

Expression and characterization of cytochrome c_{553} from *Heliobacterium modesticaldum*

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Abstract Cytochrome c_{553} of *Heliobacterium modesticaldum* is the donor to P_{800}^+ , the primary electron donor of the heliobacterial reaction center (HbRC). It is a membrane-anchored 14-kDa cytochrome that accomplishes electron transfer from the cytochrome bc complex to the HbRC. The *petJ* gene encoding cyt c_{553} was cloned and expressed in *Escherichia coli* with a hexahistidine tag replacing the lipid attachment site to create a soluble donor that could be made in a preparative scale. The recombinant cytochrome had spectral characteristics typical of a c -type cytochrome, including an asymmetric α -band, and a slightly red-shifted Soret band when reduced. The EPR spectrum of the oxidized protein was characteristic of a low-spin cytochrome. The midpoint potential of the recombinant cytochrome was $+217 \pm 10$ mV. The interaction between soluble recombinant cytochrome c_{553} and the HbRC was also studied. Reduction of photooxidized P_{800}^+ was accelerated by addition of reduced cytochrome c_{553} . The kinetics were characteristic of a bimolecular reaction with a second order rate of $1.53 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ at room temperature. The rate manifested a steep temperature dependence, with a calculated activation energy of 91 kJ mol $^{-1}$, similar to that of the native protein in *Heliobacillus gestii* cells. These data demonstrate that the recombinant soluble cytochrome is comparable to the native protein, and likely lacks a discrete electrostatic binding site on the HbRC.

Keywords Cytochrome c · *Heliobacterium modesticaldum* · Photosynthetic reaction center · Transient spectroscopy

Introduction

Heliobacteria are phototrophic *Firmicutes* with a homodimeric type 1 (FeS-type) reaction center (RC). They do not have a peripheral antennae system, and are unique in that they use bacteriochlorophyll g as the major pigment in their RC. The heliobacterial RC (HbRC) shares similarities with the RC of green sulfur bacteria and photosystem I of higher plants. The cofactor arrangement in these RCs are thought to be similar, although the core polypeptides do not share high levels of sequence identity beyond their topological arrangement in the membrane (Hauska et al. 2001; Oh-oka 2007). Heliobacteria are strict anaerobes (Madigan and Ormerod 1995), like many of the bacteria in the order *Clostridiales*, and can grow in the dark fermentatively. They are not autotrophic and use simple organic acids like pyruvate as carbon and electron sources.

Cytochromes are a diverse family of proteins involved in electron transfer, and are made by all eukaryotes and most prokaryotes. Typically, these proteins are associated with protein complexes bound to membranes. Among the *Heliobacteria*, the major membrane-anchored cytochromes have similar primary structures with high homology in the functional domain. These membrane-anchored cytochromes are monoheme c -type cytochromes, and are fully functional in isolated membranes. In the reduced form, they have an α -band centered at 553 nm, as is typical for c -type cytochromes. Although it is attached to the membrane, the primary structure of cyt c_{553} gave no indications of transmembrane helices. Instead, mass spectroscopy data indicated an N-terminal modification at Cys23,

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where a diglyceride is attached via a thioether linkage (Albert et al. 1998). The heme-binding motif is on the C-terminus (Albert et al. 1998), separated from the N-terminus by a ~40-residue Pro/Ala-rich sequence likely to be unstructured. The stoichiometry of cyt c_{553} to HbRC was measured to be around 5–6 per RC in *Heliobacillus mobilis* (Nitschke et al. 1995). The isolated RC from *Heliobacterium modesticaldum* contains no bound cytochrome (Sarrou et al. 2012).

The cyt c_{553} serves as the direct reductant of P_{800}^+ in whole cells and membranes of *Heliobacterium gestii*, with a redox potential of +215 mV (Albert et al. 1998), only slightly more negative than the calculated redox potential of +240 mV in P_{800}^+ in *H. mobilis* (Nitschke et al. 1995). In whole cells of *Heliobacterium chlorum*, the electron transfer rate from cyt c_{553} to P_{800}^+ is about 300 μ s, while in membranes it is considerably slower at ~3 ms (Prince et al. 1985). Electron transfer from cyt c_{553} to P_{800}^+ in membranes showed a strong dependence on both temperature and divalent cations. This is consistent with the anchoring of cyt c_{553} in the membrane and collisional mode of action.

A system was developed to express cyt c_{553} from *H. modesticaldum* in *E. coli* cells. The gene was restructured to remove the lipid attachment site and replace it with a hexahistidine tag to create a fully soluble electron donor to P_{800}^+ . The recombinant product was characterized in detail to ensure it retained the properties of the native protein. These data show that the expressed cyt c_{553} is on par with the native cytochrome and is functional for future studies.

Experimental procedures

Construction of recombinant His₆-tagged cyt c_{553}

The recombinant cytochrome c_{553} was designed by taking the predicted mature gene product and replacing the diacylglycerol attachment site (Cys23) with a hexahistidine tag. The OmpA signal-sequence (MKKTAIAIAVALAGFATVAQA) was placed upstream of the hexahistidine tag. Cleavage by signal peptidase would result in a polypeptide with an amino terminus of AHHHHHSSSS rather than (diacylglycerol)-CSSSS in the native protein (see Fig. 1). The synthesized sequence (Genscript) was cloned into pET30a (Novagen) using the NdeI and XhoI restriction sites, which were introduced at the 5' and 3' ends of the synthesized DNA, respectively. The resulting plasmid, called pET30a-*petJ*, replaces the multicloning site of pET30a with the synthetic H₆-*petJ* gene, putting it under control of the T7 promoter.

