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Rapid report

Rapid isolation of bacterial photosynthetic reaction centers with an engineered poly-histidine tag

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Abstract

A very rapid method for isolating bacterial photosynthetic reaction centers (RCs) of high purity and yield is described. A poly-histidine tag is engineered at the C-terminus of the RC M-subunit, allowing recovery of pure RCs in less than 4 h with a commercially available Ni^{+2} resin. The phenotype of the protein including absorption spectra and electron transfer kinetics are virtually identical to wild-type RCs. This method should facilitate studies of RCs by dramatically decreasing sample preparation time and eliminating the use of expensive equipment.

Keywords: Photosynthetic reaction center; Poly-histidine tag; Isolation method; (Bacterium)

The isolation and purification of transmembrane and membrane-associated proteins from the photosynthetic membranes of bacteria and higher plants is a time-consuming, labor- and equipment-intensive process. In particular, isolation of photosynthetic reaction centers (RCs) from bacteria by standard procedures [1–3] involves many steps which include cell lysis, step-wise membrane solubilization, ammonium sulfate precipitation, sucrose gradient density centrifugation, and anion-exchange chromatography including FPLC. In an attempt to simplify this procedure, a recent report [4] describes the isolation of several photosynthetic membrane proteins including RCs by separating the components of a detergent-solubilized cell lysate with perfusion chromatography [5]. Cu^{+2} affinity chromatography has also been utilized in this manner to isolate PSII RCs [6,7].

In the present communication we describe an altogether different strategy for isolating bacterial RCs based on the high affinity interaction between a genetically engineered poly-histidine (poly-His) tag and a commercially available Ni-NTA (Nitrilo-Tri-Acetic acid) resin. This method [8] has been utilized to rapidly and efficiently purify poly-His tagged globular proteins and, to a lesser extent, transmembrane proteins. The high affinity of Ni-NTA resin for poly-His tagged proteins ($K_d \cong 10^{-13}$, pH 8.0) allows one to simply add a cell lysate to the resin, wash the resin to remove the unwanted cellular contents, and elute the poly-His tagged protein of interest by displacing it from the resin with an imidazole-containing buffer. For the RC, a poly-His tag is introduced at the C-terminus of the RC M-polypeptide, and this permits isolation of pure poly-His RCs from cells in less than 4 h without the need for sophisticated equipment. The resulting RCs are shown to be indistinguishable in most respects from wild-type (WT) both in vivo and in vitro.

A poly-histidine tag was engineered at the carboxy-terminus of the RC M-subunit with an oligonucleotide-directed, insertion mutagenesis approach. The *puf*M gene was cloned from the original M13 clone into the phagemid pBluescript II KS(−) (Stratagene). Single-stranded DNA was isolated from XL-Blue MRF′ cells (Stratagene) harboring the phagemid and used as a template to anneal a 62 bp DNA oligonucleotide primer. The primer, with flanking complementary ends of 15 bp, contained an insertion of seven His codons (CAC) (5′ of the stop codon of *puf*M), and a T/C mismatch at the first base of the codon for Ala M304, changing this residue to Ser. Alanine at M304 is found on the surface of the protein, and Ala → Ser at this position removes a unique *Pfl*mI restriction site from *puf*M so that the phagemid population can be enriched for the poly-His insertion by linearizing WT background with a *Pfl*mI digest and screened for the loss of the *Pfl*mI site.

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The codons used for His and Ser (TCG) are found with a high frequency in the *Rb. sphaeroides* genome. After annealing the primer to the template, extension and ligation were carried out with native T7 DNA Polymerase (New England Biolabs) and T4 DNA Ligase (Boehringer Mannheim) in the presence of single-stranded DNA binding protein (New England Biolabs) and dNTPs, using protocols provided by the suppliers. In order to propagate the mutant strand, the double-stranded construct was transformed into chemically competent BMH 71-18 *mutS* cells (Clonetech) which are DNA mismatch repair-deficient. Phagemid ds-DNA was isolated from an overnight culture of transformed *mutS* cells and was transformed into chemically competent XL-Blue MRF′ cells after linearizing the wild-type background with a *Pfl*mI restriction digest. Colonies were picked and phagemids screened for the loss of the *Pfl*mI site. A $> 90\%$ mutation yield was obtained. To confirm the presence of the poly-His sequence, the *pufM* gene was sequenced by standard automated dideoxy methods. The poly-His *pufM* gene was cloned into the *puf* operon, pRKSCH, which contains a His → Glu mutation at residue 32 of *pufA* and the construct, named pRKSCH/pHisRC, was shuttled into the antenna-containing RC deletion strain of *Rb. sphaeroides*, ΔLM 1.1, via conjugation. This new strain, SMpHis, will be made available to the scientific community. M13 clones, pRKSCH, ΔLM 1.1, and protocols/maps were generously provided by Dr. JoAnn Williams [9,10].

