarizes the relative intensities of CD signals from all six interacting pairs formed by the four \( b \)-hemes.

### 3.3. Preferential Reduction of the Intramonomer \( b_n-b_p \) Pair; Mechanism of Dithionite Reduction.

Using a common measuring beam in the combined spectrophotometer/polarimeter described in Materials and Methods, simultaneous measurement was made of the kinetics of the absorbance changes (Figure 2D) at 432 nm (red, triangles) associated with \( b \)-heme reduction, and of the ellipticity changes of the Soret band split CD signal, measured at 428 nm (circles, blue) and 437 nm (squares, blue), simultaneously measured (16 s per spectrum). Measurement of all CD and OD difference spectra was started 10 (blue), 74 (pink), 138 (green), 234 (red), and 410 (brown) s after addition of dithionite. (D) Measured kinetics induced by dithionite addition to the dimeric \( b_{6f} \) complex: absorbance increase at 432 nm (triangles, black); amplitude of Soret band circular dichroism (CD) signals at 428 nm (circles, blue) and 437 nm (squares, blue).

Figure 3. Calculated CD interaction between all reduced heme pairs: \( p_1-n_1 \) or \( p_2-n_2 \) (red trace), which are the dominant interactions; calculated spectra are shown for the other heme pairs, \( p_1-n_2, p_2-n_1 \) (purple), \( p_1-p_2 \) (blue), and \( n_1-n_2 \) (green).

The major excitonic interaction responsible for the split CD spectrum arises from the two intramonomer hemes, \( b_p \) and \( b_n \). This conclusion is in excellent agreement with the CD spectra calculated from the information available from crystal structures on interheme distances and dihedral angles (Materials and Methods, section 2.6), shown in Figure 3. The major source of the split CD signal (Figure 2C, Figure S4, Supporting Information) is the interaction between the intramonomer \( b \)-heme pairs on the electrochemically positive (p) and negative (n) sides of the membrane, \( p_1-n_1 \) and \( p_2-n_2 \) (red trace). Significantly smaller CD signals are expected from all other \( b \)-heme pairs (Figure 3, Table 1). Table 1 summarizes the relative intensities of CD signals from all six interacting pairs formed by the four \( b \)-hemes.
less permeable in the hydrophobic cytochrome complex than is the physiological protonated plastoquinol or semiquinone reductant, which are also substantially weaker reductants.

The ratio of the slow and fast kinetic components characterizing the kinetics of the absorbance and CD changes (Figure 2D) is similar to the ratio of monomer to dimer complex in the fractions separated by size exclusion chromatography and analyzed by SDS-PAGE and native gel electrophoresis (Figures S2A and B, Supporting Information). The absorbance data is described by a double exponential, having rate constants for fast and slow components of 0.18 ± 0.02 s⁻¹ (22%) and 0.006 ± 0.001 s⁻¹ (78%). In addition, to explain the coincident time course of the CD and absorbance changes, an explanation is needed of the mechanism by which four electrons are transferred to the complex.

3.4. Model of the Observed CD and Absorbance Dynamics. The rate of electron transfer to the cytochrome complex, $k_5$, is assumed to be limited by the diffusion rate of the donor molecule (dithionite) to the complex and thus is independent of the redox state of the complex. Assuming that dithionite is a one-electron donor, four electron transfer steps are required to completely reduce the initially oxidized four b-heme complex, as described by eq 4 below:

$$N_i(t) \rightarrow N_i(t) \rightarrow N_i(t) \rightarrow N_i(t) \rightarrow N_i(t)$$

