High-throughput engineering to revitalize a vestigial electron transfer pathway in bacterial photosynthetic reaction centers

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Running title: Evolution of an electron transfer pathway

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Background: Bacterial reaction centers catalyze light-induced transmembrane electron transport using only one of two chemically-equivalent pathways.

Results: Multiplexed screening uncovers semi-random mutations that unexpectedly activate the unused pathway by employing ionizable residues.

Conclusion: High-throughput mutagenesis approaches reveal structure/function relationships that govern electron transfer efficiency.

Significance: Directed molecular evolution can reveal principles that enable efficient, unidirectional, transmembrane electron transfer for the design of de novo pathways or biomimetic devices.

SUMMARY Photosynthetic reaction centers convert light energy into chemical energy in a series of transmembrane electron transfer reactions, each with near 100% yield. The structures of reaction centers reveal two symmetry-related branches of cofactors (denoted A and B) that are functionally asymmetric; purple bacterial reaction centers use the A-pathway exclusively. Previously, site-specific mutagenesis has yielded reaction centers capable of transmembrane charge separation solely via the B-branch cofactors, but the best overall electron transfer yields are still low. In an attempt to better realize the architectural and energetic factors that underlie the directionality and yields of electron transfer, sites within the protein-cofactor complex were targeted in a directed molecular evolution strategy that implements streamlined mutagenesis and high-throughput spectroscopic screening. The polycistronic approach enables efficient construction and expression of a large number of variants of a heterologeric complex that has two intimately regulated subunits with high sequence similarity, common features of many prokaryotic and eukaryotic transmembrane protein assemblies. The strategy has succeeded in the discovery of several mutant reaction centers with increased efficiency of the B pathway; they carry multiple substitutions that have not been explored or linked using traditional approaches. This work expands our understanding of the structure/function relationships that dictate the efficiency of biological energy-conversion reactions – concepts that will aid the design of bio-inspired assemblies capable of both efficient charge separation and charge stabilization.

Light energy is converted to chemical energy in a series of extremely efficient electron transfer (ET) reactions performed by photosynthetic reaction centers (RCs). The structures of these transmembrane pigment/cofactor complexes from photosynthetic bacteria and higher plants reveal an axis of approximate twofold symmetry that relates both core protein subunits and functionally asymmetric sets of cofactors [Figure 1; (1-4)]. In many photosynthetic bacteria, these complexes are composed of three subunits – the homologous, integral L and M polypeptides and the membrane-tethered H polypeptide. Bacterial cofactors include a bacteriochlorophyll dimer (P) that serves as the primary electron donor, two bacteriochlorophyll monomers (B_A and B_B), two bacteriopheophytins (H_A and H_B), and two ubiquinone10 molecules (Q_A and Q_B). In type I RCs (e.g., Photosystem I), spectroscopic evidence suggests that both pathways are active in producing reduced quinone via light-induced ET [see e.g., (5-8)]. Yet, in type II RCs (e.g.,
bacterial RCs and Photosystem II), the B-pathway is silent, and possibly vestigial; only the A-branch cofactors are active.

The relative free energies of the charge-separated states are critical in determining the directionality, rates, and yields of ET in the RC. Some strategies to manipulate these factors genetically over the last twenty years [reviewed in (9)] include: addition or removal of hydrogen bonds to cofactors (10-15); addition or removal of axial ligands with attendant changes of bacteriopheophytin to bacteriochlorophyll or vice versa (16-27); modification of conserved residues or helical segments that are related by the complex’s pseudo-twofold symmetry axis (28,29); and introduction or removal of polar or ionizable residues near the cofactors (12,13,16,17,30-32). To disable ET from \( \text{H}_A \) to \( \text{Q}_A \) or from \( \text{Q}_A \) to \( \text{Q}_B \) specifically, the occupancy and environments of the quinone binding pockets have been manipulated by mutation (16,23-25,33-37).

The highest yields of ET from the excited dimer (\( P^* \)) to \( \text{H}_B \) (thus producing \( P^*\text{H}_B \)) have been achieved in mutant \( R. \text{capsulatus} \) RCs (17,31,32,35,38). In mutant RCs lacking \( \text{Q}_A \), reduction of \( \text{Q}_B \) via sole activity of the B-branch cofactors has been observed unambiguously (16,24,35,37,39-41). No cases yet reproduce any of the (essentially) 100% yields characteristic of the native A-side ET reactions. Rather, mutant RCs have demonstrated the magnitude of the B pathway’s inefficiency. The combination of a few site-specific mutations has not managed to modulate the energetics (or electronic factors) of the B-branch cofactors to provide for rapid electron transfer. At present, we lack the understanding needed to use rational design to accomplish the drastic leap required to redesign the RC to use the B pathway exclusively and efficiently.

Construction of such a re-engineered RC requires sampling of a much larger number of variants than has been examined previously. Thus, an approach has been adopted that combines semi-random mutagenesis and rapid screening techniques that require minimal sample volumes. Several methods have been streamlined by implementation of an automated liquid handler, and purification of mutant RCs has been semi-automated. A high-throughput spectroscopic assay evaluates the competency of candidate RCs for \( \text{Q}_B \) reduction via the B-side cofactors. This approach has been made feasible by advances in synthetic biology; favorable mutations that have been identified can be fed into directed molecular evolution experiments for additional optimization of the ET pathway. Techniques that have been developed here are likely to be applicable to other multi-subunit protein complexes with similar gene architectures.

