Insights into the binding behavior of native and non-native cytochromes to Photosystem I from *Thermosynechococcus elongatus*

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**Abbreviations:** bRC, bacterial reaction center; Chl, chlorophyll; cyt \(c\)\(_{HH}\), cytochrome \(c\) from horse heart; cyt \(c_6\), cytochrome \(c_6\); DDM, dodecyl-\(\beta\)-D-maltoside; DLS, dynamic light scattering; ITC, isothermal titration calorimetry; PS I, photosystem I; RC, reaction center; \(R_H\), hydrodynamic radius, \(W(A655)\) and \(W(B632)\), tryptophan 655 and 632 from PsaA and PsaB, respectively.

**Running title:** Binding of cytochromes to PS I

**Keywords:** photosynthesis, photosystem I, cytochrome \(c\), complex, docking, crystallography

**Abstract**

The binding of photosystem I (PS I) from *Thermosynechococcus elongatus* to the native cytochrome (cyt) \(c_6\) and cyt \(c\) from horse heart (cyt \(c\)\(_{HH}\)), is analyzed by oxygen consumption measurements, isothermal titration calorimetry (ITC), and rigid body docking combined with electrostatic computations of binding energies. While PS I has a higher affinity for cyt \(c\)\(_{HH}\) than for cyt \(c_6\), the influence of ionic strength and pH on binding is different in the two cases. ITC and theoretical computations reveal the existence of unspecific binding sites for cyt \(c\)\(_{HH}\), besides one specific binding site close to P\(_{700}\). Binding to PS I is found to be the same for reduced and oxidized cyt \(c\)\(_{HH}\). Based on this information, suitable conditions for a co-crystallization of cyt \(c\)\(_{HH}\) with PS I have been found, resulting in crystals with a PS I\text{-}cyt \(c\)\(_{HH}\) ratio of 1:1. A crystal structure at 3.4 \(\AA\) resolution has been obtained, but cyt \(c\)\(_{HH}\) cannot be identified in the electron density map because of unspecific binding sites and/or a high flexibility at the specific binding site. Modeling the binding of cyt \(c_6\) to PS I reveals a specific binding site where the distance and orientation of cyt \(c_6\) relative to P\(_{700}\) is comparable to cyt \(c_2\) from purple bacteria relative to P\(_{870}\). This work provides new insights into the binding modes of different cytochromes to PS I, thus facilitating...
steps towards solving the PS I - cyt $c_6$ co-structure and a more detailed understanding of natural electron transport processes.

**Introduction**

Photosystem I (PS I) from the thermophilic cyanobacterium *Thermosynechococcus* (*T.* elongatus) is a membrane-bound, trimeric, 1 MDa multi-pigment-protein supercomplex. It converts light to electrochemical energy with a quantum efficiency of almost 100%. Due to its high stability, it is a suitable system for biotechnological applications. Thus, the protein complex has been used in photobioelectrodes for the generation of photocurrents and the production of biofuels (1–4). The structure of PS I from *T.* elongatus was solved at 2.5 Å resolution in 2001 (5) and very recently for plants at 2.6 Å resolution (6). The cyanobacterial PS I consists of 9 transmembrane and 3 cytoplasmic subunits harboring 127 cofactors per monomer. The two core subunits PsaA and PsaB bind the majority of cofactors, including reaction center (RC) and antenna pigments. In the RC, light induced charge separation starts at the primary electron donor P$_{700}$, a dimer of strongly interacting chlorophylls (Chl). The electron transport chain consists of two branches, with one of the branches being more active than the other (7). From either branch, the electrons are transferred to the iron-sulfur cluster F$_X$. The electrons are finally accepted by the terminal iron-sulfur clusters F$_A$ and F$_B$ bound by the extrinsic subunit PsaC.

In cyanobacteria and green algae, the soluble redox mediators cytochrome $c_6$ (cyt $c_6$) and plastocyanin (PC) donate electrons to oxidized P$_{700}$ at the luminal side of the thylakoid membrane. The alternative expression of these homologous proteins is regulated by the availability of copper (8). In plants, however, solely PC occurs, whereas the cyanobacterium *T.* elongatus contains only cyt $c_6$ (8, 9). Mutagenesis studies indicated that optimal binding of both electron mediators for electron transfer to P$_{700}^+$ occurs at a hydrophobic binding site, which is formed by two parallel tryptophan residues W655 from PsaA (W(A655)) and W632 from PsaB (W(B632)) (10). Besides this hydrophobic site, a second binding site exists in plant and algal PS I, which is based on electrostatic interactions due to positively charged side chains of PsaF. After binding to the charged site, PC re-orientates itself to bind to the hydrophobic area and form the active complex (11, 12). This binding model is based on kinetic data. Because of the strong, charged binding-site, plant and algal PS I form a stable complex with PC (13). In contrast, PsaF does not contribute to the binding of PC or cyt $c_6$ in most cyanobacteria.

For the latter organisms, kinetic data and NMR perturbation experiments (14) allowed elucidating the binding patch on cyt $c_6$ for binding to PS I. However, no detailed structural information about the binding of cyt $c_6$ to PS I is available. Such information is not only of fundamental scientific interest, it could also be helpful to improve biotechnological applications. PS I from different organisms has been used in this context for creating photobioelectrodes or light-switchable biosensors. In some of these systems, cytochromes have been utilized to achieve electron transport to PS I (3, 15). Recently, it has been found that the mitochondrial cyt $c$ from horse heart (cyt $c_{HH}$) can be used to couple PS I to electrodes in an efficient way (1, 2, 16). On account of the demonstrated functionality of these non-native hybrid systems, the question arises of how cyt $c_{HH}$ interacts with PS I, and whether this interaction is different from native cyt $c_6$ under physiological conditions.

Structural information is a prerequisite for answering these questions. In particular, X-ray crystallography requires cyt $c$-PS I co-crystals, in which cyt $c$ is located in the specific binding site for electron transfer. For co-crystallization, conditions have to be found, under which a stable complex is formed. To this end, we focus in this study on an investigation of the binding properties of PS I from *T.* elongatus with cyt $c_{HH}$ and cyt $c_6$ under a variety of buffer conditions for elucidating the binding site.