$N_i(t)$ represents the time-dependent population state of the dimeric complex with subscript $i$ denoting the number of reduced b-hemes. The system of kinetic equations can be solved analytically, as shown for the case of one-electron transfer (eq S3, Supporting Information). Since the time scale of reduction of the complex by the donor (~100 s) is much slower than intracomplex electron transfer between the b-hemes (~1 ms), it is assumed that the electrons in the complex adopt the thermodynamically most probable configuration, i.e., that with the lowest free energy. The absorbance (OD) signal, OD(t), reflects the total population of the reduced b-hemes, i.e.,

$$OD(t) \sim \{N_1(t) + 2N_2(t) + 3N_3(t) + 4N_4(t)\},$$

and does not depend on the order in which the b-hemes are reduced. The CD signal kinetics, CD(t), however, depend on the order in which the b-hemes are reduced, because reduction of the intramonomer $b_n$ and $b_p$ hemes is uniquely required for a CD signal of significant amplitude (Table 1, Figure 3). If these two intramonomer b-hemes are the first to be reduced, the CD signal will increase faster than if any other heme pair is populated first (Figure 4). Thus, two models are considered:

(i) "n−n" model (Figure 5A): the lowest doubly reduced state of the dimer corresponds to two electrons residing on the two $b_n$ hemes on the electrochemically negative side of the membrane that belong to different subunits. In that case, three electron transfer steps are needed to observe a significant increase in the CD signal that would arise from a reduced n−p pair.

(ii) "n−p" model (Figure 5B): the lowest doubly reduced state of the dimer corresponds to two electrons residing on the $b_n$ and $b_p$ hemes belonging to the same monomer. In this case, two electron transfer events will result in a significant increase of the CD signal.

The CD signal kinetics for different models are calculated according to the population of different pairs of b-hemes and their CD yields (Table 1, eq S5 and Figure S5, Supporting Information). The measured absorbance difference was fit using kinetic scheme 4, assuming the presence of two different pools of $b_{n}$ complexes with different rate constants, $k$. The best fit is shown in Figure 4. In addition, the CD signals expected for the two models are plotted and compared against the measured CD kinetics. The faster rate, which is comparable with the time resolution of the experiment, is attributed to “contaminant” monomer complex in the dimer preparation (Figure S2B, Supporting Information, native gel). Black curve: fit to measured absorbance changes (triangles). Blue curve: expected CD kinetics in the “n−p” model where the intermediate doubly reduced intramonomer (Figure 5B; state $N_3$) dimer has a lower free energy than the intermonomer doubly reduced state in which heme $b_n$ is reduced in both monomers (Figure 5A; state $N_2$). Red function: expected time course of the CD change in the “n−n” model in which the intramonomer n1−n2 doubly reduced state has the lowest free energy.

Figure 4. CD (circles, squares, blue) and optical density changes (triangles, black) were measured simultaneously, as described in the Materials and Methods section. Solid curves are kinetic simulations as described in the Results section, with the assumption that dithionite acts as a one-electron donor; the predicted time course for dithionite acting as a two-electron donor is shown in Figure S7 (Supporting Information). Electron transfer to the $b_{n}$ dimer is described by two rate constants: $k_5 = 0.017 s^{-1}$ (70%) and $k_5 = 0.33 s^{-1}$ (30%); the larger rate constant is attributed to “contaminant” monomer complex in the dimer preparation (Figure S2B, Supporting Information, native gel). Black curve: fit to measured absorbance changes (triangles). Blue curve: expected CD kinetics in the “n−p” model where the intermediate doubly reduced intramonomer (Figure 5B; state $N_3$) dimer has a lower free energy than the intermonomer doubly reduced state in which heme $b_n$ is reduced in both monomers (Figure 5A; state $N_2$). Red function: expected time course of the CD change in the “n−n” model (Figure 4, blue curve).

Dithionite can also act as a two-electron donor (Figure 5A and B), in which case the following kinetic scheme (eq 5) describes the OD and CD signals:

$$N_1(t) \rightarrow N_2(t) \rightarrow N_3(t) \rightarrow N_4(t)$$

The analytical solution for the respective system of kinetic differential equations is provided in the Supporting Information (eq S7), and the respective fit of OD and CD signals for the “n−p” and “n−n” models is shown (Figure S6, Supporting Information). The predicted delay in the time course of the CD signal in the “n−n” model is almost identical for the case of a one- and two-electron donor (compare Figure 4 and Figure S6, Supporting Information), and disagrees with the experimental data. It is important to note that, if dithionite acts as a mixture of one- and two-electron donors, a close fit of the time course of OD and CD signals can be achieved within the “n−p” model, while the “n−n” model contradicts the data (Figure S7, Supporting Information).

