Tuning of Hemes b Equilibrium Redox Potential Is Not Required for Cross-Membrane Electron Transfer*

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Received for publication, December 22, 2015, and in revised form, February 2, 2016. Published, JBC Papers in Press, February 8, 2016, DOI 10.1074/jbc.M115.712307

In biological energy conversion, cross-membrane electron transfer often involves an assembly of two hemes b. The hemes display a large difference in redox midpoint potentials ($\Delta E_m(b)$), which in several proteins is assumed to facilitate cross-membrane electron transfer and overcome a barrier of membrane potential. Here we challenge this assumption reporting on heme membra electron transfer and overcome a barrier of membrane potential. We propose that changes do not impose overly endergonic steps on downhill electron transfer from substrate to product. We link this concept with a natural flexibility in occurrence of several thermodynamic configurations of the direction of electron flow and the direction of the gradient of potential in relation to the vector of the electric membrane potential.

Redox midpoint potential ($E_m$) is a key property of any redox active cofactor in proteins. It regulates biological functions via thermodynamic and kinetic control of electron exchange reactions. Because these reactions must take place in a variety of cellular compartments, both outside and inside the biological membrane, the structures of redox proteins have evolved to meet physicochemical requirements of these various environments to achieve assemblies that secure functionally competent $E_m$ values.

Within the group of cytochromes, molecular factors that modulate $E_m$ include types of heme axial ligation (1–3). The residues that are most commonly recruited as axial ligands for the heme iron are His and/or Met (4). A binding of hemes b within the membranous proteins is accomplished by an assembly of transmembrane $\alpha$-helices that provide His axial ligands for the heme-iron. In fact, the heme binding $\alpha$-helix bundle represents a common motif of several bioenergetic complexes (5–7). It has even been used as a prototype to construct human-made versions of heme binding proteins (protein maquettes) (8–10).

The $\alpha$-helix bundle can bind one or two hemes b. In several proteins, an assembly of two b type hemes, each facing different sides of the membrane, supports electron transfer across biological membranes crucial for energy conservation in many systems. Intriguingly, the two hemes differ largely in their redox midpoint potentials (the $E_m$ difference, $\Delta E_m(b)$, is typically in the range of 100 mV); however, the thermodynamic rationale behind the existence of $\Delta E_m(b)$ remains unclear. This is because no general rule for the direction of $\Delta E_m(b)$ with respect to the direction of the electric field generated by the membrane potential or the direction of physiological electron transfer is evident (Fig. 1).

The cytochrome b subunit of cytochrome bc$_1$ (mitochondrial complex III) is a well known example of a protein supporting cross-membrane electron transfer by using an assembly of two hemes b, named heme $b_{14}$ and heme $b_{15}$, where subscripts H and L refer to high and low potential, respectively (for recent reviews see Refs. 11 and 12). During the catalytic cycle, the electron transfer from heme $b_1$ to heme $b_{14}$ connects the quinol oxidation site (Qo)$_2$ and the quinone reduction site (Qi). In addition, in dimeric structure of an enzyme, intermonomer electron transfer parallel to the membrane plane involving two hemes $b_{14}$ is possible (13–16). In living cells, the cross-membrane electron flow from heme $b_1$ to heme $b_{14}$ may face the barrier of the membrane potential (Fig. 1a). Thus, the fact that electrons are transferred from the cofactor of lower $E_m$ to the cofactor of higher $E_m$ provided a basis for a general assumption that $\Delta E_m(b)$ is one of the factors that facilitate cross-membrane electron transfer and perhaps is important in overcoming the barrier of potential (17, 18). However, the contribution of
**E_m of Hemes b and Cross-membrane Electron Transfer**

![Figure 1. Possible thermodynamic configurations for cross-membrane electron transfer in cytochromes b.](image)

**ΔE_m** b to the overall electron flow has not been verified experimentally. Prerequisites for such verification are variants of cytochrome bc₁ with large changes in the **E_m** of hemes b and, consequently, large changes in **ΔE_m** b. However, the mutations of cytochrome b tested so far either had a relatively small effect on the **E_m** of hemes b (19, 20) or resulted in the absence of the heme (21, 22).