In particular, we employed analysis of oxygen reduction measurements and isothermal titration calorimetry (ITC). Based on these binding studies, cyt $c_{HH}$ has been co-crystallized with PS I and a crystal structure analyzed, in which, however, cyt $c_{HH}$ is not visible. Hence, as an alternative, binding of cyt $c_{HH}$ and cyt $c_6$ has been theoretically modeled by using rigid body docking combined with electrostatic calculations of binding energies. Docking complexes are found for both cytochromes, which are likely to resemble the actual cyt $c$ binding site of cyanobacterial PS I.

**Results**

**Purity of isolated proteins**

Dynamic light scattering (DLS) reveals that the hydrodynamic radius (R$_H$) of the purified PS I
ranges from 9 to 10 nm, with a polydispersity of less than 5%, as expected for monodisperse, trimeric PS I (17, 18). The absence of PS I monomers and dimers is further verified by BN-PAGE (Supplementary Fig. S1). The subunit composition of each PS I preparation was analyzed by mass spectrometry (Supplementary Table S1). 10 subunits of the PS I protein complex could be detected. Most of them are post-translationally modified (for more details see (19)). However, subunits A and B could only be detected by SDS-PAGE because of their high mass (data not shown).

The cloning of the open reading frame \textit{tll1383} encoding cyt c6 resulted in a recombinant protein that carried a 6x His-tag at the C-terminus. The protein was extracted from the periplasm of \textit{E. coli}, purified using a Ni-NTA column and, subsequently, an anionic exchange chromatography. 1 L of \textit{E. coli} cells yields 5 mg protein. The purified protein was analyzed by SDS-PAGE (Supplementary Fig. S2) and mass spectrometry. The mass of the purified cyt c6 determined by MALDI-TOF shows the presence of a single peak at 11063 m/z, which is in good agreement with the calculated mass of 11061 g/mol (cyt c6 with a 6x His-tag and a heme group).

\textit{Interaction of PS I with cyt c\textsubscript{HH} and cyt c\textsubscript{6}}: \textit{Dependence on pH and ionic strength}

In order to evaluate the interaction of cyt c\textsubscript{HH} and cyt c\textsubscript{6} with PS I, we analyzed their capability to act as an electron donor for the photocatalytic complex. Here, oxygen reduction has been used as detection tool. We investigated a pH range from 6 corresponding to physiological conditions (luminal pH) (20) to pH 8 for potential crystallization setups. This range also includes conditions under which photobioelectrodes are often used (1, 2).

By analyzing the concentration dependent behavior of both proteins it is found that the Michaelis-Menten model is well suited to describe the kinetics. Here, the enzyme is PS I, the substrate is cytochrome, the pre equilibrium is between PS I and cytochrome, and the catalytic reaction involves all electron transfer reactions. The turnover number (\textit{k_{cat}}) is represented by the rate of oxygen reduction. For cyt c\textsubscript{HH}, \textit{k_{cat}} and the Michaelis-Menten constant \textit{K_{M}} are highly dependent on pH (Table 1). In phosphate buffer at pH 6 to pH 8, \textit{k_{cat}} increases from 7 s\textsuperscript{-1} to 35 s\textsuperscript{-1} and \textit{K_{M}} from 12 \textmu M to 31 \textmu M. Besides pH, the type of buffer also affects the binding affinity. In Tricine buffer, pH 8, \textit{K_{M}} is decreased by a factor of 6 compared to phosphate buffer. The turnover number is identical in both buffer types at pH 7.5 and pH 8.

To assess which buffer type is suitable to achieve high \textit{k_{cat}} and/or low \textit{K_{M}}, the oxygen reduction rate of PS I with 16 \textmu M cyt c\textsubscript{HH} was analyzed in different buffer types at pH 8 (Supplementary Fig. S3). Because \textit{k_{cat}} remains constant, the change in the reduction rate results from the change in the \textit{K_{M}} value. For each buffer used, the reduction rate decreases linearly with increasing buffer concentration in the range from 5 to 100 mM. The rate is highest in Tricine and Tris buffer, followed by HEPES, MOPS and lastly, phosphate buffer. This order seems to correlate with the ionic strength of the buffer solutions. Ions of different charge affect the binding properties between proteins differently. Therefore, the reduction rate of PS I with cyt c\textsubscript{HH} was analyzed in the presence of NaCl, KCl, NH\textsubscript{4}Cl, Na\textsubscript{2}SO\textsubscript{4}, CaCl\textsubscript{2}, MgCl\textsubscript{2} and MgSO\textsubscript{4}. None of these ions induces a specific effect, but rather results in a decreased reduction rate. This appears to originate from the increasing ionic strength (Fig. 1). Consequently, divalent ions lead to a stronger decrease than monovalent ions at identical molar concentration.

All these experiments demonstrate that increasing salt concentrations decrease the reduction rate by strongly altering \textit{K_{M}}, while \textit{k_{cat}} still remains constant. This clearly points to an electrostatic nature of the interaction between PS I and cyt c\textsubscript{HH}.

An opposing trend is found for the interaction of PS I with its native electron donor cyt c\textsubscript{6}. In this case, the oxygen reduction rates of PS I in the presence of cyt c\textsubscript{6} without additional salt-ions can be increased by decreasing the pH (Fig. 1). An increase of the ionic strength at pH 6 leads to a decrease of the reduction rate, whereas, at pH 8 an increase of the reduction rate was measured with a larger magnitude for divalent cations at 100 mM ionic strength than for monovalent ions or divalent anions. The addition of CaCl\textsubscript{2} leads to a decreased reduction rate above 100 mM. Therefore, the highest reduction rate can be obtained at pH 8 at high ionic strength, except for CaCl\textsubscript{2}. The increase in reduction rate originates from an increasing \textit{k_{cat}} as well as a decreasing \textit{K_{M}} (Table 1).

\textit{Co-crystal structure of PS I with cyt c\textsubscript{HH}}

PS I possesses a high affinity for cyt c\textsubscript{HH} at low ionic strength, and it can be crystallized by...
“salting in” at low pH (21, 22). We have combined this knowledge and crystallized PS I in the presence of cyt cHH with MES-NaOH, pH 6.0 and low MgSO₄ concentrations. Green crystals grow within a week. Each crystal contains both PS I and cyt cHH as analyzed by MALDI-TOF (Supplementary Fig. S4). The cyt cHH content of the crystals was analyzed for crystal batches grown at different cyt cHH:PS I-ratios (Supplementary Fig. S5). Crystals containing a 1:1 ratio of both proteins are achieved by growing at a 5 fold excess of cyt cHH. Crystals do not grow at higher cyt cHH concentration. At 10 fold excess of cyt cHH, no nucleation occurs even at 0 mM MgSO₄.