Therefore, it is possible that dithionite donates one or two electrons, the “$b_n−b_p$” model ("n−p" in Figure 5) uniquely predicts the relative time course of the absorbance and CD

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Figure 5. Summary of possible electron transfer routes and heme reduction states in the b_{i/f} complex. Two different models are considered: (A) n1—n2 model: the doubly reduced state of lowest energy of the dimer corresponds to two electrons residing on the two b_{i} hemes belonging to different subunits (this state produces a weak negative CD signal (Figure 3). (B) n—p model: the lowest doubly reduced state of the dimer corresponds to two electrons residing on the b_{i} and b_{p} hemes belonging to the same subunit (in this state, the amplitude of the positive CD signal is significantly larger than that of any other heme pair). The sequence of four electron transfer events in these two models is illustrated. Reduced hemes are shown as red spheres. State N_{0} in panels A and B denotes fully oxidized hemes in dimeric complex. N_{1} represent states of the dimeric complex in which subscript "i" represents the number of reduced hemes. States N_{1} and N_{2} are bypassed if dithionite acts as a 2 e^− donor. (C) Summary of conceivable two electron half-reduced states, of which the three states marked by "X" are inferred to be substantially less probable, although they have been documented to exist (refs 14, 15, 17, 19, 21, and 26).

4. DISCUSSION

4.1. The Preferential "b_{n}−b_{p}" (n—p) Model; Thermodynamic Considerations Modified by Electrostatic Interactions. The contradiction to the expectation, based on elementary thermodynamics, that the two higher potential b-hemes (b_{n}) provide the most stable two-electron-reduced state of the heme pairs in the dimeric cytochrome complex lies in the electrostatic interactions that result from injection of electronic charge into a low dielectric medium. The preferred residence of electron pairs on one monomer is not a consequence of electron transfer to the other monomer in the dimer being blocked in any conceivable way but results from the reduced intramonomer b-heme configuration being the lowest energy state for the two-electron-reduced dimer.

4.2. Consequences for Heterogeneity of Dielectric Constant in the Cytochrome b_{i/f} Complex. The preferential reduction by dithionite of the intramonomer hemes b_{n} and b_{p} can be explained only if the dielectric constants are not equivalent between the different b-heme pairs summarized in Figure 5, and if the local dielectric constant between the intramonomer hemes b_{n} and b_{p} is relatively high (Figure 6). Thus, the structure of the photosynthetic b_{i/f} complex is characterized by internal dielectric heterogeneity. The nature of the dielectric phenomena in the interior of proteins has been discussed. Dielectric heterogeneity in hetero-oligomeric membrane protein complexes involved in photosynthetic energy transduction has been inferred previously in the hetero-oligomeric bacterial and photosystem I reaction centers.

4.3. Boundary Conditions for Dielectric Heterogeneity. The data indicate that the two electrons transferred to a monomer (Figure 5B, N_{2} panel) do not spill over to the neighboring monomer (Figure 5C) within the several minute time span of the measurements. Because intermonomer electron transfer between the two b-hemes in the dimer is expected to be on the order of microseconds (edge-to-edge distance, 12.8 Å), there is sufficient time for energetic equilibration between all possible forms of a doubly reduced dimer (Figure 5C), unless there is an energetic mechanism that precludes electron transfer between the monomers. Since the latter is improbable, it is inferred that the intramonomer reduced heme b_{p}−b_{p} pair (Figure 5B, N_{2} panel; Figure 5C, state b) corresponds to the lowest energy configuration. The total energy $E_{q}$ of this pair can be expressed as eq 6:

