Unexpected Heme Redox Potential Values Implicate an Uphill Step in Cytochrome \textit{b}_6\textit{f}

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Abstract: Cytochromes \textit{bc}, key enzymes of respiration and photosynthesis, contain highly-conserved two-heme motif supporting cross-membrane electron transport (ET) that connects the two catalytic quinone-binding sites (\textit{Q}_\text{i} and \textit{Q}_\text{p}). Typically, this ET occurs from the low-to high-potential heme \textit{b}, but in photosynthetic cytochrome \textit{b}_6\textit{f} the redox midpoint potentials ($E_m$) of these hemes remained uncertain. Our systematic redox titration analysis based on three independent and comprehensive low-temperature spectroscopies (continuous wave and pulse EPR and optical) allowed for an unambiguous assignment of spectral components of hemes in cytochrome \textit{b}_6\textit{f} and revealed that $E_m$ of heme \textit{b}, is unexpectedly low. Consequently, the cross-membrane ET occurs from the high- to low-potential heme introducing an uphill step in the energy landscape for catalytic reaction. This slows down the ET through low potential chain which can influence the mechanisms of reactions taking place at both \textit{Q}_\text{i} and \textit{Q}_\text{p} sites and modulate efficiency of cyclic and linear ET in photosynthesis.
Experimental Procedures

Materials

Buffers, salts and decylplastoquinone were purchased from Sigma. Detergents UDM and OG were purchased from Glycon (Germany). DDM was purchased from Anatrace. Propyl-Sepharose resin was prepared using activated Sepharose-CNBr powder purchased from Cytiève (Sweden). The activated Sepharose-CNBr was modified through reaction with propylamine (Sigma) according to the manual delivered by the supplier.

Detailed protocol of cyt6f isolation

Dimeric cyt6f complex was isolated from market spinach (S. oleracea) leaves using a large-scale protocol which was adapted from Baniulius et al.1 and Romanowska2. Spinach leaves were homogenized in buffer 1 (50 mM Tris pH 8, 50 mM NaCl, 200 mM sucrose, 0.2 % antifoam A) using a whole slow juicer. The resulting solution was filtered through a sieve and a must filter. After filtering, the solution was centrifuged at 17 000 g for 20 min, 4°C. The pellet containing chloroplasts was suspended in buffer 2 (10 mM Tris pH 7.5, 10 mM NaCl). The solution was thoroughly mixed with buffer 5 until the eluent was colorless. A greenish-brown band containing cyt6f was eluted with buffer 6 (buffer 3 with 1 mM UDM). The sample was loaded onto a propyl-sepharose column pre-equilibrated with buffer 5 (buffer 3 with 1 mM undecyl α-D-maltoside (UDM) and 37% of AS saturation). The column was washed thoroughly with buffer 5 until the eluent was colorless. A greenish-brown band containing cyt6f was eluted with buffer 6 (buffer 3 with 1 mM UDM and 20% of AS saturation). The eluent was again brought to 37% of AS saturation and centrifuged at 24 000 g for 10 min, 4°C. After filtering through a 220 nm membrane, the supernatant was loaded onto a second propyl-sepharose column pre-equilibrated with buffer 5. The column was washed stepwise with buffer 5 containing decreasing amounts of AS (saturation decreasing from 37% to 30%), before a brownish band was eluted with buffer 6. The eluate was concentrated using microconcentrators (Merck Millipore) and the buffer was exchanged to buffer 7 (buffer 3 with 1 mM UDM). The sample was loaded onto a 10-25% continuous sucrose gradient in buffer 7 and ultracentrifuged at 141 000 g for 16 hours, 4°C. A brown band in the middle of the gradient containing pure cyt6f was carefully collected and the buffer was exchanged to buffer 7. Further biochemical analysis (including enzymatic activity, optical and EPR spectroscopy, electrophoretic analysis) indicated that this fraction contained a full complement of the cyt6f subunits and was enzymatically active.