The crystals diffract to 3.4 Å resolution with 97 % completeness. Unit cell parameters are identical to the ones from PS I-crystals grown without cyt cHH (Supplementary Table S2). We cannot yet assign an electron density for cyt cHH at 3.4 Å resolution (Supplementary Fig. S6). Nevertheless, we are able to detect the subunit cyt cHH after the X-ray measurements of PS I-cyt cHH crystals by subsequent MALDI-TOF analysis of these crystals (Supplementary Fig. S7). In contrast to cyt cHH, no PS I-cyt c6 co-crystals with high cyt c6 saturation were achieved.

**Different binding modes of cyt cHH and cyt c6**

We used ITC to analyze the binding behavior of cyt c to PS I. The proteins need to be soluble throughout the measurement and in high concentration. 25 mM NaCl at pH 8.0 was found suitable for ITC measurements (Supplementary Table S3).

To test the influence of the redox state of cyt cHH on the binding, the measurements were performed either in the presence (reduced cyt cHH and PS I) or absence (oxidized cyt cHH and PS I) of sodium ascorbate. Due to the low binding affinity and protein concentration, the number of binding sites (n) cannot be derived with certainty. For both redox conditions, a fit to the binding curve with n = 1 or n = 2 binding sites results in a large error (Fig. 2). This means that at least a second type of binding sites is necessary to describe the experimental data (Table 2). The heterogeneity of the binding can also be visualized by depicting the data in a logarithmic binding curve (Supplemental Fig. S8). Assuming a dissociation constant \( K_D \) of the specific binding site, where the electron transfer occurs, to be equal to the \( K_M \) value from the Michaelis-Menten kinetic analysis, a reasonable set of parameters for a model of two types of binding sites can be obtained (Table 2). These data suggest that the majority of the produced heat originates from the specific binding site. For the second type of binding sites, \( n_2 > 1 \) is obtained, suggesting a rather complex binding behavior. The cyt cHH binding seems to be independent of the redox state with equal numbers of binding sites and dissociation constants of 19 and 25 µM for the oxidized and reduced proteins, respectively.

In contrast to cyt cHH, the heat of cyt c6 binding is exothermic indicating a different binding mechanism. Also, the binding properties of cyt c6 to PS I are dependent on the oxidation state: while a binding is found for reduced cyt c6, the thermogram of oxidized cyt c6 equals the heat of dilution (Fig. 3). The integrated heat signals of reduced proteins saturate at a lower cyt c6 : PS I ratio compared to cyt cHH, indicating a higher affinity. The values calculated from a fit assuming one binding site are found in Table 2, but due to the low heat of binding compared to the high heat of dilution, absolute values should be taken with care. An increased PS I concentration at elevated ionic strength (200 mM MgSO₄) did not improve the signal (Supplementary Fig. S9).

**Analysis of unspecific binding sites of cyt cHH and cyt c6**

In order to investigate this complex binding behavior, potential binding sites (further referred to as docking sites) were calculated by FTDock and pyDock3 (23, 24). Figure 4 and Supplementary Figure S10 give an overview of the positions of docking sites with negative binding energy. The binding energy ranges from -14.4 to +123.4 kcal/mol and from -28.3 to +55.8 kcal/mol for docking sites of cyt cHH located at the cytoplasmic and luminal side, respectively. This result suggests that binding of cyt cHH to PS I occurs preferentially at the luminal side. Accordingly, the binding sites identified by ITC, including the specific and unspecific ones, can be expected to be located at the luminal side. Although, cyt cHH is a non-native electron donor to PSI, an accumulation of docking sites (henceforth denoted as cluster) at the luminal side of PS I close to PSII is found (Fig. 4, left).

Similarly, docking sites of cyt c6 are found on both, the luminal side (-31.6 to +51.0 kcal/mol) and cytoplasmic side (-20.7 to +65.9 kcal/mol). The docking sites of cyt c6 at the luminal side with strongly negative binding energies are less dispersed compared to the ones for cyt cHH with
the majority of these sites organized in a cluster close to P700 (Fig. 4, right). As expected for cyanobacterial PS I, none of the docking sites are in close vicinity to PsaF.

Elucidating the specific cyt c binding site of PS I

The most interesting binding site is the one where the electron transfer from cyt c to P700 occurs (specific binding site). At this site, the heme group of cyt c and P700 have to be in close proximity. In the case of cyt c6, the 100 docking sites with the strongest interaction display binding energies in the range of -31 to -15 kcal/mol. For 25 out of these 100 sites, the smallest distance between carbon atoms of the heme group of cyt c6 and tryptophan residues W(A655) and W(B631) of PS I is below 10 Å. An NMR analysis of cyt c6 - PS I interaction in Nostoc sp. PCC 7119 revealed certain amino acid residues of cyt c6, which are likely part of the binding interface (14). Nostoc sp. cyt c6 shares a high sequence identity with cyt c6 of T. elongatus. 13 of the 25 docking sites identified above are in agreement with the NMR results, with heme-tryptophan distances of 2.5 to 8.9 Å.

Binding of cyt c6 and PS I is dependent on ionic strength and pH, as shown above. Therefore, the electrostatic binding energy for these 13 docking sites was calculated for three different values of ionic strengths at pH 6 and 8 using the Poisson-Boltzmann equation (Supplementary Fig. S11). Re-calculating the electrostatic binding energy reveals that the binding energy of most of the docking sites is decreased to less positive values by increasing the ionic strength at pH 8 but not at pH 6 (Supplementary Fig. S11). The closest of these docking sites has a heme-tryptophan distance of 2.5 Å and a binding energy of -15.5 kcal/mol (Fig. 5, bottom). The distance between the iron from the heme group and the magnesium of the two P700 chlorophylls is 21.4 Å and 21.3 Å, respectively. In this specific docking site, cyt c6 is in close proximity to a luminal loop of PsaA. The negatively charged amino acid residue D628 from this loop is at 7.4 Å distance from the negatively charged residue E34 from cyt c6 leading to a repulsive interaction at low ionic strength (Fig. 5). The amino acid residues which form the interface between T. elongatus cyt c6 and PS I are shown in Supplementary Table S4. It has to be mentioned that the absolute distances shown in Supplementary Table S4 have to be taken with caution, because the expected perturbation of amino acid residues upon binding is not described by rigid body docking. Out of the 19 amino acid residues shown in this table, only 3 are not perturbed in cyt c6 from Nostoc sp. upon binding to PS I (14).