Here we mutated the native bis-His coordination pattern for heme b₁ and/or heme b₄ into the Hist-Lys pattern, to our knowledge, providing the first His-Lys coordinated hemes b₁ in a transmembrane protein. The hemes remain low spin as in a native enzyme but have markedly elevated **E_m** b. The mutations of cytochrome b tested so far either had a relatively small effect on the **E_m** of hemes b (19, 20) or resulted in the absence of the heme (21, 22).

**Experimental Procedures**

**Bacterial Strains, Plasmids, and Growth Conditions—Rhodobacter capsulatus and Escherichia coli** (HB101, DH5α) were grown in liquid or solid MPYE (mineral-peptone-yeast extract) and LB (Luria Bertani) media, at 30 and 37 °C, respectively, supplemented with appropriate antibiotics as needed. Respiratory growth of *R. capsulatus* strains was achieved at 30 °C in the dark under semiaerobic conditions. Photosynthetic growth abilities of mutants were tested on MPYE plates using anaerobic jars (GasPak™ EZ Anaerobe Container System; BD Biosciences) at 30 °C under continuous light. The *R. capsulatus* strains used were: pMTS1/MTRbc₁, which overproduces wild-type cytochrome bc₁ from the expression vector pMTS1 (contains a copy of petABC operon coding for all three subunits of cytochrome bc₁), and MTRbc₁ which is a petABC operon deletion background (23). The mutagenized pMTS1 derivatives were introduced to *R. capsulatus* MTRbc₁ via triparental crosses as described (23). Plasmid pPET1 (a derivative of pBR322 containing a wild-type copy of petABC) was used as a template for PCR and in some of the subcloning procedures.

**Construction of Lys Mutants—Spontaneous Ps⁺ revertant of the H212N mutant originally described in Ref. 22 was obtained on MPYE plate containing tetracycline after ~7 days of cultivation under photosynthetic conditions.** The DNA sequence analysis of the plasmid DNA isolated from the revertant strain revealed a single base pair change replacing the mutated Asn into Lys at position 212 of cytochrome b. The Xmal/Sful fragment containing the reversion (mutation H212K) and no other mutations was exchanged with its counterpart on expression vector pMTS1 carrying the wild-type copy of the petABC operon. Expression of this vector in the MTRbc₁ background strain confirmed that cells bearing single mutation H212K display the photosynthetically competent Ps⁺ phenotype. The Ps⁺ reversion H212K occurred also in the double mutant A181T/H212N cultivated on MPYE plate containing kanamycin (in this case H212N in the cytochrome b subunit was accompanied by a mutation A181T in cytochrome c₁ described originally in Ref. (24)). Because A181T/H212N, unlike original H212N, contained cytochrome b already equipped with the Strep-tag II attached to its carboxyl end, the plasmid obtained from the revertant of A181T/H212N was used to construct the mutants used in further analysis. First, the Xmal/Sful fragment containing the reversion (H212K) and the sequence coding for Strep-tag II (ST), and no other mutations were exchanged with its counterpart on expression vector pMTS1 carrying the wild-type copy of the petABC operon. This created pMTS1-ST-H212K. Second, the same Xmal/Sful fragment was cloned into pPET1 creating pPET1-ST-H212K. Mutation H198K and the double mutation H212K/H198K were constructed by the QuikChange site-directed mutagenesis kit from Stratagene using pPET1-ST (25) and pPET1-ST-H212K plasmids as templates, respectively, and the mutagenic forward H198K-F (5'-GGGCAAGAATTTTCCGAGAAGAGCGG-3') and reverse H198K-R (5'-TTTTTCTCGCTGAAATATCTGCTGCCTTCC-3') oligonucleotides. After sequencing, Xmal/Sful fragments of pPET1 plasmids bearing the desired mutations, and no other mutations were exchanged with their wild-type counterparts in pMTS1. This created the plasmids pMTS1-ST-H198K and pMTS1-ST-H212K/H198K. Plasmids pMTS1-ST-H212K, pMTS1-ST-H198K, and pMTS1-ST-H212K/H198K were inserted into *R. capsulatus* MTRBC1 cells, creating mutants H212K, H198K, and H212K/H198K, respectively. These mutants are listed in Table 1. In each case, the presence of introduced mutations was confirmed by sequencing the plasmid DNA reisolated from the mutated *R. capsulatus* strains.