Concentration and activity measurements

Concentration of cyt6f was determined spectrophotometrically by measurement of ascorbate-reduced minus ferricyanide-oxidized absorption spectra at 554 nm, relative to isosbestic point 543 nm, using differential extinction coefficient of heme $f_{554,543}=25 \text{mM}^{-1} \text{cm}^{-1}$. The enzymatic activity of cyt6f was determined by measuring the cyt6f-mediated reduction of plastocyanin. Preparation of substrates for activity measurement involved several procedures. Plastocyanin was oxidized with potassium ferricyanide which was subsequently removed by concentration-dilution cycle on microconcentrators. Decylplastoquinone was dissolved in ethanol and
reduced to decylplastoquino (dPQH₂) with hydrogen gas in the presence of platinum on carbon as the catalyst. After reduction, dPHQ₂ was diluted 3 times with dimethyl sulfoxide.

The enzymatic reaction was carried out in buffer 3 (from isolation protocol) containing 20 μM of oxidized PC and 100 μM dPQH₂. The total volume of the reaction mixture was 1 mL. The enzymatic reaction was initiated by injection of cyt b₆f to final concentration of 20 nM (concentration calculated per cyt b₆f) and the reaction progress was monitored using a Biologic Diode Array spectrophotometer. The reduction rate of plastocyanin was determined from the initial slope after the addition of cyt b₆f to the cuvette. The turnover rate of the sample was estimated to be approximately 120/s. Concentration and enzymatic activity of cyt b₆c₁ was assessed as described in 5.

Results and Discussion

Statistical analysis

Figures S1 and S2 show the statistical analysis of equilibrium redox titration data obtained for cyt b₆f and cyt b₆c₁. As it is not possible to precisely assess the uncertainty of each of the external redox potential (Eh) value, it was assumed that the uncertainty is the same for all data points. The maximum value of uncertainty was assumed to be ± 15 mV, which was converted to standard uncertainty (u(Eh) = 8.66 mV) and used to create horizontal error bars.

Figure S1 shows the analysis of the results obtained by decomposition of optical spectra. Due to the fact that standard errors of the amplitudes of reduced hemes were negligible, the points are indicated only as horizontal error bars. Figure S2 shows the analysis of the results obtained by analysis CW EPR spectra. Vertical error bars represent the uncertainties in determination of the amplitudes of the oxidized hemes. The uncertainty was calculated as standard uncertainty arising from both: the normalization of spectra on the amplitude of 2Fe2S and the noise present in the spectra.

The statistical significance of the model with respect to data was assessed by constructing confidence intervals at confidence level of 95%. Confidence intervals are depicted as blue confidence bands. Values of confidence intervals at each data point were calculated using the expression

\[
Y_i = \hat{Y}_i \pm t(1−a/2, n−p) \cdot \text{RMSE} \cdot \sqrt{\frac{1}{n} + \frac{(X_i - X_m)^2}{S_{xx}}}
\]

where: \( \hat{Y}_i \) is the value of fitted function at given Eh \( X_i \), \( t \) is the 100*(1-a/2) percentage point of Student’s t distribution on \( n-p \) degrees of freedom \( n \) = number of data points, \( p \) = number of parameters, RMSE is the Root Mean Squared Error, \( X_m \) is the mean of the \( E_n \) values and \( S_{xx} = \sum (X_i - X_m)^2 \).

Figure S1. Statistical analysis of the results, shown in Figure 2 in Main Text, obtained by decomposition of the optical spectra. a) and b) show the Nernst curves fitted to the reduced fractions of hemes \( b_p \) and \( b_n \) of cyt b₆f, respectively. c) and d) show the same type of
analysis for hemes $b$ and $b_h$ of cytbc$_1$, respectively. Data points are represented only by horizontal error bars for clarity. Data was fitted with the least squares method (Levenberg-Marquardt) using the Nernst function. Fitted values are given with the standard error calculated from the diagonal elements of covariance matrix. Confidence intervals at 95% confidence level are shown as blue bands.