For RC expression, *Rb. sphaeroides* was grown both semi-aerobically in the dark and anaerobically in the light under photosynthetic pressure. For bacteria grown in the dark, 4.5 liters of YCC media were shaken at ~ 225 rpm in 6.0 liter flasks for 6 days without control of the oxygen tension. Growth in the light was carried out in parafilm-sealed bottles filled with Gunars media which were exposed to two 150-Watt light bulbs for 6 days. Ni-NTA resin, composed of a high surface concentration of the Ni-NTA attached to Sepharose CL-6B, was obtained from Qiagen. FPLC columns contained DEAE Toyopearl 650-S. The following buffers were used: buffer 1 was composed of 10 mM Tris (pH 8.0), 100 mM NaCl; buffer 2 was composed of 10 mM Tris (pH 8.0), 0.1% LDAO (*N,N*-dimethyl-dodecylamine *N*-oxide); buffer 3 was composed of 10 mM Tris (pH 8.0), 0.1% LDAO, 40 mM imidazole. $P^+Q_A^-$ recombination kinetics were measured as described previously [11]. The detector-limited time response of the apparatus was ~ 1 ms. Spontaneous fluorescence decays were measured in Q_A-reduced RCs in Triton X-100 at 85K as described previously [12,13]. Horse heart cytochrome *c* (Type VI, Sigma) was purified with Sephadex G-50 following reduction with sodium dithionite.

Poly-His RCs of high purity and yield are obtained easily from *Rb. sphaeroides* cells grown both semi-aerobically in the dark and anaerobically in the light with the Ni-NTA resin in a one half-day procedure that eliminates most of the tedious labor and equipment-intensive steps found in previous RC purification protocols. The procedure is outlined below.

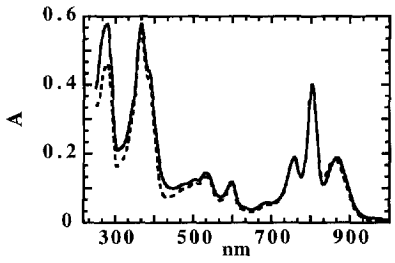

Fig. 1. Absorption spectra of poly-His RCs after elution with buffer 3 on Ni-NTA resin (solid line) and after further purification on DEAE with an FPLC (dotted line) as described in the text. Spectra were recorded at room temperature in buffer 2. The $OD_{280/804}$ of the solid line is 1.45 and that of the dotted line is 1.15.

Rb. sphaeroides cells are centrifuged at $\sim 10000 \times g$, suspended in buffer 1, centrifuged again, and resuspended in buffer 1. The cells are lysed with a French Press in the presence of DNAse and Mg^{+2} and centrifuged at $\sim 10000 \times g$ for 30 min to pellet lysed cell debris and unlysed cells [1]. The chromatophore-containing supernatant is diluted so that the $OD_{850nm,\ 1cm} \leq 50$. LDAO (0.5%), imidazole (5 mM) and buffer 2 equilibrated Ni-NTA resin are added to the supernatant, and this is stirred in the dark at 4°C for 30 min [2]. The slurry is poured into an empty column and washed with buffer 2 until absorption spectra reveal that the elutant is clear of pigment (primarily antenna, free bacteriochlorophyll, and carotenoid) and other proteins. Poly-His RCs are eluted with buffer 3. An absorption spectrum of the eluted poly-His RCs is shown in the solid line of Fig. 1. The ratio of the optical density measured at 280 nm divided by that measured at 804 nm ($OD_{280/804}$) is a good overall measure of the purity of RCs [1]. The $OD_{280/804}$ of poly-His RCs after the Ni-NTA resin elution is found to vary between 1.3–1.5. Stirring the solubilized cell lysate and Ni-NTA resin without imidazole yields RCs of considerably lower purity with an $OD_{280/804}$ of approx. 1.7–1.8, implying that the low concentration of imidazole reduces non-specific protein binding to the Ni-NTA resin. In the presence of 5 mM imidazole, the capacity of the Ni-NTA resin is approx. 8–10 ODV or 2.4–3.0 mg RC protein/ml dry resin, comparable to Qiagen's specifications for the Ni-NTA resin (5–10 mg/ml) [3].