**Isolation of Membranes and Proteins—Chromatophore membranes were isolated from *R. capsulatus* as described previously (26).** The cytochrome bc₁ complexes were isolated from detergent-solubilized chromatophores by affinity chromatography using the procedure described previously (27). SDS-PAGE of purified complexes was performed as described before (28).
Optical and EPR Spectroscopy—Optical spectra measurements of isolated complexes and determination of protein concentration were performed on UV-2450 Shimadzu spectrophotometer. Cytochrome bc1 samples were suspended in 50 mM Tris, pH 8.0, 100 mM NaCl, 0.01% (m/m) dodecyl maltoside, and 1 mM EDTA, an appropriate amount of ferricyanide was added to fully oxidize complexes; then solid ascorbate and solid sodium dithionite were added to reduce samples, and spectra were recorded right after oxidation and after each step of reduction. Concentration of cytochrome bc1 was determined as described (26). EPR measurements were performed on Bruker Elexys ES80 spectrometer. X-band CW-EPR spectra of hemes were measured at 10 K, using SHQE0511 resonator combined with ESR900 Oxford Instruments cryostat unit, using 1.595 mT modulation amplitude, and 1.543 milliwatt of microwave power. For EPR measurements, cytochrome bc1 samples were dialyzed against 50 mM Tris, pH 8.0, 100 mM NaCl, 20% glycerol (v/v), 0.01% (m/m) dodecyl maltoside, and 1 mM EDTA. Final concentration of cytochrome bc1 in EPR samples was 50 μM. Antimycin A was used in 5-fold molar excess over the concentration of cytochrome bc1.

Redox Potentiometry—Midpoint potentials of hemes b were determined by dark equilibrium redox titrations on chromatophores according to the method described in Ref. (29). Chromatophores were suspended in argon-equilibrated 50 mM MOPS buffer (pH 7.0) containing 100 mM KCl and 1 mM EDTA. Immediately before the titration, the following redox mediators were added: 100 μM tetrachlorohydroquinone, 100 μM 2,3,5,6-tetramethyl phenylenediame (Ea, 260 mV), 100 μM 1,2-naphtoquinone-4-sulfonate (Em, 210 mV), 100 μM 1,2-naphtoquinone (Em, 130 mV), 50 μM phenazine methosulfate (Em, 80 mV), 50 μM phenazine ethosulfate (Em, 50 mV), 100 μM duroquinone (Em, 5mV), 30 μM indigotrisulfonate (Em, −90 mV), 100 μM 2-hydroxy-1,4-naphthoquinone (Em, −152 mV), 100 μM anthroquinone-2-sulfonate (Em, −225 mV), and 100 μM benzyl viologen (Em, −374 mV). Dithionite and ferricyanide were used to adjust ambient redox potential. During the titrations, samples of 150–200 μl were taken and transferred to EPR tubes anaerobically and frozen by immersion into cold ethanol. The Em values of hemes b were determined by fitting the amplitudes of appropriate EPR g transition to the Nernst equation for one-electron couple (for WT, H212K, and H198K) or for two one-electron couples (in case of H212K/H198K mutant).

Flash-induced Electron Transfer Measurements—Measurements of flash-induced turnover kinetics of cytochrome bc1 were performed on a home-built double wavelength time-resolved spectrophotometer as described in previous work (24). Chromatophores for measurements were suspended in 50 mM MOPS, pH 7.0, 100 mM KCl, 3.5 μM valinomycin, and redox mediators: 7 μM 2,3,5,6-tetramethyl-1,4-phenylenediame, 1 μM phenazine methosulfate, 1 μM phenazine ethosulfate, 5.5 μM 1,2-naphtoquinone, and 5.5 μM 2-hydroxy-1,4-naphthoquinone (valinomycin and redox mediators were added immediately before the measurement). Dithionite and ferricyanide were used to adjust ambient redox potential. Inhibitors antimycin A and myxothiazol were used at final concentration of 7 μM. Transient hemes b reduction kinetics were followed at 560–570 nm (for WT and H198K) or 557–570 nm (for H212K and H212K/H198K). Transient hemes c oxidation and re-reduction kinetics were followed at 550–540 nm. Single flash activation measurements were initiated by a short saturating flash (10 μs) from a xenon lamp, and multiple flash activation measurements were initiated by a series of short (10 μs) saturating flashes every 20 ms. In order to measure kinetics in the presence of the membrane potential, valinomycin was omitted. Rates of flash-induced heme b reduction were determined by fitting transient kinetics data to a single exponential equation.