Strain construction and protein expression

The pET30a-*petJ* and pRGK333 (Feissner et al. 2006) plasmids were co-transformed into BL21(DE3) cells (Invitrogen)

and grown on Luria–Bertani (LB) plates supplemented with 100 mg L⁻¹ ampicillin (Sigma-Aldrich) and 75 mg L⁻¹ kanamycin (Fisher Scientific). A single colony was picked to inoculate a 50-mL starter culture. Cells were grown aerobically in LB media (Difco) containing 100 mg L⁻¹ ampicillin and 75 mg L⁻¹ kanamycin by shaking at 250 rpm at 37 °C. The overnight culture was diluted 1:100 into 4 L of LB the following morning. Cells were grown to an OD₆₀₀ of 0.5. At this point, δ -aminolevulinic acid (δ -ALA; Goldbio) was added to 1 mM to support heme synthesis, and isopropyl β -D-1-thiogalactopyranoside (IPTG) was added at 300 μ M to induce synthesis of both H₆-PetJ and the cytochrome maturation machinery. After 3 h, cells were harvested by centrifugation for 10 min at 5,000 \times g, and the cell pellet was stored at -20 °C.

Purification of cyt c_{553} and HbRC

Cells were thawed on ice and resuspended with 2 pellet volumes of wash buffer (50 mM sodium phosphate, 300 mM NaCl, pH 7.4) and sonicated with a Microson XL 2000 on ice for 5 rounds of 1 min at 50 % power with 30 s between each round. The cell lysate was centrifuged at 25,000 \times g for 30 min at 4 °C, and the supernatant was re-spun under the same conditions. The resulting supernatant was passed through a 0.22- μ m filter. Clarified extract from 4 L of culture was loaded onto a column containing 1.5 mL His60 Ni Superflow resin (Clontech), which was then washed with 20 volumes of wash buffer, followed by 20 volumes of wash buffer + 3 mM imidazole. The cytochrome was eluted with 10 column volumes of wash buffer + 300 mM imidazole. The red fraction was collected and concentrated with Viva-Spin20 5-kDa molecular weight cut-off centrifugal filters (Sartorius) followed by buffer exchange in the centrifugal filters with 50 mM MOPS (pH 7.0).

The HbRC from *H. modesticaldum* was purified as described (Sarrou et al. 2012). The native cytochrome c_{553} was collected as the flow-through fraction from the carboxymethyl-Sepharose ion-exchange column during purification of the HbRC from *H. modesticaldum*, followed by concentration in 5-kDa molecular weight cut-off centrifugal filters.

Mass spectrometry

Native and recombinant cytochrome c_{553} (both 200 μ m) were diluted 1:10 in saturated sinapinic acid. Mass spectra were recorded on an Applied Biosystems DE-STR MALDI-TOF operating on electron reflector mode at 25,000 V and a laser rep rate of 20 Hz.

UV–Vis spectroscopy

The UV–Vis reduced-minus-oxidized difference spectrum of recombinant cyt c_{553} was collected by subtracting the

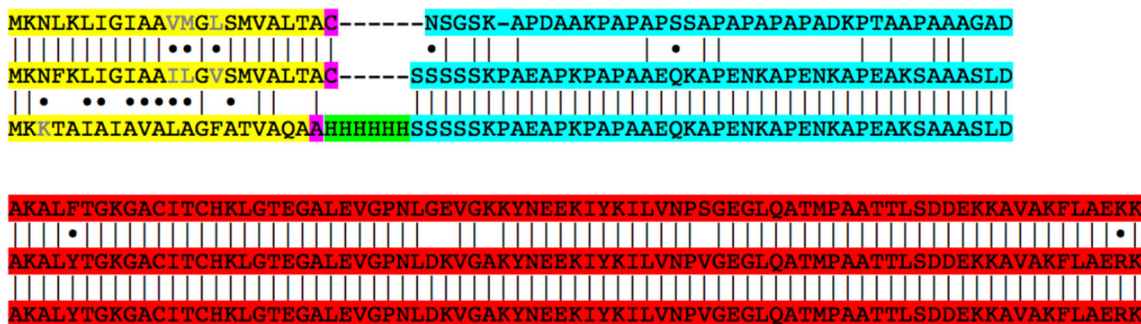


Fig. 1 Alignment of cyt *c*₅₅₃ from *H. gestii* (top sequence) and *H. modesticaldum* (middle sequence) along with the recombinant *H. modesticaldum* cyt *c*₅₅₃ (bottom sequence). Highlighting indicates the signal sequence (yellow), N-terminal residue of mature protein (magenta), linker region (cyan), and cytochrome domain (red). The

recombinant cyt *c*₅₅₃ uses the signal sequence of *OmpA* (yellow) and contains a hexahistidine tag (green). The linker domains are poorly conserved between *H. gestii* and *H. modesticaldum* beyond a propensity for Pro and Ala

spectrum of fully oxidized protein (purified and stored in air) from that of a fully reduced sample (after addition of excess sodium dithionite). Spectra were collected on a Perkin Elmer Lambda35 spectrometer.