[1] It may be possible to eliminate the French press step by lysing the cells using detergent or enzymes. For example, it was found that stirring the cells in the presence of 1% LDAO also lysed the cells, but the yield of isolated RCs was four-fold less.

[2] The supernatant is stirred with the Ni-NTA resin as opposed to pouring the LDAO-solubilized lysate onto a resin-filled column because it was determined, using purified poly-His RCs, that RCs bind to the Ni-NTA resin slowly. Thus the flow rate would have to be very slow in order to quantitatively bind RCs which is not practical. This procedure can also readily be carried out in parallel on separate samples.

[3] ODV, a measure of an amount of RCs, is the OD804 nm, 1 cm × milliliters.

WT RCs, purified by standard protocols, do not bind to the Ni-NTA resin. A final FPLC step is employed to simultaneously remove imidazole from the buffer (which can also be done by dialysis) and improve the purity of the RCs. An absorption spectrum of the FPLC purified RCs is shown in the dotted line of Fig. 1. It is evident that this final purification step removes unpigmented proteins and does not alter the near infrared spectrum of the RC. After FPLC, the $OD_{280/804}$ of the poly-His RCs is found between 1.1 and 1.3, within an acceptable range for further use. The total yield of purified poly-His RCs obtained is ~ 1–2 ODV/gram of cells (wet weight), as good and often better than yields obtained by standard RC purification procedures [1–3].

The phenotype of the poly-His RC is identical to the WT RC, indicating that the poly-His tag and the Ser → Ala mutation at M304 are not deleterious to the structure or function of the RC. Absorption spectra were recorded of poly-His RCs, isolated from semi-aerobically grown cells, at room temperature in buffer 2 and at 77K in 1:1 (v/v) glycerol/buffer 2. The spectra in the Q_Y region are identical to those of WT RCs (data not shown). The Q_X and carotenoid region of the absorption spectrum is compared with that of WT in Fig. 2 and reveals small differences in the region assigned to the carotenoid. The carotenoid composition of WT RCs isolated from semi-aerobically grown *Rb. sphaeroides* cells is heterogeneous, containing both spheroidenone and spheroidene in a ratio of approx. 3.5:1 [14]. This region of the spectrum is a linear combination of the spectra of the bound carotenoids weighted by their percent composition [15]. The small differences in this region of the spectra between poly-His and WT RCs observed in Fig. 2 are consistent with a lower ratio of spheroidenone/spheroidene for the poly-His RC compared to WT. The extent of this difference was found to vary for different semi-aerobically grown cultures. It is well known that the carotenoid composition of *Rb. sphaeroides* is regulated by the oxygen tension of the growth medium [14], and this was not controlled during cell growth, so the variations in the carotenoid composition of the RC may be due to differences in oxygen tension during cell growth. Interestingly, these variations have not been observed for WT RCs isolated by standard procedures.

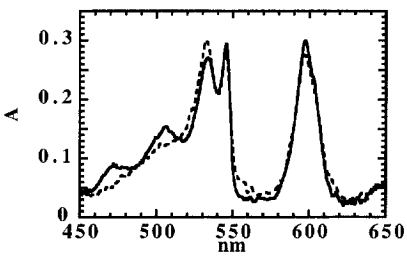

Fig. 2. Absorption spectra of poly-His (solid line) and WT (dotted line) RCs in the Q_X and carotenoid region of the spectra. Spectra were recorded at 77K in 1:1 glycerol/Buffer 2 and normalized to the Q_Y peak at 802 nm.