Since cyt cHH is a non-native binding partner, it does not necessarily have to bind in the native binding site. The 300 cyt cHH docking sites with strongest binding have binding energies in the range of -28 to -15 kcal/mol. 36 of these 300 docking sites have heme-tryptophan distances of less than 10 Å between carbon atoms. After re-calculating the electrostatic binding energy by using the Poisson-Boltzmann equation, 7 docking sites remain, which show pH and ionic strength dependence in good agreement with the analysis of kinetic parameters (see above). The electrostatic binding energy is strongly negative in the absence of salt-ions and increases to about 0 kcal/mol at an ionic strength corresponding to 100 mM MgSO4 (Supplementary Fig. S11). Out of these 7 cyt cHH docking sites, the one with the most negative binding energy (-25.8 kcal/mol) is the one in closest proximity to P700. Here, the distance between the heme group and the parallel tryptophan residues W(A655)/W(B631) is 4.5 Å (Fig. 5, top). The distances of the iron from the heme group and the magnesium ions from P700 is 24.3 and 24.9 Å, respectively. The distances between the closest side chains of PS I and cyt cHH are shown in Supplementary Table S5. There is no salt bridge between residues, suggesting that the electrostatic interactions are mainly non-specific.

Discussion

Activity and affinity of PS I for cyt c

The PS I oxygen reduction rate with both cytochromes is highly dependent on the pH and the ionic strength. These effects are in agreement with the P700− re-reduction rates from time resolved spectroscopy with cyt c6 (25, 26). The binding affinity of PS I for cyt c6 is increased by increasing ionic strength at pH 8, but not at pH 6, which is close to the physiological pH (20). The isoelectric point (pI) of 6x His-tagged cyt c6 can be estimated to 6.5 based on the amino acid sequence and assuming a reduced heme group using the compute pI tool from ExPASy (27). Without the His-tag, the pI is estimated to be 5.5. Thus, in both cases, cyt c6 is close to zero net charge at pH 6, while it is negatively charged at pH 8. If we assume that the luminal side of PS I is negatively charged at both pH values (given, that it is negatively charged at pH 7 (16)), it follows that there is a repulsive interaction between PS I and...
cyt c₆ at pH 8 which is almost absent at pH 6. This can explain the ionic strength dependence found for the two different pH values.

At both, pH 8 and pH 6, increasing the ionic strength decreases the binding affinity of cyt cHH to PS I. As the pI of cyt cHH is 10.5 (28), the protein is positively charged at the investigated pH values. Therefore, decreasing the ionic strength favors binding of cyt cHH. In this study, $K_M$ values of T. elongatus PS I of up to 33 µM (pH 8, high ionic strength) and 5 µM (pH 8, low ionic strength) could be achieved for the native and non-native cytochrome, respectively (Table 1). Both values are comparable to the affinity of plastocyanins and cytochromes in plants, algae and other cyanobacteria (7 to 125 µM, (14, 29–32)).

The binding affinity of the homologous cytochrome C₂ (cyt c₂) to photosynthetic, bacterial reaction centers (bRC) is 1 µM (33). In this case, co-crystallization of cyt c₂ with bRC was successful (34). The latter results indicate how strong the affinity has to be for a successful co-crystallization. The present data confirm that cyt cHH can form a stable complex with PS I at low ionic strength (2). This motivated us to attempt a co-crystallization of cyt cHH with PS I. The low ionic strength necessary for complex formation matches the known crystallization conditions of PS I (5).

**Binding affinity of oxidized and reduced cyt c to PS I**

To elucidate why cyt cHH is not identified in the crystal structure, we have analyzed the binding behavior of cyt cHH by ITC. Cyt cHH binds to PS I at more than one site. The positive enthalpy (Table 2) reveals that the binding of cyt cHH to PS I is endothermic. Positive enthalpies for the electrostatic binding of cyt cHH were reported and are likely to originate from the displacement of bound water molecules (35). Another observation by ITC is that the binding is independent of its redox state in contrast to cyt c₆. This behavior renders cyt cHH a suitable redox mediator in biotechnological applications.

**Co-crystal structure of PS I with cyt cHH**

A co-crystal-structure of cyt cHH with PS I was solved, but no electron density was found for cyt cHH. This may have the following reasons.

In the crystal, cyt cHH is highly disordered or flexible. In Supplementary Figure S12, a part of the PS I crystal lattice is shown. Here, the PS I trimers form layers with the membrane planes oriented parallel to each other. The crystal contacts are formed by the cytoplasmic subunit PsaE and luminal helices of the subunit PsaF. A volume is present between the trimers, in which no electron density is visible. Part of this volume is occupied with detergent belts (17). The remaining volume contains an aqueous phase, including an area close to the luminal surface of PS I, highlighted in blue in Supplementary Figure S12. The cyt cHH can be expected to be located in this volume. As illustrated by the randomly chosen docking state shown in Supplementary Figure S12, cyt cHH cannot form protein contacts with other PS I trimers. In such flexible environments, a high resolution crystal structure is usually necessary to visualize the co-crystallized protein (36). If there is more than one binding site for cyt cHH, the occupancy of the specific binding site at P₇₀₀ will not be 100% even in a 1:1 co-crystal. In this respect, variation of the protein ratio could have an influence as shown for cyt cHH-peroxidase co-crystals (37, 38). By using isothermal titration calorimetry and rigid body docking, we have revealed that there is more than one cyt cHH binding site at PS I under low ionic strength. These binding sites are likely to spread over the whole luminal side of PS I (Fig. 4) and mostly would not interfere with the crystal contacts. Increasing the cyt cHH : PS I ratio might be necessary to achieve a full occupancy for the binding site at P₇₀₀. However, cyt cHH disturbs the crystal formation. Therefore, saturating the binding site at P₇₀₀ is not possible under the crystallization conditions used in this study.

Even if cyt cHH is bound to the site close to P₇₀₀ to 100%, it could occur at different conformers or orientations rendering it invisible in the crystal structure. This possibility is supported by the theoretical binding studies (Supplementary Fig. S10).