Electrophoresis and staining for protein or heme

A 20 % BisTris-SDS–polyacrylamide gel was loaded with solubilized native and recombinant cyt *c*₅₅₃ samples corresponding to 125 ng of heme (calculated from spectra of reduced samples). The gels were run at 175 V and rinsed in de-ionized water for 5 min. For heme staining, the gel was soaked in 12.5 % trichloroacetic acid for 30 min, washed in de-ionized water for 30 min, and then soaked in a solution of 4 mM *o*-dianisidine, 10 % acetic acid, 10 % 0.5 M sodium citrate (pH 4.4), and 0.1 % hydrogen peroxide. After development of heme-containing polypeptides, the gel was washed in de-ionized water and photographed. For silver staining of polypeptides, the protocol of (Morrissey 1981) was used.

Redox titration of recombinant cyt *c*₅₅₃

Recombinant cytochrome *c*₅₅₃ at 200 μM in a buffer containing 50 mM MOPS (pH 7.0) and 150 mM NaCl was allowed to become fully reduced by exposure to an atmosphere of ~3 % H₂ in N₂ for several days at room temperature. The redox potential of the sample was monitored with a digital auto-range multi-meter (VWR) with flexible platinum and silver electrodes as the anode and cathode, respectively. The solution was slowly rendered more oxidizing by addition of small amounts of potassium ferricyanide while monitoring the potential and taking the spectrum after the potential had stabilized following each addition (The use of mediators led to technical difficulties with our voltage measurements. Extra care was taken with the

addition of potassium ferricyanide). The amount of reduced cytochrome *c*₅₅₃ was estimated by the absorption of the α-band at 553 nm (Each spectrum was fitted as a weighted sum of the spectra of fully oxidized and fully reduced cyt *c*₅₅₃ in order to calculate the reduced/oxidized ratio).

Transient absorption spectroscopy

Transient absorbance spectroscopy was performed with a JTS-10 transient spectrometer (Bio-Logic). Purified HbRC samples were diluted to 0.5 μM in a buffer containing 50 mM MOPS (pH 7.0), 5 mM MgSO₄, 10 mM ascorbate, 5 mM benzyl viologen (BV), and 0.03 % *n*-dodecyl β-D-maltoside, and were loaded into a sealed anaerobic cuvette (1-cm pathlength). A baseline was taken for 1.2 s before excitation and linear regression was used to subtract any baseline shift. A frequency-doubled Nd:YAG laser (Surlite Mini-Lite, Continuum) delivered a 6-ns saturating flash (20 mJ at 532 nm) to generate the P₈₀₀⁺ cation. The redox state of the special pair was probed with 10-μs LED flashes centered at 803 nm, commencing 350 μs after the laser flash. Placing a 532-nm notch filter and a 780-nm high-pass filter in front of both the reference and measurement detectors minimized effects of the actinic flash. Recombinant cyt *c*₅₅₃ was added up to 108 μM. For the temperature-dependence experiments, the cuvette was equilibrated at temperatures varying from 10–40 °C for 5 min before absorption traces were collected. For each transient experiment, the decay of P₈₀₀⁺ was fit to the sum of 3 exponential decay components, with the equation of $y = A_1^{(x/t1)} + A_2^{(x/t2)} + A_3^{(x/t3)}$.

EPR spectroscopy

Continuous wave electron paramagnetic resonance (EPR) spectra were recorded at the EPR facility of University of

Arizona. The magnetic field modulation frequency was 100 kHz with a field modulation of 2 mT peak-to-peak. The microwave power was 0.2 mW, the microwave frequency was 9.337 GHz, and the temperature was 6 K.

EPR spectra were simulated using EasySpin (v 4.5.0), a computational package developed by Stoll and Schweiger (2006) and based on Matlab (The MathWorks, Natick, MA, USA). The model used for the EPR simulations considered a single low-spin Fe^{3+} ion ($S = 1/2$). The fitting parameters were the g values (g_x , g_y , and g_z) and the line widths (ΔB_x , ΔB_y , and ΔB_z). The fitting procedure was similar to the one previously described by (Flores et al. 2007).

Results

Expression and purification of recombinant cyt c_{553}

The native cyt c_{553} from *H. gestii* was found to be attached to a diacylglycerol molecule via a thioether linkage to Cys23, which becomes the N-terminal residue after translocation and cleavage of the signal peptide (Albert et al. 1998). Mass spectroscopic analysis of the cyt c_{553} from *H. modesticaldum* was consistent with the same type of N-terminal modification (Sarrou et al. 2012). We replaced the native signal peptide and Cys23 with the signal peptide of OmpA followed by the sequence AHHHHHH. This would result in replacement of Cys23 with AH₆ after signal peptide cleavage. We did not expect this change to have a large influence upon the structure of the cyt c_{553} , beyond rendering it soluble, as the first ~40 residues of these polypeptides are not well conserved between *H. gestii* and *H. modesticaldum*, beyond a propensity for Pro, small (Ala and Ser) and charged (Lys, Asp, Glu) residues (Fig. 1). This is consistent with the primary role of this sequence being an unstructured link between the membrane integration site at the N-terminus and the C-terminal cytochrome domain.