$$E_{q} = E_{i} + E_{j} + V_{ij}$$

where $E_{i}$ and $E_{j}$ are the energies of noninteracting electrons in sites i and j, respectively, and $V_{ij}$ is the Coulombic interaction energy between these two electrons, which, as shown below, can be comparable or larger than the difference.
in redox energy between the two hemes in the complex. If the dielectric constant is \( \varepsilon = 2 \), then the electrostatic interaction energy between two hemes in the complex is on the order of \( 0.2 \) to \( 0.3 \) eV.

The indices \( i \) and \( j \) correspond to the reduction of one of the four \( b \)-hemes: \( i, j = n_1, p_1, n_2, p_2 \), where \( n_1, p_1 \) denote hemes \( b_n \) and \( b_p \) in one monomer and \( n_2, p_2 \), the pair in the second monomer of the dimeric complex. The electrostatic interaction energy in a point-charge approximation, described by Coulomb’s law, is obtained from eq 7 below:

\[
V_{ij} = \frac{1}{4\pi\varepsilon_0\varepsilon_j} \frac{e^2}{R_{ij}}, \quad i \neq j
\]

where \( e \) is the charge of an electron, \( R_{ij} \) is the distance between two electrons, \( \varepsilon_0 \) is the permittivity of free space, and \( \varepsilon_j \) are the effective dielectric constants that operate between any heme pair, \( b_i \) and \( b_j \). The above eq 7 can then be rewritten as eq 8:

\[
V_{ij} \text{[eV]} = \frac{14.4 \times [\text{Å} \cdot \text{eV}]}{\varepsilon_j R_{ij}}, \quad i \neq j
\]

with the units of \( R_{ij} \) in Å, the resulting \( V_{ij} \) expressed in electron-volts (eV), and the dimensions of the numerator, Å·eV. The \( R_{ij} \) are the center-to-center distances between the respective hemes: \( R_{n_1p_2} = 21 \) Å, \( R_{n_2p_2} = 34 \) Å, \( R_{n_1n_2} = 35 \) Å, and \( R_{p_1p_2} = 22 \) Å (Table 1); \( \varepsilon_j \) is the corresponding dielectric constant between these heme pairs. The consensus values of midpoint redox potentials, \( E_m \), of hemes \( b_p \) and \( b_n \) in isolated active \((\geq 200 \) electrons transferred/cytochrome \( f \)-s) cytochrome \( b_6f \) complex, measured in vitro and in situ, differ by 0.05 to \( 0.1 \) V, with heme \( b_p \) having the more negative potential.\(^{19,20,55,56,72}\) Thus, defining the energy of the reduced heme \( b_{n/p} \) as \( E_{n/p} = E_{n/p} = 0 \), the energy of the reduced heme \( b_{p/n} \) will be \( E_{n/p} = E_{n/p} = 0.05\) to \( 0.1 \) eV corresponding to the difference, \( \Delta E_m \), in midpoint potential of hemes \( b_p \) and \( b_n \). Using formulas 6 and 8, and taking \( \Delta E_m \) into account, the energies (in electron-volts, eV) for all possible
doubly reduced states of the dimeric complex shown in Figure 5C are obtained from eq 9.

\[
\begin{align*}
E_{n1,n2} &= \frac{14.4 \text{ Å} \text{ eV}}{\varepsilon_{n1,n2}\mathbf{R}_{n1,n2}} \quad \text{(state a)} \\
E_{n1,p1} &= E_{n2,p2} = \Delta E_m \text{ [eV]} + \frac{14.4 \text{ Å} \text{ eV}}{\varepsilon_{n1,p1}\mathbf{R}_{n1,p1}} \quad \text{(state b)} \\
E_{n1,p2} &= E_{n2,p1} = \Delta E_m \text{ [eV]} + \frac{14.4 \text{ Å} \text{ eV}}{\varepsilon_{n1,p2}\mathbf{R}_{n1,p2}} \quad \text{(state c)} \\
E_{p1,p2} &= 2\Delta E_m \text{ [eV]} + \frac{14.4 \text{ Å} \text{ eV}}{\varepsilon_{p1,p2}\mathbf{R}_{p1,p2}} \quad \text{(state d)}
\end{align*}
\]