Steady-state Kinetics Measurements—Steady-state enzymatic activities of cytochrome bc1 complexes in chromatophores were determined spectroscopically by the decylubiquinol-dependent reduction of bovine heart cytochrome c (Sigma-Drich) as described before (23). Conditions used in assays were as follows: 50 mM Tris-HCl (pH 8), 100 mM NaCl, 20 μM decylubiquinol, 20 μM oxidized cytochrome c. Errors were calculated as standard deviation of the mean of nine measurements. Chromatophores were treated with KCN (final concentration in sample was 0.5 mM) before experiments. Decylubiquinol was obtained as described (30).

Results

His-Lys Ligation for Hemes b in a Low Spin State Is Possible in Transmembrane Cytochrome b—Changes in the coordination pattern of heme iron are expected to exert a large influence on the redox properties of hemes (1). Thus, to impose large shifts in Em values of hemes b in cytochrome b, we created three variants in which one of the His ligands to heme iron was replaced by Lys (Lys mutants): single mutants H212K (for heme bL) and H198K (for heme bH) and the double mutant H212K/H198K combining both single mutations (Fig. 2a).

The mutated complexes contained all three catalytic subunits, as indicated by the SDS electrophoretic profiles (Fig. 3a). Optical (UV-visible), and electron paramagnetic resonance spectroscopy (EPR) showed that the mutants contained all redox active cofactors: heme c1, hemes b (bL and bH), and 2Fe-2S cluster (Figs. 2b and 3, b and c). Although the spectral and redox properties of 2Fe-2S and heme c1 remained unchanged in the mutants, the properties of hemes b were modified. We emphasize the results of EPR analysis (Fig. 2b), which in this case is most informative because, unlike UV-visible spectroscopy, it allows for a complete spectral separation of g transitions originating from each of the hemes b: in native enzyme g = 3.78 and 3.44, corresponding to g transition of heme bL and heme bH, respectively (31).

The mutationally imposed changes in ligation pattern resulted in the disappearance of the g transition of the targeted heme at the position characteristic for a native enzyme with concomitant appearance of new transitions at g = 3.22 and 3.15 (Fig. 2b, black). More specifically, these new transitions replaced g = 3.78 in H198K, g = 3.44 in H212K, and both g = 3.78 and 3.44 in the double mutant H212K/H198K. In general, these shifts reflect a lowering of the symmetry and changes from a highly axial to a more rhombic low spin heme (32). The new transitions g = 3.22 and 3.15 are assigned to g transitions of low spin hemes b coordinated by His-Lys. Support for this assignment comes from the differential effect of antimycin, an
inhibitor that binds to the $Q_i$ site in the proximity of heme $b_{14}$, and is known to affect the EPR spectrum of heme $b_{14}$ (33). Antimycin clearly affects the $g = 3.22$ and 3.15 of heme $b_{14}$ in H212K and H212K/H198K, whereas in H198K it has a weak or no effect (Fig. 2b, gray). In H212K/H198K, for which both hemes $b_1$ and $b_{14}$ contribute to 3.22 and 3.15 transitions, the effect of antimycin is intermediary between the largest and smallest effect seen in H212K and H198K, respectively.

**FIGURE 2.** His-Lys coordinated hemes $b$ contain low spin heme iron and have markedly increased $E_{m}$ values. This increase reaches 50 mV for heme $b_1$ in H212K and almost 160 mV for heme $b_1$ in H198K (Fig. 4 and Table 1). At the same time, there was no significant change of $E_{m}$ of Q pool, orange lines indicate calculated $E_{m}$ of Q/SQ and SQ/Q couples (upper and lower line, respectively), and the red line indicates the resulting average $E_{m}$ of Q at the $Q_i$ catalytic site.

**FIGURE 2.** His-Lys coordinated hemes $b$ contain low spin heme iron and have markedly increased $E_{m_b}$ values. $b$—Dark equilibrium redox titrations revealed that changing the ligation pattern from bis-His to His-Lys in hemes $b$ leads to a significant increase in their $E_{m}$ values. This increase reaches 50 mV for heme $b_1$ in H212K and almost 160 mV for heme $b_1$ in H198K (Fig. 4 and Table 1). At the same time, there was no significant change of $E_{m}$ of Q pool, orange lines indicate calculated $E_{m}$ of Q/SQ and SQ/Q couples (upper and lower line, respectively), and the red line indicates the resulting average $E_{m}$ of Q at the $Q_i$ catalytic site.