The synthetic gene, codon-optimized for *E. coli*, was expressed using the T7 promoter in BL21(DE3) cells, in which T7 RNA polymerase can be induced by addition of IPTG. In order to maximize expression of the recombinant cyt c , we co-expressed the *ccmA-H* genes (cytochrome biogenesis system I); (Feissner et al. 2006). H₆-tagged cytochrome was purified from the clarified lysate by immobilized metal affinity chromatography (IMAC; see “Experimental procedures” section). Purified protein exhibited a 280/410 ratio of 1.5, and the calculated yield of the purification was 4.6 mg protein per liter, with an overall yield of 37 %.

UV–Vis spectroscopy

The spectrum of cyt c_{553} reduced by dithionite is characterized by the appearance of an α -band peaking at 553 nm and a

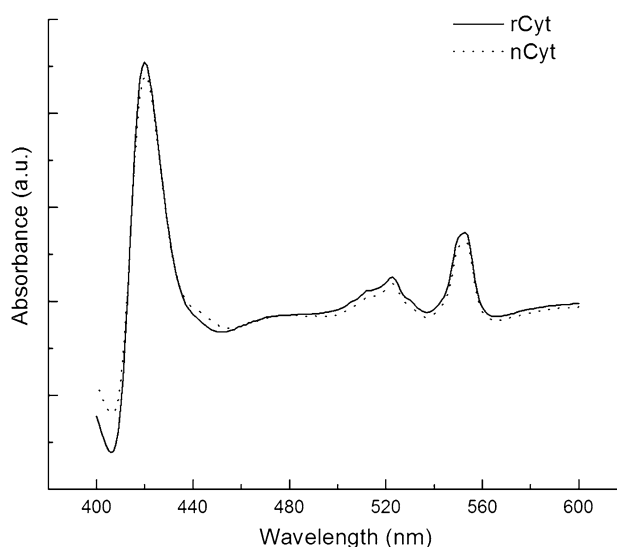


Fig. 2 UV-visible difference spectra. Reduced-minus-oxidized difference spectra of recombinant cyt c_{553} (solid) and native cyt c_{553} (dotted) at room temperature. Samples were diluted to 1 μM , based on heme concentration. Separate spectra were collected for reduced (excess dithionite) or oxidized (air purified) conditions, and the oxidized spectrum was subtracted from the reduced spectrum

broad β -band centered at 524 nm (Fig. 2). The Soret band peaks at 412 nm in the oxidized state and at 417 nm in the reduced state (Online Resource 1). These properties are identical to those of cyt c_{553} from *H. gestii* (Albert et al. 1998), and are typical for c -type cytochromes. The extinction coefficient of reduced protein at 417 nm was calculated to be $23.4 \text{ mM}^{-1} \text{ cm}^{-1}$ using the pyridine hemeochrome method essentially as described by (Berry and Trumpower 1987).

Electrophoretic and mass spectrometric analyses

Recombinant and native cytochromes were loaded on an SDS-PAGE gel on the basis of equal heme (as estimated by the spectrum of reduced protein). The native and recombinant proteins had apparent molecular masses of 13 and 14 kDa, respectively. The slightly higher mobility of the native protein is probably due to its slightly shorter sequence and binding of SDS to the attached lipid moiety. The gels were stained for protein (Fig. 3a) or for heme (Fig. 3b). Their equivalent staining intensity indicates that the heme is covalently attached in the recombinant cyt c_{553} with identical stoichiometry to that of the native protein (i.e., 1:1). The lack of other silver-staining bands in the recombinant protein preparation demonstrates that IMAC was sufficient to purify it to homogeneity.

The native cyt c_{553} prep contained 4 additional bands in the 30–42 kDa range, which are resolved poorly in this gel; none of them stain for heme (Fig. 3b), as seen before (Sarrou et al. 2012). The prep in *H. gestii* had a similar cohort of additional polypeptides, and it was suggested that

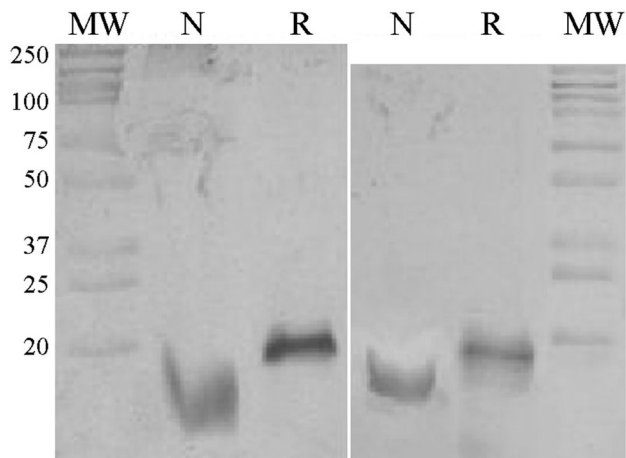


Fig. 3 SDS-PAGE analysis of cyt c_{553} from *H. modesticaldum*. Equal amounts (equivalent to 125 ng of heme) of native cytochrome c_{553} (left lane; “N”) and recombinant cyt c_{553} (right lane; “R”) were run on 20 % SDS-PAGE gels. Gels were subsequently silver-stained (a) or stained for heme (b)

these formed a complex with the cytochrome (Albert et al. 1998). However, mass spectrometric analysis of these polypeptides from *H. modesticaldum* revealed them to be ABC transporters (Sarrou et al. 2012). Thus, they are unlikely to be involved with the function of cyt c_{553} , despite their tendency to co-purify with it.