**Figure S2.** Statistical analysis of the results, shown in Figure 3 in Main Text, obtained by analysis of CW EPR spectra. a) and b) show the Nernst curves fitted to the oxidized fractions of hemes $b_p$ and $b_h$ of cytbc$_1$, respectively. Data points are represented only by error bars for clarity of presentation. Data was fitted with the least squares method using the Nernst function. Fitted values are given with the standard error calculated from the diagonal elements of covariance matrix. Confidence intervals at 95% confidence level are shown as blue bands.

**Analysis of optical spectrums**

Low-temperature optical spectra of cytbc$_2$ and cytbc$_1$ were analyzed using GAF procedure. The analysis was performed with a self-written Python program utilizing SciPy, NumPy and Pandas packages. Each spectrum was fitted with a combination of three components: baseline, two-gaussian function and one-gaussian function. Baseline consisted of a spectrum recorded at high $E_h$ where only $h_f$ and $h_c$ are reduced and a linear function to correct for slope deviation. Two-gaussian component was used to fit $h_b$ and $h_h$ while one-gaussian component fit $h_c$ and $h_b$. Other variations of components including any combination of two or more one- or two-gaussian functions were also examined but the best fit was obtained by fitting one two-gaussian component ($h_b$ and $h_h$) and one one-gaussian component ($h_c$ and $h_b$).

Implementation of GAF procedure was achieved by dividing the parameters into two sets: global, that should be held the same for all spectra and local, which could vary from spectrum to spectrum. The set of local parameters consisted of the amplitudes of the components and linear function arguments. The peaks widths, positions and relative amplitude ($h_b$ and $h_h$) were fitted as global parameters, since those values determined the shape of the component and should be the same for all spectra. Model consisting of 3 components with both global and local parameters was fitted to the data matrix using Levenberg-Marquardt algorithm. Reasonable boundary conditions were applied in the fitting procedure. The model fit the data very well as depicted in Figure 1 (Main text). Components for hemes $b$ of both cytbc$_1$ and cytbc$_2$ with optimized values are shown in Table 1 (Main text).

**Analysis of EPR spectrums**

While EPR spectra of cytbc$_1$ has been extensively studied and the EPR transitions of LS hemes $b$ have been recognized, the presence of HS $h_c$ in cytbc$_1$ creates additional EPR transitions originating from spin-spin exchange interactions with $h_b$. Typical CW EPR spectra of cytbc$_1$ measured at 3 different $E_h$ are shown in Figure S3. A rhombic EPR spectrum at $1.76 < g < 2.02$ originates from the reduced 2Fe2S cluster. The signal of $h_f$ ($g = 3.51$) was not detected, as this heme was fully reduced. The transition at $g = 3.67$ was previously ascribed to $g_1$, transition of the oxidized, highly axial low-spin (HALS) $h_b$. The signals at $g > 4.3$ were ascribed to $h_c$, which is spin-coupled to $h_b$. An additional component at $g \sim 6$ of unknown species, suggests a presence of a HS heme of axial symmetry. It is likely that it originates from either partially denatured $h_f$ or a heme $b$, or from a small fraction of $h_c$, which is not
coupled to hbp. Similar axial HS signal of heme was detected in cytochrome c oxidase, despite the presence of spin-spin exchange interaction with the copper ion.

Well separated EPR signals ascribed to the pair of hcn and hbn were found at g = 12.4 and 4.73, thus they were used as a measure of the redox-dependent changes in the amplitude of oxidized hcn, to construct the Nernst curves for this particular pair of hemes.

Figure S3. CW EPR spectra of cyt b6 poised at the high (black), intermediate (green) and low $E_h$. The arrows indicate g values of EPR transitions of the oxidized hemes $b_p$, (3.67), $c_n$ spin-coupled to heme $b_n$ (4.73, 6.7, 12.4), an axial H-S heme species (6.0) and the reduced 2Fe2S cluster (1.76, 1.91 and 2.02).