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Em of Hemes b and Cross-membrane Electron Transfer

Changes in ΔEm_b Affect Equilibria of Partial Reactions—Kinetic traces measured when the Q pool is fully oxidized before activation (ambient potential of 200 mV at pH 7) are compared in Fig. 5b. Under these conditions, the amount of quinol molecules after activation is limited, and consequently, approximately only one quinol is oxidized in every Qo site. It follows that a difference between the level of reduction of heme b14 in the absence and presence of antimycin reports equilibration of electron between heme b143+/2b142+ and quinone/semiquinone (Q/SQ) couples at the Qi site. In native enzyme and H198K mutant, this difference is large, indicating that the electron resides mostly on semiquinone at the Qo site (~80%). However, in H212K mutant, the amplitude of reduced heme b14 in the absence of inhibitors is significantly larger than in wild type. This indicates that equilibrium between heme b143+/2b142+ and Q/SQ is shifted so that the electron resides mostly on heme b14 (at the expense of semiquinone). This shift is a consequence of an elevated Em of heme b14 in H212K, which exceeds the Em of Q/SQ at pH 7 (Fig. 2a).

Another way to monitor electron equilibration between heme b14 and the occupant of the Qo site benefits from the occurrence of reverse reaction in the Qo site (Fig. 5c). When the Q pool is fully oxidized before light activation, and the Qo site is blocked by an inhibitor (myxothiazol), light-induced reduction of heme b14 reports electron transfer from quinol that entered the Qo site to heme b14. However, in a native enzyme, this reaction cannot be observed at pH 7 (Fig. 5c, blue), which seems consistent with the fact that Em of heme b14 is lower than Em of the Q pool. On the other hand, at this pH, the reverse electron transfer from Qo site quinol to heme b14 is prominent in H212K (Fig. 5c, blue). Furthermore, the amplitude of heme b14 reduction in this reaction (i.e. in the presence of myxothiazol) is almost as large as the amplitude of heme b14 reduction in the absence of any inhibitor.

Equilibration of electrons between heme b14 and the occupant of the Qo site in H212K mutant under the conditions described in Fig. 5 (b and c) allowed us to estimate the Em values for the SQ/QH2 and Q/SQ redox couples at the Qo site at pH 7. The extent of forward reaction (electron transfer from heme b14 to Q) monitored in Fig. 5b will reflect the difference between the Em of heme b143+/2b142+ and the Em of Q/SQ couples, whereas the extent of the reverse reaction (electron transfer from quinol to heme b14) monitored in Fig. 5c will reflect the difference between the Em of heme b143+/2b142+ and the Em of SQ/QH2 couples. Based on these assumptions, and considering Em = 130 mV for heme b14 in an H212K mutant, we estimate values of Em for the Q/SQ couple to be ~114 mV, and Em for the SQ/QH2 couple to be 147 mV. The average redox midpoint potential for Q/QH2 (Em for Q/QH2) is then 130 mV, which makes it isopotential with the Em of heme b14 in an H212K mutant and ~30 mV higher than the Em of Q in the Q pool reported in the literature. The split in quinone redox couples defines the stability constant for SQ (35, 36); thus, our estimates of Em for Q/SQ and Em for SQ/QH2 indicate the stability constant (log(K)) = [Em(Q/SQ) - Em(SQ/QH2)]/60) for SQ, at the level of 3 x 10^-1.
**FIGURE 4.** Midpoint potentials of hemes \(b\) determined via EPR-monitored redox titrations. Each blue line represents the Nernst titration curve. The respective \(E_m\) values are given in the middle of each plot. Titrations were performed on isolated chromatophores at pH 7.0. Amplitudes of heme \(b_L\) and heme \(b_H\) were monitored at respective \(g\) values: 3.78 and 3.44 in WT, 3.22 and 3.44 in H198K, 3.78 and 3.2 in H212K, and 3.2 in H212K/H198K.