Mass spectrometric analysis of the recombinant cyt c_{553} was consistent with covalent attachment of heme to the polypeptide. We obtained a mass of $14,041 \pm 14$ Da, compared to an expected mass of 14,023 Da (Online Resource 2).

EPR spectroscopy of native and recombinant cyt c_{553}

The oxidized native cyt c_{553} exhibited a low-temperature EPR spectrum typical of a low-spin cytochrome (Fig. 4), with g -values of 3.05, 2.235, and 1.36 (Table 1). Reduction by ascorbate eliminated this spectrum entirely. These g -values are very similar to those reported for the g_x and g_y values (3.048 and 2.238, respectively) for the cytochrome from *H. gestii* by (Albert et al. 1998), although they were unable to observe the low-field trough shown in this work. The recombinant protein behaved identically, although the g -tensor was slightly less anisotropic, with g -values of 2.97, 2.285, and 1.435 (Fig. 4; Table 1).

Redox titration of recombinant cyt c_{553}

A potentiometric titration was performed to determine the midpoint potential (E_m) of the recombinant cyt c_{553} . The spectrum of the cytochrome solution was measured as the potential was varied. The potential of the sample was lowered by addition of small amounts of potassium

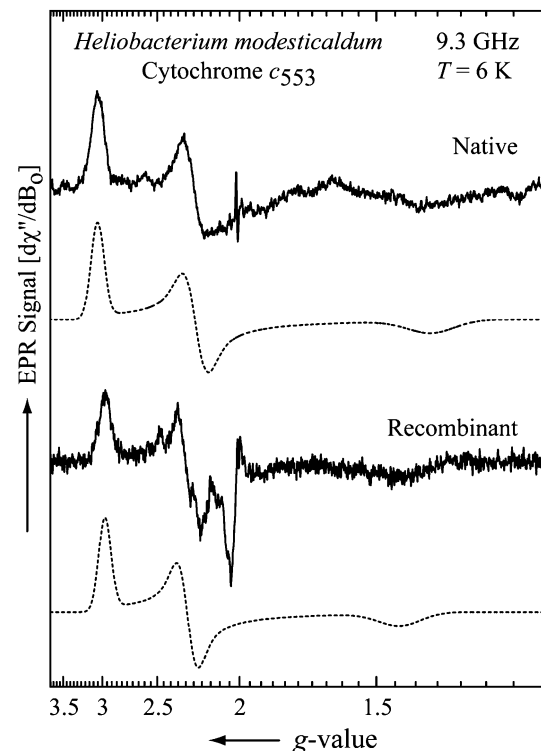


Fig. 4 EPR spectra of native and recombinant cyt c_{553} . Oxidized-minus-reduced difference cw-EPR spectra of recombinant cytochrome (bottom) and native cytochrome (top) at 6 K. Microwave frequency and power were 9.337 GHz and 0.2 mW, respectively; field modulation frequency and amplitude were 100 kHz and 2 mT, respectively. Simulations are shown below each spectrum as dotted lines. (See text for details; parameters of simulations are in Table 1)

Table 1 Summary of parameters used in the simulations of X-band EPR spectra of cytochrome c_{553} from *H. modesticaldum*

Parameters ^a	Source of cytochrome c_{553}	
	Native	Recombinant
g_z	3.050	2.970
g_y	2.235	2.285
g_x	1.360	1.435
ΔB_z (MHz)	530	490
ΔB_y (MHz)	600	510
ΔB_x (MHz)	850	800

^a The simulation parameters were the following: the three g -values, g_x , g_y , and g_z , and the three line widths, ΔB_x , ΔB_y , and ΔB_z

ferricyanide and was monitored with an electrode. After the potential had stabilized, the spectrum was taken. Spectra were fit as a weighted sum of the spectra of the fully oxidized and fully reduced protein, to estimate the fraction of reduced cytochrome. When fit to the Nernst equation, the titration plot (Fig. 5) was consistent with a single titrating species with $n = 1$ (single electron oxidation/reduction) and $E_m = +217 \pm 0.6$ mV versus NHE. This is