The equations for states (b–d) contain two terms: the first term describes the difference in midpoint redox potential, \(\Delta E_{m}\), in units of eV (states b, c), between the n- and p-side b-hemes, and \(\Delta E_m\) (state d), the total difference in midpoint potential between the two p-side and n-side b-hemes in the complex. The second term on the right-hand side of the formulas for states b–d, and the term on the right sides of the formula for state a, describes the Coulombic interaction between the b-hemes in a point charge approximation.

The constraint on the dielectric constants that result in a Coulombic interaction energy \(E_{n1,p1}\) or \(E_{n2,p2}\) level between the two trans-membrane hemes \(b_n\) and \(b_p\) in each monomer being lower than that between the two high potential hemes, \(E_{n1,p1}\), is

\[\varepsilon_{n1,p1} > \varepsilon_{n2,p2}\]

where \(\varepsilon_{n1,n2}\) is the dielectric constant between the two hemes \(b_{n}\). \(\varepsilon_{n1,n2}\) is assumed to be close to the minimum value inferred to exist in a protein, \(\varepsilon = 2.5\), because (i) the protein is embedded in a membrane, and (ii) the intermonomer cavity between the hemes \(b_n\) contains a significant lipid dipole, as depicted in Figure 6. Values of \(E_{n1,p1}\) or \(E_{n2,p2}\) that satisfy this inequality for \(\Delta E_m\) values of 0.05, 0.075, and 0.1 V are >6.1, 7.8, and 10.8 (Table 2). For the same set of \(\Delta E_m\) values, the local dielectric constants that would render most probable (a) the intermonomer, interheme pathways, are \(\varepsilon_{n1,p2}\), \(\varepsilon_{n2,p1}\) > 3.7, 4.7, and 6.6; for \(\Delta E_m\) values of 0.05 and 0.075 V, \(\varepsilon_{p1,p2}\) > 10.1 and 44. It is important to note that the energetic preference for reduction of the intramonomer heme pairs \(b_{n1}, b_{p1}\) and \(b_{n2}, b_{p2}\) implies that the values of the dielectric constants, \(\varepsilon\), between the other heme pairs, \(b_{n1}, b_{p1}\) and \(b_{n2}, b_{p1}\), and \(b_{n1}, b_{p2}\) are smaller than the values presented in Table 2.

4.4. The Preferential Intramonomer Electron Transfer Pathway; Interheme Distance Considerations. If the elevated dielectric constant (>6.1) between the intramonomer hemes \(b_n\) and \(b_p\) provides the explanation for the preferential heme reduction of the intramonomer heme pair, the question arises as to why reduction of the intermonomer heme pair, \(b_{n1} - b_{p2}\) or \(b_{n2} - b_{p1}\), is disfavored if the constraint on this interheme dielectric constant, \(\varepsilon > 4\), is less stringent. The low probability of utilization of the latter electron transfer pathway is a consequence of the steep donor–acceptor distance dependence of intraprotein electron transfer and the much greater distance, 22.3 Å, between the intermonomer heme rings compared to 8.1 Å between the intramonomer pair (Figure 1B). Although the interdistance, 12.8 Å (Figure 1B), does not preclude interheme electron transfer, preferred reduction of the heme \(b_{n1} - b_{p2}\) pair is rendered extremely unlikely because of the requirement of a dielectric constant of \(\geq 44\), substantially approaching that of bulk water. This extreme constraint is a consequence of the large free energy surplus in the two hemes \(b_p\) resulting from the appreciable (75 mV in this calculation) redox potential difference between hemes \(b_n\) and \(b_p\). See the note in Table 2 regarding the case of \(\Delta E_m = 100\) mV. It should be noted that the above dielectric constants are not absolute but are referenced to an assumed dielectric constant of 2.5 in the lipid-containing intermonomer space between the two hemes \(b_n\), whose rationale is discussed above.