**TABLE 1**

Selected properties of wild-type and Lys mutants

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<th>Form of (bc_1)</th>
<th>Phenotype</th>
<th>(E_m)</th>
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\(E_m\) of Hemes \(b\) and Cross-membrane Electron Transfer
Discussion

We have examined the effect of large changes in $E_m$ on electron transfer between hemes $b$ in cytochrome $bc_1$. These changes were implemented by significant increases in the $E_m$ values of the hemes, which came as a result of mutating the native bis-His coordination of the heme iron into the His-Lys coordination. Our results indicate that the natural difference in $E_m$ values of the two hemes $b$ ($\Delta E_m^b$) of 170 mV (Q pool oxidized) is increased to 210 mV (in H212K) or diminished to almost 0 (in H198K and double mutant H212K/H198K), and the complex still remains functional in vivo, retaining the catalytically relevant electron transfer from the Qo to Qi sites measured in vitro under the absence or presence of membrane potential. The previously reported moderate decrease in $E_m$ for heme $b_1$ in yeast cytochrome $bc_1$ falls into this category of changes, i.e. ones not imposing overly endergonic steps, and thus, consistent with our observations, did not affect significantly the measured turnover rate of the enzyme (20).

Considering the direction of electron flow and the direction of the gradient of $E_m$ in relation to the vector of the electric membrane potential, four configurations are possible (Fig. 1). In cytochrome $bc_1$, the high potential heme $b$ is located at the negative side of the membrane (Fig. 1a), which at first would seem to be in line with the concept of the obligatory difference in $E_m$ to overcome membrane potential. However, in formate dehydrogenase N (40, 43, 44) and probably in membrane-bound [Ni-Fe] hydro-
a

b

FIGURE 7. Localization of low and high potential hemes \( b \) with respect to the quinone binding site in cytochrome \( b \) subunits of enzymes involved in cross-membrane electron transfer. \( a \), in cytochromes \( b \) with one Q binding site, low potential heme \( (b_L) \) is adjacent to the Q binding site, whereas high potential heme \( (b_H) \) faces the opposite side of the membrane, \( b \). In cytochromes \( b \), the cytochrome \( b \) subunit contains two Q binding sites and heme \( b_L \) is adjacent to one site \( (Q_L) \), whereas heme \( b_H \) is adjacent to the other \( (Q_H) \). Brown diamonds, hemes \( b \); yellow hexagons, Q binding sites. Numbers indicate \( E_m \) values (in mV) for pH 7. Superscripts A, B, and C refer to \( E_m \) values for pH 7.6, 7.2, and 7.5, respectively. Types of natively used quinones are given below the superscripts. Localization of hemes in membrane-bound [Ni-Fe] hydrogenase \( (52–54) \) is simply an inversion of the case in Fig. 1. Other abbreviations as follow: B. c., Bacillus cereus; B. s., Bacillus subtilis; C. j., Campylobacter jejuni; C. r., Chlamydomonas reinhardtii; E. c., Escherichia coli; H. p., Helicobacter pylori; R. e., Raistonia eutropha; R. c., Rhodobacter capsulatus; T. a., Thermoplasma acidophilum; W. s., Wolinella succinogenes; MK, menaquinone; TPQ, plastoquinone; UQ, ubiquinone.

Genase \((45, 46)\), the low potential heme is located at the negative side of the membrane, and the electron is transferred against both the electric membrane and the redox potential of hemes \( b \) (Fig. 1b), which remains at odds with the concept presented above. The third possibility found in succinate-quinone reductase \((47–50)\), NADPH oxidase \((51)\), or cytochrome \( b_561 \) family \((52–54)\) is simply an inversion of the case in Fig. 1a with electron transfer from high to low potential facilitated by the presence of electric membrane potential (Fig. 1c). The fourth possibility (Fig. 1d) perhaps concerns proton motive force-driven electron transfer in thioulate reductase, if heme \( b_L \) in this enzyme is located close to the menaquinone binding site, as in formate dehydrogenase \( N \) (which seems likely, based on sequence similarities between the cytochrome \( b \) subunits of these complexes) \((55)\).