**The specific cyt c binding site at PS I**

The specific binding sites of cyt c₆ and cyt cHH are the ones with closest proximity of the heme group to W(A655) as analyzed by rigid body docking. Calculating the binding energy of the closest docking sites at different pH values and ionic strengths results in changes which are in good agreement with the measured oxygen reduction rates for both cytochromes.

Both cytochromes bind more strongly to W(A655) than to W(B631) (Fig. 5) as was also shown for cyt c₆ from Chlamydomonas reinhardtii (39). It is found that the PS I - cyt cHH-complex has a more negative binding energy than
the PS I - cyt\textsubscript{c\textsubscript{6}} complex, which is in good agreement with the higher affinity of PS I for cyt\textsubscript{c\textsubscript{HH}}. The distance between the heme group and P\textsubscript{900} is smaller for the PS I - cyt\textsubscript{c\textsubscript{6}} complex, than for the PS I - cyt\textsubscript{c\textsubscript{HH}}-complex. As the positioning of the heme group is slightly different for both complexes, different turnover numbers can be expected. Indeed, PS I has a higher turnover number using cyt\textsubscript{c\textsubscript{6}} as electron donor (Table 1).

At low ionic strength and pH 8, the binding energy of PS I and cyt\textsubscript{c\textsubscript{6}} is repulsive. This repulsive interaction partially arises from negatively charged side chains on the luminal loop of PsaA and cyt\textsubscript{c\textsubscript{6}}, as was shown for the interaction of PS I with PC (40) and as revealed by rigid body docking (Fig. 5). The screening effect is stronger for divalent cations than for monovalent ions of the same ionic strength (Fig. 1), suggesting that divalent cations can form a bridge between these side chains.

Previously, a co-structure of PS I with cyt\textsubscript{c\textsubscript{6}} was achieved by rigid body docking for the diatom \textit{Phaeodactylum (P.) tricornutum} (31), here the docking sites with the most negative energy result from interaction of cyt\textsubscript{c\textsubscript{6}} with PsaF. However, the closest docking site is different and has less negative binding energy. In contrast to this diatom, cyt\textsubscript{c\textsubscript{6}} from \textit{T. elongatus} does not show the complex kinetics that can be explained with an additional docking site at PsaF (25, 26, 41). Indeed, the docking sites described in the present work that show short heme - P\textsubscript{900} distances are found within the top 100 ranks with the most negative binding energies, and no binding site close to PsaF with a high binding energy can be identified. This is in agreement with the binding properties in most cyanobacteria (12, 42).

In contrast to cyanobacterial and algal PS I, the co-crystal structure of the bRC with cyt\textsubscript{c\textsubscript{2}} from \textit{Rhodobacter sphaeroides} is known (34). bRCs are structurally homologous to cyanobacterial photosystems (43). Fig. 6 shows a comparison between the modeled PS I – cyt\textsubscript{c\textsubscript{6}} complex and the co-crystal structure of the bRC – cyt\textsubscript{c\textsubscript{2}} complex. Both complexes differ only in a small rotation of the cytochrome, while having identical heme - P\textsubscript{700}/P\textsubscript{870} distances. This suggests that the specific binding site, where the electron transfer occurs, diverged only slightly during evolution. The positioning of the heme group relative to the active center remains conserved, while the sequence identity of the amino acid residues on the protein surface is low.

Conclusions and outlook

We have analyzed the binding behavior of a native and a non-native cytochrome to PS I from the cyanobacterium \textit{T. elongatus}. While the highest turnover number is found for the cyt\textsubscript{c\textsubscript{6}} - PS I complex, the highest affinity is detected for cyt\textsubscript{c\textsubscript{HH}}. Both proteins show a very different dependence of the interaction with PS I on the ionic strength. For cyt\textsubscript{c\textsubscript{HH}}, this points to a mainly electrostatically determined binding mode to the photo-active protein complex.

This information is not only of fundamental interest, but can also be used to improve biotechnological applications. Because self-assembled photobioelectrodes often need low ionic strength, cyt\textsubscript{c\textsubscript{HH}} is well suited as a mediator for the assembly of PS I. Other arguments for the use of cyt\textsubscript{c\textsubscript{HH}} are the high turnover number and the similar binding behavior of oxidized and reduced protein.

Theoretical modelling of cyt\textsubscript{c} – PS I interactions reveals docking sites for cyt\textsubscript{c\textsubscript{6}} that highly resembles the native binding site of cyt\textsubscript{c\textsubscript{2}} with bRC. In addition, the modelling provides a rationale for the inability to detect cyt\textsubscript{c\textsubscript{HH}} in co-crystals as it suggests a variety of binding sites.

To improve the modeling with regard to pH dependence and accuracy of computed binding energies, future work will also consider the protonation states of titratable groups in the proteins that may be different from the ones assumed in the present work.

Improved co-crystal structures will ultimately serve to understand the electron transfer reaction. The present data suggest that PS I should be co-crystallized with cyt\textsubscript{c\textsubscript{HH}} at higher cyt\textsubscript{c\textsubscript{HH}} concentration with low ionic strength at pH 6, which might be achieved by using an alternative precipitation agent such as polyethylene glycol. First crystals diffracting at medium resolution have been obtained. Although cyt\textsubscript{c\textsubscript{6}} binds to PS I at a conserved binding site while no unspecific binding occurs, the binding affinity of cyt\textsubscript{c\textsubscript{6}} to PS I is weaker and further investigations are needed to find suitable conditions for the co-crystallization. The present results serve as a guideline in this respect.

Experimental Procedures

Chemicals and Enzymes

All chemicals were of analytical grade or higher and purchased from Sigma Aldrich (Germany). Cytochrome \textit{c} from Sigma Aldrich (Germany) with 95 % purity
for the majority of experiments and with 99 % purity for crystal structure analysis. The detergent n-dodecyl-β-D-maltoside (DDM) was purchased from Glycon (Germany). The plasmid pEC86, harboring the genes for heme maturation, was kindly provided by L. Thöny-Meyer (44).