4.5. Origin of Heterogeneous Dielectric Constants: Reorganization Energy Associated with Electron Transfer? Heterogeneity in the Structure. The existence of heterogeneous dielectric constants in electron transferring membrane proteins is not unprecedented. The local dielectric constants in the electron transfer pathways of the multisubunit hetero-oligomeric photosynthetic reaction centers can be higher than the values (\(\varepsilon \approx 2.5\)) assigned to a dry folded protein. A value of \(\varepsilon \approx 7\) was found for the region of the protein surrounding the electron transfer chain in PS I, and local values of 4.5–4.7 were inferred along the active electron transfer branch of the bacterial photosynthetic reaction center complex.

As shown in Figure 6: \(\varepsilon_{n1,n2}\), dielectric constant between hemes \(b_{n1}\) and \(b_{n2}\), \(\varepsilon_{n1,p1}\), dielectric constant between hemes \(b_{n1}\) (or \(b_{n2}\)) and \(b_{p1}\), \(\varepsilon_{n2,p2}\), dielectric constant between hemes \(b_{n1}\) (or \(b_{n2}\)) and \(b_{p2}\), \(\varepsilon_{p1,p2}\), dielectric constant between hemes \(b_{n1}\) and \(b_{p1}\), \(\varepsilon_{p1,p2}\), dielectric constant between hemes \(b_{n1}\) and \(b_{p2}\), and \(\varepsilon_{p1,p2}\), mid-point redox potential difference between hemes \(b_{n}\), \(b_{p}\), and \(b_{n}\), given an unfavorable redox potential difference for storage of two electrons on the two hemes \(b_{n}\) there is no value of the dielectric constant that will satisfy this state.

Table 2. Threshold Interheme Dielectric Constants (\(\varepsilon\)) in the Dimeric Cytochrome \(\alpha_{sF}\) Complex

<table>
<thead>
<tr>
<th>(\Delta E_m) (mV)</th>
<th>(\varepsilon_{n1,p1})</th>
<th>(\varepsilon_{n2,p1})</th>
<th>(\varepsilon_{p1,p2})</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>≥6.1</td>
<td>≥7.8</td>
<td>≥6.6</td>
</tr>
<tr>
<td>75</td>
<td>≥7.8</td>
<td>≥4.7</td>
<td>≥43.6</td>
</tr>
<tr>
<td>100</td>
<td>≥10.8</td>
<td>≥6.6</td>
<td>*</td>
</tr>
</tbody>
</table>

"As shown in Figure 6: \(\varepsilon_{n1,n2}\), dielectric constant between hemes \(b_{n1}\) and \(b_{n2}\), \(\varepsilon_{n1,p1}\), dielectric constant between hemes \(b_{n1}\) (or \(b_{n2}\)) and \(b_{p1}\), \(\varepsilon_{n2,p2}\), dielectric constant between hemes \(b_{n1}\) (or \(b_{n2}\)) and \(b_{p2}\), \(\varepsilon_{p1,p2}\), mid-point redox potential difference between hemes \(b_{n}\) and \(b_{p}\), and \(\varepsilon_{p1,p2}\), dielectric constant associated with electron transfer, described locally in an electron transfer pair by a reorganization energy."