Global analysis fit of ESE decay curves

The model used for the global analysis fit of ESE decay curves included following assumptions:

a) only dipolar interactions within a single monomer were considered;
b) a number of fast relaxing species having different $E_m$ in cyt b6 was 3 - hemes $b_n$, $b_p$ and $c_n$;
c) dipolar ESE decay curve resulting from the interactions between 2Fe2S and a heme iron was approximated with mono-exponential function described by average $T_{dip}$;
d) effect of changes in the redox state of heme $c_n$ and $b_n$ on ESE decay of 2Fe2S were treated separately, despite existence of spin-spin exchange interactions between these two hemes;
e) for cytbc, the number of species was 2, while the second species representing heme $b_h$ was divided into two fractions with the lower and higher $E_m$ and contributions of 60% and 40%, respectively.

The first and the second assumptions were supported by the observation, that increasing the number of species from 3 to 4 did not lead to any sensible results of the fit and the obtained parameters of the redundant 4th species ($T_{dip}$, fraction and $E_m$) were always very similar to one of the remaining species with shared contribution to compensate the respective fractions.

A model used for fitting to the whole experimental set of ESE decay curves is described by the following formula:

$$A(t, E_h) = A_{base}(t) \cdot (f_{base} + f_1(E_{m1}) \cdot \exp\left(-\frac{t}{T_{dip1}}\right) + f_2(E_{m2}) \cdot \exp\left(-\frac{t}{T_{dip2}}\right) + f_3(E_{m3}) \cdot \exp\left(-\frac{t}{T_{dip3}}\right))$$

and

$$f_{base} + f_1 + f_2 + f_3 = 1$$

eq S2

where: $A(t, E_h)$ is the amplitude of ESE decay curve at time point $t$ [μs] of the ESE decay measured for a sample at particular $E_h$ [mV]; $A_{base}(t)$ is the normalized experimental ESE decay curve (the base function) measured for the sample at the lowest $E_h$; $f_{base}$ is the fraction of the base function at given $E_h$; $f_1$, $f_2$ and $f_3$ are the fractions of oxidized heme iron ions, that contribute to the enhancement
of the phase relaxation of 2Fe2S; $T_{\text{dip1}}$, $T_{\text{dip2}}$ and $T_{\text{dip3}}$ are the semi-quantitative time constants associated with the enhancement of the phase relaxation imposed on 2Fe2S by a respective fast relaxing species; $\tau$ and $E_n$ are independent variables representing the time axis of a single ESE decay curve and the external redox potential, respectively.

The fractions $f_1$, $f_2$ and $f_3$ ($f_x$) were calculated on the basis of the Nernst equation, as a function of $E_m$, $n$ and $E_n$ for a particular sample:

$$f_x = \frac{10^{0.01666 \cdot n \cdot (E_m - E_{mx})}}{1 + 10^{0.01666 \cdot n \cdot (E_m - E_{mx})}}$$

where: $f_x$ - fraction of species $x$, $E_{mx}$ - the redox midpoint potential of species $x$. For the reasons given in the main text the parameter $n$ was fixed at 1.

By combining eqs. S2 and S3, we obtained the model, in which all experimental ESE decay curves, at different $E_m$, were described by 9 global, free adjustable parameters: 3 $E_m$, 3 $n$ and 3 $T_{\text{dip}}$ values.

### Measurement of $E_m$-dependent enhancement of the phase relaxation of 2Fe2S in cyt$b_f$.

Global fitting of the model to ESE decay curves of cyt$b_f$ with all 9 parameters to optimize was found to be unstable resulting mostly in unreliable values of $n$, which entails random combinations of $E_m$ and $T_{\text{dip}}$. Fixing $n$ values at 1 greatly improved the stability of the optimization. The convergence criteria was tested by performing 1000 independent optimizations, starting from randomized set of initial guesses for $T_{\text{dip}}$, $E_m$ and fixed $n$ = 1. Values of $T_{\text{dip}}$ were randomly drawn from the range 0.1 to 20 $\mu$s, while $E_m$ values were randomly drawn from the range -200 to +200 mV. Among 1000 optimizations 573 were successful, while 424 did not reached the global minimum, showing that a probability of successful optimization was $0.573 \pm 0.016$. All successful optimizations yielded nearly the same values of $T_{\text{dip}}$ and $E_m$ for respective species. The results of the fitting was shown in Table 3 in the Main Text.