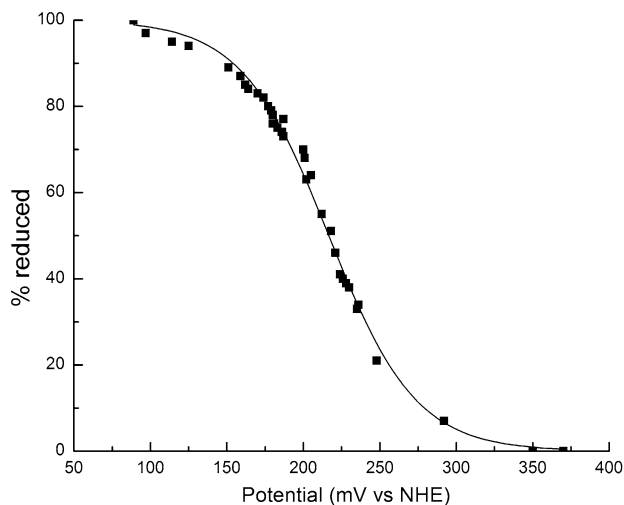


Fig. 5 Potentiometric titration of recombinant cyt *c*₅₅₃. Squares are the measured data, and the line represents the best fit to the Nernst equation with no constraints, which yielded $E_m = +217 \pm 0.6$ mV and $n = 0.91$

very similar to the value obtained for the native cyt *c*₅₅₃ from *H. gestii* (Albert et al. 1998).

Reduction of purified HbRC by recombinant cyt *c*₅₅₃

Transient spectroscopy was used to test the ability of the soluble recombinant cyt *c*₅₅₃ to function as an electron donor to the HbRC ($P_{800}^+ + c_{553}^{red} \rightarrow P_{800} + c_{553}^{ox}$). Mixtures containing the HbRC and cyt *c*₅₅₃ with ascorbate as electron donor and BV as electron acceptor were loaded anoxically into sealed cuvettes, and the oxidation state of P_{800} was monitored with 10- μ s flashes at 803 nm. The HbRC was excited with a saturating laser flash at 532 nm, which resulted in instantaneous bleaching of P_{800} (within the timescale of the measurement) as P_{800} was photooxidized, followed by decay of the bleaching as P_{800}^+ was reduced (Fig. 6a). The decay of P_{800}^+ was fit to the sum of 3 exponential decay components. The fastest component (~ 15 ms) representing ~ 10 % of the decay is assigned to charge recombination of the $P_{800}^+F_X^-$ state (Heinzel and Golbeck 2007; Sarrou et al. 2012), and is attributed to HbRCs from which the electron does not escape to BV. The slowest component (>3 s) is assigned to the slow reduction of P_{800}^+ by ascorbate.

After addition of cyt *c*₅₅₃, a component of intermediate rate appeared, which can be attributed to P_{800}^+ reduction by cyt *c*₅₅₃. As the concentration of cyt *c*₅₅₃ was increased, this component increased in amplitude at the expense of the slow component; it represented ~ 75 % of the decay at the highest concentrations tested. Addition of a fourth component did not increase the quality of the fit (data not shown). The calculated rate of reduction of P_{800}^+ by cyt *c*₅₅₃ is plotted

against cyt *c*₅₅₃ concentration in Fig. 6b. Based on this, we estimate the second order rate for this reaction ($P_{800}^+ + c_{553}^{red} \rightarrow P_{800} + c_{553}^{ox}$) to be 1.53×10^4 M⁻¹ s⁻¹ at 22 °C.

In membranes of *H. gestii*, the rate of P_{800}^+ reduction by the native *c*₅₅₃ was strongly dependent upon temperature and Mg²⁺ concentration (Oh-Oka et al. 2002a). We wished to see if these were characteristic of the intrinsic HBRC:cyt *c* interaction or of the attachment of cyt *c*₅₅₃ to the membrane. The temperature dependence of P_{800}^+ reduction by *c*₅₅₃ was assessed by varying the temperature from 10 to 40 °C and measuring the kinetics of P_{800}^+ reduction as before. The reduction of P_{800}^+ by cyt *c*₅₅₃ was found to be highly temperature dependent, with a 40-fold increase in rate over the 30 °C span of measurement. The apparent activation energy calculated by an Arrhenius plot was 91 kJ mol⁻¹ (Fig. 7). This is very similar to what was seen in membranes from *H. gestii* (Oh-Oka et al. 2002a). In contrast, however, the rate of P_{800}^+ reduction by the soluble recombinant *c*₅₅₃ was invariant over a Mg²⁺ concentration ranging from 5 μ M to 50 mM (data not shown).

Discussion

Our analysis of the native cyt *c*₅₅₃ from *H. modesticaldum* revealed no differences between it and the protein from *H. gestii* in terms of heme attachment, UV-Vis spectrum, or EPR spectrum (Albert et al. 1998), confirming that the orthologs from these two species are very similar. One would expect this, given the high sequence conservation in their cytochrome domains (74 out of the 80 C-terminal residues; see Fig. 1). Based on its visible and EPR spectra, reduction potential, and other properties, the recombinant cyt *c*₅₅₃ is almost identical to the native proteins of *H. modesticaldum* and *H. gestii*. This confirms our hypothesis that the N-terminus would be a good place to insert the hexahistidine tag without altering the properties of the cytochrome.