The occurrence of all these configurations suggests that there is no a universal rule for the arrangement of \( E_m \) values of hemes \( b \) among various transmembrane cytochromes \( b \). This, however, becomes understandable in light of our observation that large \( \Delta E_m \) \( b \) and fine tuning of \( E_m \) values of hemes \( b \) of cytochrome \( b_{561} \) family \((52–54)\) is simply an inversion of the case in Fig. 1a with electron transfer from high to low potential facilitated by the presence of electric membrane potential (Fig. 1c). The fourth possibility (Fig. 1d) perhaps concerns proton motive force-driven electron transfer in thioulate reductase, if heme \( b_L \) in this enzyme is located close to the menaquinone binding site, as in formate dehydrogenase \( N \) (which seems likely, based on sequence similarities between the cytochrome \( b \) subunits of these complexes) \((55)\).

Extrapolating all these observations to other cytochromes \( b \), it can be proposed that, in all those proteins, hemes \( b \) simply act as electronic connectors for the catalytic sites with no fine tuning in \( \Delta E_m \) \( b \) required for efficient electron transfer. It follows that the existence of \( \Delta E_m \) \( b \) in transmembrane embedded hemes \( b \) is not an element in the control of electron flow across the membrane. Rather, it may be a consequence of a higher probability of coexistence of two cofactors having different \( E_m \) in comparison to the case when the two cofactors have similar \( E_m \). Intriguingly, we found one resemblance for enzymes with one quinone binding site: from two hemes \( b \) of different potentials, the one with the lower potential is adjacent to the quinone binding site (Fig. 7) \((38, 39, 42, 44–47, 49, 50)\). Further studies are required to verify whether this has any functional relevance or is just a consequence of structural constraints. Nevertheless, this resemblance may be useful in predicting the location of low and high potential heme \( b \) in quinol-binding cytochromes, for which such assignments are yet to be made.

The demonstrated robustness of cytochrome \( b \) to changes in ligation pattern and associated changes in \( \Delta E_m \) \( b \) raises an interesting question as to whether variation in the heme ligation patterns exists in natural membrane proteins of similar design and/or function. Such variation is evident in the group of cytochromes \( c \) for which bis-His \((4, 56, 57)\), His-Met \((4, 56, 57)\), His-Lys \((58)\), His-Cys \((59–61)\), or even His-Tyr \((62, 63)\) patterns ligation heme iron are observed. However, in most known cytochromes \( c \), the heme binding domains are water-soluble or solvent-exposed (so far, the heme \( c \) from cytochrome \( b \) is the only known exception \((5, 64, 65)\)), whereas in cytochromes \( b \) these domains can be located either in membrane or in aqueous phase \((5)\). It may seem that the location of the heme binding motifs outside the membrane environment is one of the factors increasing structural flexibility to accommodate diverse heme ligation. Indeed, all rare cases of His-Met \((66, 67)\), Lys-Met \((68)\), or bis-Met \((69, 70)\) ligations in cytochromes \( b \) are relevant only to water-soluble domains. In fact, to our knowledge, no cases have been reported so far of Met or Lys serving naturally as axial ligands for hemes bound within the integral membrane proteins.

However, our Lys mutants prove that His-Lys ligation for hemes \( b \) can occur within the transmembrane helix bundle of cytochrome \( b \), yielding functional hemes that contain a low spin form of iron ion. Notably, the H212K mutant was originally isolated as a reversion for H212N, the so-called heme \( b_4 \) knockout, with impaired electron transfer at the level of the heme \( b_4/Q \) site. Likewise, the His-Met heme \( b \) mutant of \( B. subtilis \) succinate-quinone reductase was isolated as a reversion of non-functional Leu mutant \((48)\). This all indicates that an assembly
of functionally active low spin heme b present within the transmembrane segment of the protein and coordinated by His and Lys or Met is feasible from a protein engineering perspective. If this is the case, one should expect that there are natural cases of His-Lys and His-Met ligation patterns for membranous heme proteins (71) that still await identification, especially because the range of scrutinized heme proteins is currently continuously widening.

Author Contributions—S. P. performed most of the biochemical and spectroscopic experiments and analyzed data; P. K. performed light-induced electron transfer measurements; E. C. constructed mutants and contributed preliminary results; A. B. performed enzymatic activity assays; and S. P., M. S., and A. O. designed the experiments, interpreted the data, and cowrote the paper. All authors reviewed the results and approved the final version of the manuscript.

Acknowledgments—We thank Dr. Wolfgang Nitschke for useful discussions and Dr. Robert Ekiert for technical assistance.

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