We also performed another fit with parameters of $E_m$ and $n$ fixed at values determined by optical and CW EPR spectroscopy (Figures 2 and 3): $E_m = -111$ mV and $n=1$ (species 1 - heme $b_1$); $E_m = -80$ mV and $n = 0.7$ (species 2 - heme $b_2$), $E_m = +46$ mV and $n = 1$, (species 3 - heme $c_2$). In this case, the only adjustable free parameters were $T_{\text{dip}}$.

In both these cases $T_{\text{dip}}$ values are the lowest for the species 1 with the lowest $E_m$ suggesting, that it is more remote from the 2Fe2S cluster than the species with intermediate $E_m$ value.

### Measurement of $E_m$-dependent enhancement of the phase relaxation of 2Fe2S in cyt$c_i$.

We compared the results of measurement of ESE decay in cyt$b_f$ to the data obtained in similar manner for cyt$c_i$. In this case the model was slightly modified to account for the presence of two fractions of heme $b_h$ with higher ($b_{h1}$) and lower ($b_{h2}$) $E_m$. These two fractions of the heme $b_h$ were expected to have the same dipolar effects on the phase relaxation of 2Fe2S as they correspond to the same structure, distance and paramagnetic properties. The fit with fixed $E_m$ and $n$ parameters (drawn from optical spectroscopy data) yielded $T_{\text{dip}}$ of heme $b_h$ less than that of heme $b_b$ which could be expected as both these hemes possess similar paramagnetic properties and the difference in dipolar effect on phase relaxation of 2Fe2S results from heme $b_b$ being closer to 2Fe2S than heme $b_h$.

The result of fitting of the above discussed models to ESE decay curves of cyt$b_f$ and cyt$c_i$ are presented in Figure S4.
Figure S4. Results of application of GAF to ESE DC curves obtained for cytbc6 and cytbc6f. a) the concatenated ESE DCS measured for cytbc6f at different $E_b$ (blue) and the GAF curves (red). b) Difference between ESE DCS and the fitted model. Panels c) and d) represent the same analysis as for a) and b), respectively but obtained for cytosome bc6. The x-axes represent consecutive points of ESE DCS, that were concatenated starting from the lowest (left) to the highest $E_b$ (right). Before analysis each ESE DC was normalized by division of ESE DC amplitude by estimated amplitude at the zero time point (the onset of the relaxation process). Initial 440 ns of the ESE DC are missing as being obscured by the resonator dead time.

Overall, the analysis of ESE decay curves measured for cytbc6f provided estimates of $E_m$ for the 3 species but gave no explicit information on which heme was represented by the respective species. However, comparison of $E_m$ values obtained by the analysis of relaxation curves with those obtained from optical and CW EPR spectroscopy (see Figures 2 and 3 in the Main Text) suggests that $E_m$ of species 1 is similar to $E_m$ of heme $b_1$ (species having $E_m$ around -129 mV (Figure 2b)) and therefore it is most probably heme $b_1$. Species 2, with $E_m$ - 96 mV has similar $E_m$ value to heme $b_2$ (Figure 2a). Both these comparisons correspond well with the spatial arrangement of hemes in the structure of cytbcf given that the $T_{	ext{rel}}$ obtained for species 1 is lower than that of species 2 and heme $b_1$ is placed more remotely than 2Fe2S than heme $b_2$. Species 3 was optimized with $E_m$ value around +20 mV which is close to the value obtained for heme $c_6$ (Figure 3b).