**Isolation of Proteins**

Cultivation of *T. elongatus* and membrane protein extraction were performed as reported previously (45). For the purification of PS I, the protein extract was applied to two steps of anion exchange chromatography. In the first step, PS I was separated from PS II by a column packed with Toyo Pearl DEAE 650 S (GE Healthcare, Germany) equilibrated with buffer A (20 mM MES-NaOH, pH 6.0, 5 % glycerol (v/v), 20 mM CaCl₂ and 0.02 % DDM (w/v)). After washing with buffer A containing 5 mM MgSO₄, proteins were eluted with a linear gradient from 5 to 100 mM MgSO₄ in buffer A. PSII was eluted at 30 mM MgSO₄, whereas PS I was eluted at 55 mM MgSO₄. The PS I fractions were pooled and diluted with buffer B (5 mM MES-NaOH, pH 6.0, 0.02 % DDM) to a conductivity of 6 mS/cm and applied to a Q-Sepharose™ (GE Healthcare, 6.0, 0.02 % DDM) to a conductivity of 6 mS/cm and diluted with buffer B (5 mM MES-NaOH, pH 6.0, 5 % glycerol (v/v), 20 mM CaCl₂ and 0.02 % DDM (w/v)). After washing with buffer A containing 5 mM MgSO₄, proteins were eluted with a linear gradient from 5 to 100 mM MgSO₄ in buffer A. The addition of IPTG was not necessary. Harvested cells were incubated in 20 % sucrose (w/v), 1 mM EDTA, 25 mM Tris-HCl, pH 8.0, for 30 min on ice. Subsequently the cells were centrifuged at 12,000 g for 10 min at 4 °C. The cell pellet was re-solubilized in cold 10 mM Tris-HCl, pH 8.0 containing 5 mM MgSO₄ to isolate the periplasmatic proteins. After centrifugation (10000 g, 10 min, 4 °C), the supernatant was adjusted to buffer C (500 mM NaCl, 20 mM imidazole and 20 mM phosphate buffer, final pH = 7.5) and applied to a Ni-NTA column (Rotigaure-His/Ni, Carl-Roth, Germany). The column was washed with 10 volumes of buffer C and the protein was eluted with a linear gradient at 140 mM imidazole. The cyt c₆ containing fractions were pooled and dialyzed against 1 mM Na-ascorbate in 25 mM Tricine-NaOH, pH 7.2. For further purification, cyt c₆ was applied to a Toyo Pearl DEAE 650 S (GE Healthcare, Germany), washed with 5 column volumes of 25 mM Tricine-NaOH, pH 7.2 and 10 mM NaCl. Cyt c₆ was eluted with a linear gradient of 10-30 mM NaCl in 25 mM Tricine-NaOH, pH 7.2. Cyt c concentration was spectrophotometrically determined in the presence of 5 mM Na-ascorbate (ε₅₅₀ = 29.5 mM⁻¹cm⁻¹ for cyt c₆H and ε₅₅₅ = 25 mM⁻¹cm⁻¹ for cyt c₆ (42, 47)).

**Polyacrylamide Gel Electrophoresis**

Purity of the isolated cyt c₆ was verified by SDS-PAGE with 15 % polyacrylamide using 0.5 to 10 µg protein according to (48). PS I was analyzed by BN-PAGE with a polyacrylamide gradient from 3 % to 9 % according to (49). PS I crystals corresponding to 5 µg Chl were dissolved in solubilization buffer containing 0.2 % DDM and 100 mM NaCl. The gel was destained by 10 % acetic acid.

**Dynamic light scattering (DLS)**

Homogeneity of purified trimeric PS I samples was verified by DLS. PS I crystals were dissolved isolated from *T. elongatus* served as a template. The resulting PCR product was subcloned in a pJET1.2 vector (Thermofisher, Germany) and verified by DNA sequencing (SMB, Germany). Subsequently, cloning was performed into a pET22b expression vector (Novagen, Germany) and transformed into E. coli BL21-Star strain. For the maturation of cytochrome c₆ in *E. coli*, the pEC86 vector was also introduced. For heterologous expression, cells were grown in 1 l of LB media containing 100 µg ml⁻¹ ampicillin and 10 µg ml⁻¹ chloramphenicol at 37 °C for 16 h. The concentration of the RC (equivalent to the concentration of P₇₀₀) was determined by ε₅₈₀ = 5.5 µM⁻¹cm⁻¹ and the concentration of PS I-bound Chl a by ε₅₈₀ = 57.1 mM⁻¹cm⁻¹ in 25 mM Tris-HCl pH 8.0, 100 mM NaCl and 0.02 % DDM (46).

Cloning and expression of cytochrome c₆ in *E. coli*

The coding gene for cytochrome c₆ from *T. elongatus* (tll1283) was amplified by PCR using the primers 5’-CTCGAGGCTGGCCAACCTT-3’ and 5’-CATATGGCTGACCTAGCCCATGCT-3’ containing restriction sites for *NdeI* and *Xhol* (underlined), respectively. Chromosomal DNA
in 25 mM Tricine-NaOH, pH 8.0, 100 mM NaCl, 0.02 % DDM to a protein concentration between 5 and 10 µM P700 and were filtered through a 0.45 µm membrane. Measurements were performed on a DynaPro NanoStar (Wyatt, USA) with a 787 nm laser at 20 °C in a disposable 4 µl cuvette.

**MS-Analysis**

The subunit composition of PS I samples was analyzed by MALDI-TOF. 0.5 µl of 2 µM purified PS I was mixed with 0.5 µl of sinapinic acid in 40 % (w/v) acetonitrile and 0.1 % (v/v) TFA on the target. MALDI-TOF mass spectra were recorded on a Microflex spectrometer (Bruker, Germany) in linear, positive-ion mode.

**PS I activity measurements**

The oxygen reduction rate of PS I was measured with a Clark type electrode (Oxygraph+, Hansatech, Germany). Except where stated, the standard reaction mixture contained 25 mM Tricine-NaOH, pH 8.0, 0.02 % DDM, 2 mM Na-ascorbate and 300 µM methyl viologen with either 16 µM cyt cHH or 2 µM cyt c6 at 20 °C. The reaction mixture was stirred under illumination of > 500 µmol photons*m⁻²*s⁻¹ for 30 s. The reaction was started by addition of PS I (5 µg/ml chlorophyll) and the initial velocity of the reaction was determined. For the determination of $K_M$ and $k_{cat}$, the data were analysed in terms of Michaelis-Menten kinetics, using cyt cHH or cyt c6 as substrate. All measurements were done with three different PS I preparations.