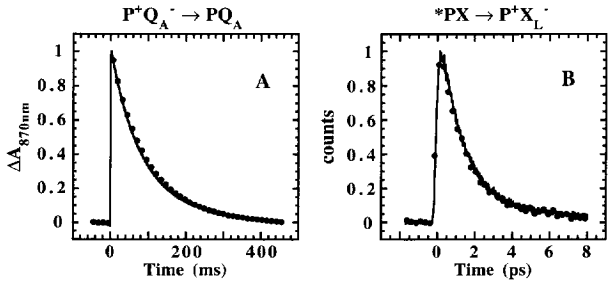

Fig. 3. Electron transfer kinetics of poly-His (dotted line) and WT RCs (solid line). (A) $P^+Q_A^-$ recombination data on the millisecond time scale at room temperature; (B) $^*P$ spontaneous fluorescence decays on the picosecond time scale at 85K. Data were collected as described in the text and, in both cases, normalized to the peak of the signal.

The $P^+Q_A^-$ recombination yield and millisecond kinetics of poly-His RCs were measured with a sub-saturating actinic flash at room temperature in buffer 2 with 1 mM 1,10 o-phenanthroline. The data are compared with WT in Fig. 3A and found to be virtually identical [4]. Because the poly-His tag was engineered at the carboxy-terminus of the M-subunit and this is in proximity to the cytochrome *c* (Cyt *c*) docking site of the RC (see below), the ability of Cyt *c* to reduce P^+ was qualitatively measured. The kinetics of the Cyt *c* reduction of P^+ have been well studied (see review in Ref. [16]) and found to occur on the order of μs, three orders of magnitude faster than the time resolution of the apparatus used here to measure $P^+Q_A^-$ kinetics. Therefore, Cyt *c* reduction of P^+ is detected as a decrease in the $P^+Q_A^-$ recombination yield after a single actinic flash. The $P^+Q_A^-$ recombination yields of both poly-His and WT RCs were measured with a single actinic flash at room temperature in buffer 2, before and after the addition of a 5-fold molar excess of Cyt *c* dissolved in 10 mM Tris at pH 8.0. The amplitude of the signal was found to decrease to zero upon addition of the Cyt *c* (data not shown). This was the minimum amount of Cyt *c* needed for a complete reduction of the yield for both poly-His and WT RCs. This verifies that the poly-His tag, to a first approximation, does not interfere with Cyt *c* binding. Spontaneous fluorescence decays on the picosecond timescale were measured for poly-His and WT RCs at 85K (excitation at 800 nm, emission detected at 920 nm) to verify that the initial electron transfer rate $^*PX \rightarrow P^+X_L^-$ is not effected by the mutations (X_L is the initial electron acceptor, either B_L or H_L). The decays are overlaid in Fig. 3B and found to be identical.

The *Rb. sphaeroides* deletion strain, ΔLM 1.1, complemented with the poly-His RC containing *puf* operon,

[4] In the absence of 1,10 o-phenanthroline, poly-His RCs contain a similar fraction of Q_B as WT RCs isolated with standard procedures. Poly-His RCs containing nearly 100% Q_B were obtained by adding a molar excess of exogenous Q_{10}; poly-His RCs were successfully Q_A depleted by standard procedures. Detergent exchange can be readily performed while the poly-His RCs are bound to the Ni-NTA resin.

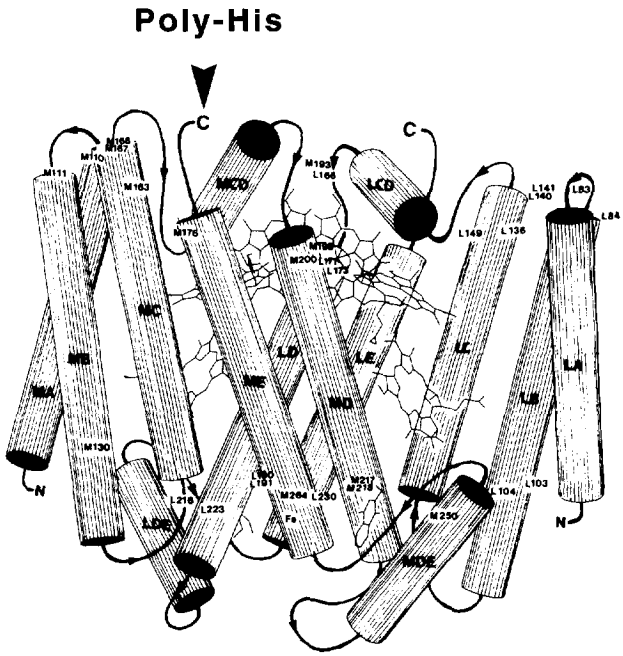

Fig. 4. Schematic diagram of the L- and M-subunits of *Rp. viridis* RCs adapted from Fig. 13 of [17]. The arrow shows the approximate position of the engineered poly-His tag at the carboxy-terminus of the M subunit in the RC from *Rb. sphaeroides*.