We observed very few differences between the native and recombinant cyt *c*₅₅₃, beyond the slower rate of P_{800}^+ reduction by the recombinant protein, which was almost two orders of magnitude slower than the native cyt *c*₅₅₃ in membranes (Oh-Oka et al. 2002a). We attribute the higher rate to the incorporation of native cyt *c*₅₅₃ in membranes, and this is unsurprising, as the effective concentration of cyt *c*₅₅₃ in membranes would be much higher than what we can obtain with the recombinant protein in solution; in addition, it may be constrained to interact with the RC in ways that tend to be more productive for electron transfer between them. Consistent with this, we found that the solubilized native protein was also very slow at reducing the purified HbRC, although we were unable to test it at as high a concentration as we did with the recombinant protein (data not

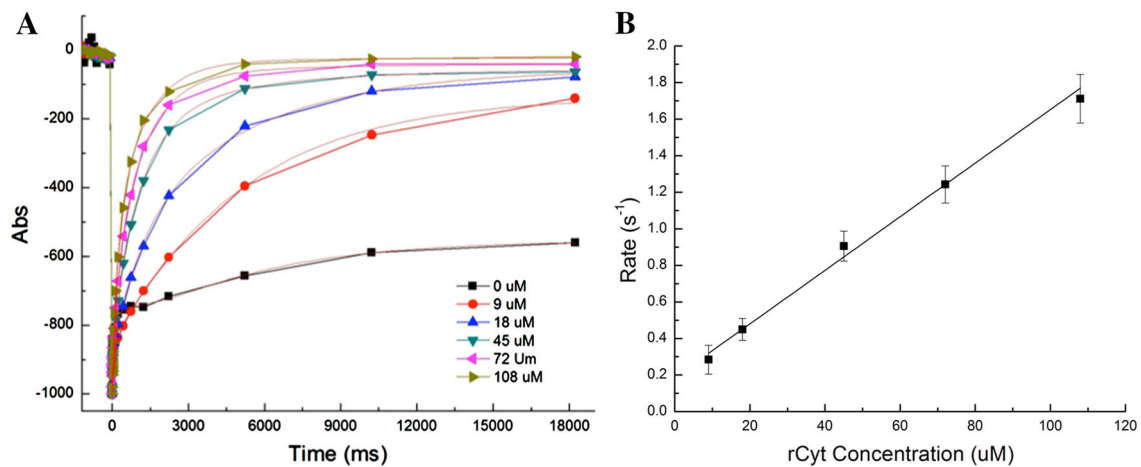


Fig. 6 Use of recombinant cyt c_{553} as electron donor to P_{800}^+ . HbRCs (at $0.5 \mu\text{M}$) were given a saturating 532-nm laser flash (30 mJ, 6-ns duration), and absorption was monitored at 803 nm using 10- μs flashes from an LED. Ascorbate (10 mM) was used as a sacrificial electron donor, and BV (5 mM) was used as an artificial

electron acceptor. *Panel A* shows the raw data of P_{800} photobleaching and recovery at each cyt c_{553} concentration, along with the three-component fitting (see text for details). In *panel B*, the calculated rate of P_{800}^+ reduction by cyt c_{553} is plotted vs cyt c_{553} concentration

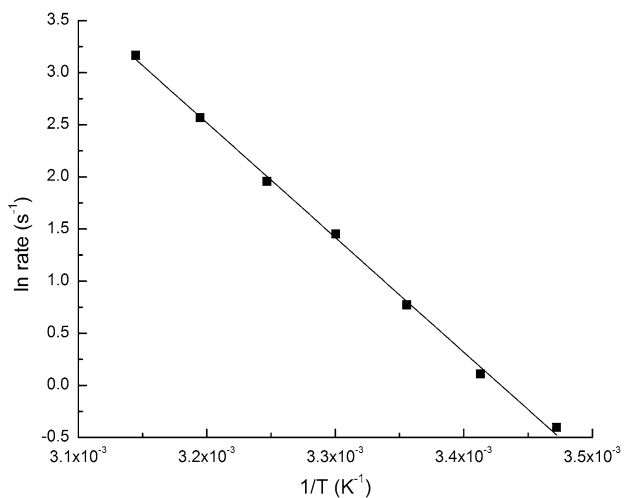


Fig. 7 Arrhenius plot of reduction of P_{800}^+ by recombinant cyt c_{553} . The anaerobic cuvette was equilibrated at temperatures varying from 10 to 40 °C, and the reduction of P_{800}^+ by cyt c_{553} was followed as in Fig. 6. The calculated E_a was 91.4 kJ mol^{-1}

shown). Oh-Oka et al. (2002a) observed an approximate fourfold increase in the P_{800}^+ reduction rate upon addition of MgCl_2 to membranes in the range of 0.3–3 mM. This effect of divalent cations on electron donation rate is somewhat similar to what had been seen with the interaction of plastocyanin and P_{700}^+ (Tamura et al. 1981). However, we saw no difference in the rate of electron donation from soluble cyt c_{553} to P_{800}^+ in the range of 0.005–50 mM MgCl_2 using the soluble version of cyt c_{553} (data not shown). Therefore, the dependence of this rate upon divalent cations must be an effect upon membranes and unrelated to the interface between cyt c_{553} and the HbRC.