**Isothermal titration calorimetry (ITC)**

For the ITC measurements, PS I crystals were washed twice with H2O containing 0.02 % DDM and subsequently dissolved to buffer D (25 mM Tricine-NaOH, pH 8.0, 0.02 % DDM, 25 mM NaCl). To remove remaining PS I crystals in the solution, the sample was centrifuged for 5 min and filtered through a 0.45 µm membrane. Cyt cHH powder was dissolved in buffer D. Cyt c6 was dialized against buffer D in a centrifugal concentrator (5000 MWCO), except for the 0.02 % DDM which was added after the final concentrating step. Experiments under reducing conditions were carried out by addition of 5 mM Na-ascorbate (final concentration) in the dark. Experiments were performed at 20 °C with VP-ITC (MicroCal, Northampton, USA) in a 1.45 ml cell. Baseline subtraction was done by NITPIC 1.1.5 (50, 51), and data analysis with Origin 7 software.

**Co-crystallization of the PS I - cyt cHH supercomplex and X-ray diffraction analysis**

For co-crystallization, 7.5 µM P700 were mixed with 37.5 µM cyt cHH in buffer B containing 40 mM MgSO4. Samples were dialyzed successively against buffer B (5 mM MES-NaOH, pH 6.0 with 0.02 % DDM) containing 5, 3 and 2 mM MgSO4 in dialysis buttons (Hampton Research, USA) with a 2000 MWCO membrane (CarlRoth, Germany) at 20 °C. 200-500 µm long, but thin and often hollow, needle shaped crystals grew within 3 - 5 days and were used for microseeding: The crystals were crushed in a seed tool kit (Hampton Research, USA) by vortexing for 5 min. The seed stock was centrifuged for 5 min at 16,000 g and the supernatant was diluted hundredfold with buffer B. 1 µl of the diluted supernatant was added to 40 µl of 7.5 µM P700 with 37.5 µM cyt cHH in buffer G containing 3 mM MgSO4. Crystals grown over night were needle-shaped with dimensions from 200 x 30 µm to 800 x 100 µm and diffracted up to 3.4 Å resolution. For cryo-protection the buffer was exchanged against buffer B containing 0.25 to 1.75 M sucrose in 0.25 M steps with 5 – 10 min incubation time for each step and 2 h for the last step. Crystals were frozen in liquid nitrogen.

Diffraction data were collected from single crystals at beamline 14.1 at BESSY II electron storage ring, operated by Helmholtz-Zentrum Berlin (Berlin-Adlershof, Germany) and at beamline 8.2.1 at the Advanced Light Source (ALS, Berkeley, USA) by guest on July 25, 2018 at 20 °C in a disposable 4 µl cuvette. Baseline subtraction was done by NITPIC 1.1.5 (50, 51), and data analysis with Origin 7 software.

**Analysis of cyt cHH content in PS I - cyt cHH co-crystals**

The presence of cyt cHH in the co-crystals was qualitatively analyzed by MALDI-MS. Single PS I - cyt cHH co-crystals with sizes from 200 x 30 to 800 x 100 µm from different batches were selected and transferred to buffer B. Each crystal was washed 6 times by exchanging the supernatant against buffer B (dilution factor > 10, each step) and subsequently dissolved in buffer B containing 20 mM MgSO4. The supernatant from these crystallization batches was treated in the same manner as control samples. MS analysis
was done as described above. Additionally, for one crystal an X-ray diffraction dataset at 3.5 Å resolution was measured and the sucrose concentration in the crystal was successively reduced afterwards by washing with 1.5, 1.0, 0.5, 0 and 0 M sucrose in buffer B for analysis by MALDI-TOF. The supernatant of the single crystals was exchanged for 10 µl buffer B containing 10 mM MgSO₄ for solubilization. 0.5 µl of each sample was measured as described in MS-analysis.

Quantitative analysis of the cyt cHH content was carried out by washing a batch of crystals 6 times in buffer B, subsequently dissolving the batch in buffer B containing 100 mM MgSO₄ and separating the cyt cHH from PS I using a centrifugal concentrator with a 100,000 MWCO membrane (Sartorius Stedim, Germany). The PS I and cyt cHH concentrations were analyzed by their chlorophyll and heme absorption bands at 680 and 550 nm, respectively.

Protein-Protein Docking Simulation

The cyt c binding site of PS I was analyzed by rigid body docking using FTDock and rescoring by pyDock3 (23, 24).

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Conflict of Interest

The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions

M.H. conceived and performed the cloning of the heterolog cyt c6 in E. coli. A.K. and M.H. isolated the proteins. A.K. carried out the analysis solely or in collaboration with M.H. (MALDI-TOF, oxygen reduction rates), J.F.K. (crystal structure), F.M. and K.R.S. (docking). The experiments were designed...
by A.K., M.H., H.L., S.C.F., F.L. and A.Z. All authors reviewed the results and approved of the final version.

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Table 1

Oxygen consumption measurements of PS I with cyt c_{HH} and cyt c_{6} as electron donor in either 5 mM phosphate buffer or 25 mM Tricine buffer at specified pH and addition of ions. Kinetic constants have been determined by applying the Michaelis-Menten model. Standard deviations result from three independent measurements.

| Buffer                  | pH   | K_{M} [µM] | k_{cat} [s^{-1}] |
|-------------------------|------|------------|-----------------|
| cyt c_{HH}              |      |            |                 |
| phosphate buffer        | 6.0  | 11.5 ± 2.8 | 7.1 ± 1.3       |
| phosphate buffer        | 6.5  | 14.2 ± 2.7 | 8.5 ± 0.7       |
| phosphate buffer        | 7.0* | 22.8 ± 2.8 | 20.3 ± 1.0      |
| phosphate buffer        | 7.5  | 23.5 ± 2.3 | 28.9 ± 1.1      |
| Tricine                 | 7.5  | 4.9 ± 0.5  | 27.7 ± 1.5      |
| phosphate buffer        | 8.0  | 30.5 ± 3.0 | 34.9 ± 3.1      |
| Tricine                 | 8.0  | 5.0 ± 0.7  | 34.3 ± 1.9      |
| Tricine + 25 mM NaCl    | 8.0  | 10.8 ± 1.7 | 34.8 ± 1.4      |
| Tricine + 10 mM MgSO_{4} | 8.0 | 44.7 ± 3.6 | 31.2 ± 0.9     |
| cyt c_{6}               |      |            |                 |
| Tricine                 | 8.0  | 290 ± 45   | 55 ± 9          |
| Tricine + 10 mM MgSO_{4} | 8.0 | 65 ± 9     | 143 ± 11        |
| Tricine + 200 mM MgSO_{4}| 8.0| 33.3 ± 1.3 | 159 ± 2         |

*Value taken from (2).