The structural origin of the heterogeneity of the dielectric constant of the cytochrome \(\alpha_{sF}\) complex can be attributed to several features of the protein complex: (a) The arrangement of the \(\alpha\)-helices in the structure is intrinsically anisotropic, as is true of all helical membrane proteins; (b) a heterogeneous environment could arise from the nonuniformity introduced by lipids intercalated between subunits in these proteins, as documented for the \(\alpha_{sF}\) and \(\alpha_{ci}\) complexes. In the case of the dimeric \(\alpha_{sF}\) complex, the intramonomer hemes \(b_{p}\) and \(b_{n}\) are embedded within a protein environment, while the two \(b_{n}\) hemes are separated by a large, apolar intermonomer cavity (Figure 6), which is expected to lower the effective dielectric constant between the \(b_{n}\) hemes. Thus, it is expected that the reduction of the intramonomer heme \(b_{n}\) pair would be energetically more favorable than the reduction of the intermonomer heme \(b_{p}\), thereby precluding reorganization associated with electron transfer.
4.6. Consequences of Dielectric Heterogeneity for Efficiency of the Trans-Membrane Electron Transfer Pathway. It has been found that electrons can cross over from one monomer to the other in the dimeric cytochrome bc
dcomplex.\textsuperscript{40,41,43,47} In the present study, it is inferred that the
most probable pathway for transfer of the two electrons donated by plastoquinol on the p-side of the complex to the n-
side, where they are utilized for quinone reduction, is via a
transfer that is effectively pairwise through the heme pair, \(b_p\)
and \(b_n\). The existence of a preferred intramonomer pathway
does not contradict the experimental documentation that
electrons can cross over between the two monomers of the
dimeric complex\textsuperscript{40,41,43,47} at the level of heme \(b_p\). Electrons
injected into different monomers in the dimeric complex on the
p-side are expected to merge to one of the monomers, the most
stable doubly reduced thermodynamic state.

4.7. Further Consequences for Function. It is concluded
that, if two electrons are injected into different monomers of the
dimeric complex, the electrons tend to cross over into one
monomer, which represents the most stable thermodynamic
state. Thus, electron transfer across the trans-membrane
domain of the \(b_{cf}\) complex is expected to proceed through the
monomer to the quinone bound on the n-side, whose
reduction would then require only two p-side quinol oxidation
events. Alternatively, if reduction of the two \(b_p\)-hemes
represented the most stable thermodynamic state of a
two-electron-reduced \(b_{cf}\) dimer, then up to three p-side quinol
oxidation events could be required to provide two electrons for
n-side quinone reduction. The p-side quinol deprotonation–
oxidation reactions are known to contain the slow, millisecond
rate-limiting charge transfer step in cytochrome \(bc\) complexes.\textsuperscript{6,79} Due to the more stable environment for an electron
within the \(b_f\) monomer, the requirement for slow p-side quinol
reactions is decreased from three to two, thereby
increasing the efficiency of the complex. As described
elsewhere, electron transfer within the cytochrome complex
may thus be essential to reducing the lifetime of unpaired
electrons, which can leak to oxygen to form possibly deleterious
superoxide.\textsuperscript{57}

\section*{ASSOCIATED CONTENT}

\section*{Supporting Information}
Documentation is presented underlying a new perspective, a
mapping of internal dielectric constants, for analyzing the
internal structure of hetero-oligomeric integral membrane
proteins. In a framework describing the b-heme centers in the
core of the cytochrome \(b_{cf}\) complex, and electrophoretic and
spectrophotometric characterization of the complex,
simulations are presented of the time course of formation of
absorbance and CD changes arising from dithionite reduction
of the hemes, which document that the intramonomer heme pair is preferentially reduced. This material is available free of
charge via the Internet at http://pubs.acs.org.

\section*{AUTHOR INFORMATION}

Corresponding Authors
*Phone: 765-494-0706. E-mail: sergei@purdue.edu.
*Phone: 765-494-4956. E-mail: waclab@purdue.edu.

Notes
The authors declare no competing financial interest. 
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NOTE ADDED IN PROOF

During the revision of the current manuscript, a 2.5 Å electron density map of the cytochrome $b_{6}f$ complex (PDB ID 4OGQ) has been published, which shows an extensive lipid component within the inter-monomer cavity.