The cyt c_{553} of *H. modesticaldum* seems to fall in line with other small *c*-type cytochromes of similar function. *Rhodobacter sphaeroides*, *Chlorobaculum tepidum*, and *H. gestii* all contain *c*-type cytochromes that donate to their respective RCs. These cytochromes all have low-spin hemes (Drepper and Mathis 1997; Azai et al. 2010; Albert et al. 1998) and share similar spectral characteristics, including an asymmetric α -band, and a slightly red-shifted Soret band when reduced (Oh-oka et al. 1998; Albert et al. 1998; Dutton et al. 1975); Fig. 2). They also have similar midpoint potentials, all being within the range of +170 to +220 mV. Thus, the cyt c_{553} of *H. modesticaldum* seems very much like the other heliobacterial *c*-type cytochromes, which are themselves fairly typical members of this family. Furthermore, the EPR parameters obtained in this work for both native and recombinant cyt c_{553} of *H. modesticaldum* suggest that they belong to the family of low-spin ferriheme proteins containing two histidines as axial ligands (Walker et al. 1984). The small differences in the observed *g*-values (Table 1) likely reflect slightly different orientations of such ligands.

The interaction of cyt c_{553} with the HbRC of *H. modesticaldum* is very similar to that seen in other type-I photosynthetic RC systems. Interestingly, the cytochromes in *Chlorobium* and *Heliobacteria* have midpoints that give a relatively small gap in potential when compared to the special pair of the corresponding organism. The gap in *C. tepidum* may be as small as 45 mV (Okumura et al. 1994), while it is 55 and 50 mV in *H. chlorum* (Prince et al. 1985) and *H. mobilis* (Nitschke et al. 1995), respectively. Given the highly conserved nature of the photosynthetic proteins within *Heliobacteria*, it is likely that the gaps in potential are similar

for *H. modesticaldum* and *H. gestii*. Even the donor proteins for photosystem I, plastocyanin and cyt c_6 , typically have a gap of less than 100 mV with respect to the special pair (Nakamura et al. 2011). These ranges likely indicate the minimum driving force for an electron transfer rate that does not limit overall electron flow, while optimizing energy conservation. The activation energies of electron transfer from the c -type cytochrome to its native RC are also similar, with $E_a = 88 \text{ kJ mol}^{-1}$ for *H. gestii* (Oh-Oka et al. 2002a) and $E_a = 101 \text{ kJ mol}^{-1}$ for *C. tepidum* (Oh-oka et al. 1997) in their native context. We determined an activation energy of 91 kJ mol^{-1} when using the soluble version of *H. modesticaldum* c_{553} (Fig. 7).

The theoretical isoelectric points were calculated from the functional portion of the mature protein (the signal sequence and transmembrane helices were omitted to approximate the functional portion of the protein). In addition, the cysteine residues involved in heme binding were replaced in the calculations by glutamic acids to more appropriately simulate the propionates on the heme. The fully matured recombinant cyt c_{553} has a calculated pI of 7.8. The cyt c_{553} proteins from different heliobacteria are: *H. gestii* (7.1), *H. modesticaldum* (native) (7.3), and *H. mobilis* (5.9). The soluble cytochrome from *R. sphaeroides* has a calculated pI of 6.1, while the cytochrome of the green sulfur bacterium *C. tepidum* (pI = 9.4) has a somewhat more basic isoelectric point, perhaps indicating a larger dependence on electrostatic interactions for optimal cooperation with the acceptor. In both the chlorobial and purple bacterial systems, the cytochrome is pre-bound to the RC, while in the heliobacterial system the cytochrome seems to depend on a collisional mode for electron transfer.

The rate of electron transfer from cyt c_{553} to P_{800}^+ is $\sim 3 \text{ ms}$ in membranes from *H. gestii* (Oh-Oka et al. 2002a). This is much slower than that seen with *R. sphaeroides* (20–40 μs) (Dutton et al. 1975) or *C. tepidum* (110 μs) (Oh-oka et al. 1995). Although the rate is increased when the reaction is measured in whole cells of *H. gestii* (0.3 ms) (Oh-oka et al. 2002b) or *H. modesticaldum* (0.5 ms) (Redding et al. 2013), the order of magnitude difference is maintained when compared to the electron transfer kinetics of *C. tepidum* (7 μs) (Hauska et al. 2001), and *R. sphaeroides* (1 μs) (Wang et al. 1994). The lack of membrane tether in the recombinant cytochrome is likely the dominant factor influencing the disparity in electron transfer rate when compared to the native cytochrome. Water-soluble cytochromes likely have a stronger dependence on electrostatic interactions to orient correctly from a longer range, as they are faster to associate and slower to dissociate. This was strikingly demonstrated in a study comparing the ability of the soluble cyt c_2 , membrane-attached cyt c_y , and a mutant of cyt c_y in which the membrane tether was removed, to support respiratory

or photosynthetic electron transfer in *Rhodobacter capsulatus* (Ozturk et al. 2008). Similar to our in vitro results here, removal of the membrane-spanning helix from cyt c_y resulted in slowed electron transfer to the RC in cells, likely due to a drop in the rate of binding. When tethered to the membrane, cyt c_{553} is limited to 2-dimensional movement and does not require long-range electrostatic interactions to bind the HbRC, which allows it to facilitate electron throughput despite the lack of a tight-binding interaction.

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