Table 2

Binding parameters derived from ITC measurements. Datasets for oxidized and reduced proteins are analyzed with either 1 or 2 sets of binding sites. Standard deviations are determined from three independent measurements (n: number of binding sites, K_{D}: dissociation constant, ΔH: binding enthalpy).
Table 1. Binding parameters of PS I for cyt c₉H ± 0.1, 1* 1.5 ± 0.2, 1* 24.8 ± 1.2, 11* 7290 ± 360, 1* -1370 ± 40

|            | cyt c₉H oxidized | cyt c₉H reduced | cyt c₆ reduced |
|------------|------------------|------------------|----------------|
| n₁         | 1.5 ± 0.1        | 1.5 ± 0.2        | 1* 1.5 ± 0.2   |
| Kᵥ₁ [µM]   | 18.8 ± 0.2       | 24.8 ± 1.2       | 21 ± 3         |
| ΔHᵥ₁ [cal/mol] | 5320 ± 70    | 4530 ± 250       | 7290 ± 360     |
| n₂         | 2.0 ± 0.2        | 6.4 ± 1.2        |                |
| Kᵥ₂ [µM]   | 28.4 ± 1.0       | 39.4 ± 5.4       |                |
| ΔHᵥ₂ [cal/mol] | -480 ± 120     | -220 ± 100       |                |

^iNumber of types of binding sites, presumed in the fit. *n₁ and Kᵥ₁ were set to 1 and 11 µM, respectively, as derived from Michaelis-Menten kinetics.

**Figure Legends**

**Figure 1.** Oxygen reduction rates of PS I with 16 µM cyt c₉H (top) and cyt c₆ (bottom) at pH 8 (left) and pH 6 (right) as a function of ionic strength. Monovalent (NaCl, black) and divalent (MgCl₂, red) cations are depicted as circles and squares, respectively. For cyt c₆, pH 8 (bottom, left) differences between the applied salts become prominent. Therefore, a further differentiation of the salts is shown: NaCl (black), Na₂SO₄ (yellow), NH₄Cl (blue), MgCl₂ (red), CaCl₂ (grey), MgSO₄ (cyan). NaCl, MgCl₂ and CaCl₂ are connected by a line in their corresponding color. All measurements were performed in either 25 mM Tricine-NaOH (pH 8) or 5 mM MES-NaOH (pH 6) with 2 mM ascorbic acid and 300 µM methyl viologen at 20 °C. The concentration of buffer ions and counter ions, which contribute to the ionic strength, was calculated by using the Henderson-Hasselbalch equation with a pKₐ of 8.2 and 6.2 for Tricine and MES buffers, respectively. Standard deviations result from three to nine independent measurements.

**Figure 2.** Isothermal titration calorimetry of PS I with cyt c₉H. A: Thermogram for exemplary background measurements (top), oxidized (middle) and reduced (bottom) proteins. B: Integrated heats of titrations after background subtraction in the presence (red) or absence (black) of 5 mM ascorbate. High cyt c₉H : P₇₀₀ ratios are omitted for a better overview. Fits (top) and residuals (bottom) are shown for 1 set of binding sites with n = 1.0 (dashed line) and for one set of binding sites with n = 1.5 (solid line). Parameters obtained from the models are shown in Table 2. Measurements were performed at 20 °C in 25 mM Tricine buffer, pH 8.0 with 25 mM NaCl and 0.02 % DDM. Each titration step consisted of 5 µl injected volume from 1 mM cyt c₉H.

**Figure 3.** Isothermal titration calorimetry of PS I with cyt c₆. A: Thermogram for exemplary background measurements (top, red), oxidized (middle) and reduced (bottom) proteins. B: Integrated heats of titrations after background subtraction in the presence (red, reduced) or absence (black, oxidized) of 5 mM ascorbate. The fit of the reduced data is shown for 1 set of binding sites with n = 1.0. Parameters obtained from the model are shown in Table 2. After substraction of the heat of dilution, the oxidized data converge to negative values at high cyt c₆ : PS I ratio (-0.1 kcal/mol, not shown) and are thus not analyzed by a model. Measurements were performed at 20 °C in 25 mM Tricine-NaOH, pH 8.0 with 25 mM NaCl and 0.02 % DDM. Each titration step consisted of 5 µl injected volume from 1 mM cyt c₆.
**Figure 4.** Molecular docking simulation of monomeric PS I with cyt cHH (left) and cyt c6 (right). Each sphere represents the position of a docked cyt c. The binding energy, calculated by pyDock, is highlighted by a color code. Docking states with less than -20 kcal/mol are highlighted by an increased sphere size.

**Figure 5.** Potential cyt cHH (top) and cyt c6 (bottom) binding site of PS I. Shown are the docking sites which most likely resemble the specific cyt c binding site of PS I. The heme group (red) of cyt cHH and cyt c6 point towards the luminal tryptophan residues W(A655) and W(B631) (blue) and P700 (green) of PS I. The distances between the heme groups and the closest tryptophan are highlighted by a black, dotted line. Cyt c6 does not interact with PsAF (purple), but is close to the luminal loop of PsA (yellow). The carboxyl group of E34 from cyt c6 is at a distance of 7.4 Å from the carboxyl group of D628 from PsA (grey, dotted line).

**Figure 6.** Superposition of the potential cyt c6 binding site to the known cyt c2 binding site of the bRC from *Rhodobacter sphaeroides* (PDB-ID: 1l9b (34)). The superposition was achieved by aligning the heme groups. Right view rotated by 90° with respect to the left view. The distance of the heme-iron from cyt c6 to the Mg2+ ions of P700 are 21.4 and 21.3 Å, respectively. This distances are identical to the distances between the heme-iron of cyt c2 and the Mg2+ ions of P870 (pink) from bRC (21.3 and 21.2 Å, respectively).
Figure 2

A

B

Figure 2
Figure 3
Figure 4

PS I with cyt $c_{\text{HH}}$ (horse heart)  
PS I with cyt $c_6$ (T. elongatus)

Luminal view

Side view

-30 kcal/mol  
0 kcal/mol
Insights into the binding behavior of native and non-native cytochromes to Photosystem I from *Thermosynechococcus elongatus*
Adrian Kölsch, Mahdi Hejazi, Kai R. Stieger, Sven C. Feifel, Jan F. Kern, Frank Müh, Fred Lisdat, Heiko Lokstein and Athina Zouni

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