Discussion on relaxation enhancement

We performed analysis of pulse EPR data of cytbc6f and the results were compared to the data obtained for structurally similar protein cytbc6. Figure 4 in the Main Text compares the distances between paramagnetic 2Fe2S center and heme iron ions in both cytochromes. In the case of cytbc6, heme $b_2$ is positioned closer to 2Fe2S (approx. 26.4 Å) than heme $b_1$ (approx. 34.2 Å), therefore assuming that these two hemes have similar paramagnetic properties the former heme should exert a stronger dipolar effect on the phase relaxation of the 2Fe2S cluster than the latter. Therefore, a shorter $T_{	ext{rel}}$ for heme b-2Fe2S interactions was expected. Similar effect was expected for cytbc6f in which heme $b_2$ is also closer to the 2Fe2S cluster than heme $b_1$ ($\sim 30.5$ Å and $\sim 39.4$ Å).

Theoretically, dipolar relaxation curves are not mono-exponential as they depend on many additional factors such as relative angles between the vectors connecting the paramagnetic centers and the external magnetic field. However, we performed a qualitative comparison of the relative dipolar relaxation time constants by taking into account only relative distances and $T_{	ext{rel}}$ values of cytbc6f and cytbc6. In the most simplified case, the strength of the dipolar effect on the phase relaxation can be described by a formula which considers distance $r$ between two interacting centers:

$$\langle T_{\text{rel}}^{-1} > \rangle = \langle a \rangle \cdot r^{-3}$$

eq S4

where: $\langle T_{\text{rel}}^{-1} >$ is the effective ESE dipolar decay rate caused by the dipolar interactions, $\langle a \rangle$ is the proportionality factor including all remaining effective contributions other than distance averaged, while $r$ is the distance between 2Fe2S cluster and respective heme iron ion. First we calculated average $\langle a \rangle$ values for interactions between hemes $b_{bc}$, $b_{f}$, $c_{f}$ and 2Fe2S in cytbc6f and corresponding $\langle a \rangle$ for interactions between heme $b_1$ and $b_2$ and the 2Fe2S cluster in cytbc6f with the use of $T_{\text{rel}}$ values obtained for the respective cytochromes. In case of cytbc6f, the averaged $\langle a \rangle$ value was estimated to $\sim 27.5$ Å$^3$/GHz, while for cytbc6 this value was similar and equal to $\sim 30.0$ Å$^3$/GHz. Using these different $\langle a \rangle$ values for calculating distances between 2Fe2S cluster and hemes in both cytochromes and respective $T_{	ext{rel}}$ values, we obtained distances $\sim 34.4$ Å or $\sim 36.8$ Å for 2Fe2S - heme $b_{bc}$, $\sim 38.6$ or $\sim 39.4$ Å for 2Fe2S - heme $b_{f}$ and $\sim 44.0$ or $\sim 41.7$ Å for 2Fe2S - heme $c_{f}$. In cytbc6f, the estimated distances were $\sim 28.5$ and $\sim 32.1$ Å for 2Fe2S - heme $b_{f}$ and 2Fe2S - heme $c_{f}$, respectively. These values however must be treated only as crude approximations since such an analysis ignores many factors that contribute to the effective enhancement of the phase relaxation of the cluster. Most of the additional factors are not available and, additionally, we must take into account the fact that the PDB structures do not provide information on the distribution of the position of 2Fe2S due to the mobility of the iron-sulfur protein, an inherent feature of cytbc6 and cytbc6f. However, despite the above-mentioned limitations in the analysis of the pulse EPR data, a general view drawn from these experiments is qualitatively in agreement with the expected larger effects of hemes $b_2$/h2 on the relaxation of 2Fe2S than hemes $b_1$/h1 due to the differences in their relative distances to the cluster and their estimated $E_m$ values. This again supports the thesis that heme $b_2$ in cytbc6f exhibits a higher $E_m$ than heme $b_1$, whereas in cytbc6, the respective heme $b_1$ and $b_2$ are low- and high-potential LS heme.

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Author Contributions

MSz isolated and purified proteins. MSz, LB and MS designed and performed experiments and analyzed data. AO acquired funding and discussed data. All authors participated in writing the manuscript.