pRKSCH/pHisRC, was photosynthetically viable and expressed poly-His RCs that were purified with the same procedure outlined above. Their properties were identical to those described above, except that the carotenoid was almost entirely spheroidene, as expected.

A schematic diagram of the RC from *Rp. viridis* adapted from Fig. 13 of [17], is shown in Fig. 4, with an arrow pointed at the carboxy-terminus of the M-subunit, the position where the poly-His was added. The M-subunit carboxy-terminus of the RC from *Rb. sphaeroides* is exposed on the surface of the protein and apparently is not involved in major tertiary or quaternary structural interactions with other residues [18]. Indeed, the last five residues of this terminus, residues M303-307, are not found in the crystallographic coordinates of the RC from *Rb. sphaeroides* [18]. The electron density for these residues is ill-defined, indicating that they are disordered in the crystal and presumably in solution. Estimates of the location of the membrane-spanning region of the RC [19] suggest that the carboxy-terminus of the M-subunit is situated in the periplasmic space, free from interaction with the lipid bilayer. Interestingly, the M-subunit carboxy-terminus of the RC from *Rp. viridis* is 18 amino acids longer [20] (not shown in Fig. 4) than that of the RC from *Rb. sphaeroides*, [21] and the crystal structure of the RC from *Rp. viridis* [22] reveals that this carboxy-terminal peptide is approximately normal to the plane of the membrane and interacts with the bound cytochrome. It has been suggested that this interaction is involved in tethering the cytochrome to the RC from *Rp. viridis* [23].

All measured observables for the poly-His RC including absorption spectra at room temperature and 77K, $P^+Q_A^-$ recombination rate and yield, and the rate of $^*PX \rightarrow P^+X_L^-$ are identical to WT, indicating that the poly-His insertion and Ser → Ala mutation at M304 have no detrimental effect on the structure and function of the RC. Furthermore, *Rb. sphaeroides* containing poly-His RCs is photosynthetically viable revealing that these perturbations do not disrupt interactions with other proteins involved in photosynthesis such as Cyt *c*, which was also found to reduce P^+ in vitro in the isolated poly-His RCs with the same efficiency as WT RCs [5]. Interestingly a recent co-crystallization and structural characterization of a *Rb. sphaeroides* RC-Cyt c_2 complex reveals that the Cyt binding site is situated in closer proximity to the carboxy-terminus of the L-subunit [24], which is consistent with our observation that the poly-His tail added to the carboxy-terminus of the M-subunit does not interfere with Cyt *c* reduction. The only difference observed for the poly-His RCs is the small difference in carotenoid composition (Fig. 2). It is not clear whether these differences are due to differences in oxygen tension during cell growth, an artifact of the new isolation procedure, a consequence of the poly-His addition and/or the Ser → Ala mutation at M304 (both of which are close to the carotenoid binding site), or reflect the *actual* native carotenoid composition of the RC (this new isolation procedure is much faster and less stringent).

In conclusion, a rapid method is described for purifying RCs of high yield and purity by engineering a poly-His tag on the carboxy-terminus of the M-subunit and utilizing a commercially available Ni-NTA resin. The phenotype of these poly-His RCs is normal, and therefore this method should facilitate studies of the structure and function of RCs by dramatically decreasing sample preparation time, frequently the rate-limiting step toward discovery, especially when large libraries of changes are to be studied. This procedure may also find general use in isolating other proteins of the photosynthetic membrane [6] and as a method for labeling components for structural studies.

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[5] This was carried out with horse heart Cyt *c* and not native Cyt c_2 from *Rb sphaeroides*.

[6] This strategy has recently been used to tag the cytochrome bc_1 complex (A. Crofts, personal communication) and the PSII RC (W. Kuhlbrandt, personal communication).

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