**EXPERIMENTAL PROCEDURES**

**Growth –** \( R. \text{capsulatus} \) strains\(^2\) were cultured on \( \text{SuperRCVPY} \) (20) medium under semi-aerobic, chemoheterotrophic conditions in the dark (165 rpm, silicone sponge closures, 33°C). \( E. \text{coli} \) strains were propagated on LB or 2xTY media (42). Plasmids were selected with kanamycin (30 \( \mu \text{g/ml} \) for both organisms).

**Molecular biology –** The expression vector used in this study, \( \text{pBBRKW2HTsLsM} \) (Figure 2; Genbank Accession Number JN565028), was constructed from \( \text{puf} \) operon genes derived as an \( \text{EcoRI-SacI} \) fragment from plasmid \( \text{pUHTMluBgl:\alpha} \) (24) that were inserted into a derivative of broad-host-range cloning vector \( \text{pBMR1MCS-2} \) (43), generating \( \text{pBBRKW2HTBALMX} \).\(^5\) The expression plasmid was then modified extensively to facilitate the approach described herein. Portions of both the \( \text{pufL} \) and \( \text{pufM} \) genes were synthesized (GenScript) to encode silent changes that introduced restriction sites flanking each of the segments chosen for mutagenesis (Figure 2). To eliminate duplicate restriction sites elsewhere, eleven restriction sites in \( \text{pBMR1MCS-2} \) and four sites in the \( \text{puf} \) operon were removed (Table S2) using either site-directed mutagenesis (QuickChange, Stratagene) or mung bean nuclease. The synthetic segment of \( \text{pufL} \) was then cloned into \( \text{pBBRKW2HTBALMX} \) (Table S2) as a \( \text{PstI-KpnI} \) fragment, and the synthetic \( \text{pufM} \) segment was cloned using \( \text{BgII} \) and \( \text{BamHI} \), thus creating \( \text{pBBRKW2HTsLsM} \). A heptahistidine tag is encoded at the C-terminus of \( \text{pufM} \). The H32R mutation in the \( \text{pufA} \) gene prevents assembly of the light-harvesting (LH) I antenna complex (44). Thus, genes expressed from this plasmid in \( R. \text{capsulatus} \) strain U43 result in the antennaless, “RC-only” photosynthetic membrane needed for quantitative extraction of RCs by mild detergents (24). A summary of substitutions carried by mutant RCs and the nomenclature used to refer to them is contained in Table S3.

Construction of the \( \text{F(L181)X+FHV, F(L216)X+YFHV} \) and \( \text{E(L212)A-D(L213)A-F(L216)X+YFHV} \) sets of mutant strains were expedited with cassette-based mutagenesis. The \( \text{W(M250)V} \) control strain was constructed by site-
directed mutagenesis of the synthetic BglII-BamHI fragment of the puf/M segment described above. Protocols for phosphorylation and annealing of oligonucleotides, ligations, transformations and plasmid screening were adapted for use with a liquid handling system (Biomek FX, Beckman-Coulter). For each mutant set, a library of oligonucleotides was designed to encode all 20 amino acids at residue “X.” Oligonucleotides for the F(L181)X+FHV set were flanked by HindIII and BbvCI restriction sites, and the oligonucleotides for the F(L216)X+YFHV and E(L212)A-D(L213)A-F(L216)X+YFHV cassettes were flanked by AccIII and Accl restriction sites (Table 1; Figure 2). Oligonucleotides were phosphorylated, annealed, and ligated to pBBRKW2HTsLSM digested with the appropriate restriction enzymes. Candidate plasmids from DH5α transformants were screened using restriction fragment analysis and sequenced. Correct plasmids from small cultures (80 ml) were lysed using a microfluidizer (Microfluidics, Inc) and mutant RCs were solubilized with Deriphat 160-C. Following overnight binding to 200 µl Ni-NTA resin (Qiagen), chromatography proceeded in two-ml, 96-well filter plates. UV-vis spectra of dark-adapted (10 min) samples were recorded (SpectraMax M5<sup>c</sup>; Molecular Devices). Based on A<sub>865nm</sub>, mutant RCs were classified into three expression categories relative to yields of WT RCs – abundant (>25%), moderate (2.5-25%), and poor (<2.5%).

Semi-automated RC purification – Mutant strains that expressed RCs abundantly or moderately were grown in 2-6 L volumes depending on the expression level. RCs were purified via semi-automated methods using ÄKTAPrep FPLCs (46).

RC Functionality Screening – Assaying mutant RCs for formation of P<sub>Q<sub>β</sub></sub> via B-branch ET was accomplished with a home-built, time-resolved spectrometer designed for 100-200 µl samples arrayed in clear-bottom 96-well plates (BD Falcon). Rapid data collection and reproducibility were facilitated via use of a computer-controlled translation stage (Aerotech) to position the sample well under collinear, vertical excitation and probing (measuring) beams. RCs were excited with a single sub-saturating, 5-ns, 532-nm flash from a Nd:YAG laser. The magnitude and decay of bleaching of the ground state absorbance of P was probed by a stable, continuous-wave, 850-nm laser diode (Melles-Griot) attenuated with neutral density filters so that no discernible actinic effects were observed. For added sample protection, the probe light was incident on the sample only during data collection and was otherwise blocked with a computer-controlled shutter. Following passage through the sample, the probe light was isolated from the collinearized pump-probe beams by dichroic and 850-nm interference filters. The isolated probe light was collected onto a near-infrared-sensitive photosensor module (Hamamatsu H-5784-20), whose output was recorded on a digital oscilloscope. The apparatus is capable of measuring transient spectroscopic signals spanning 50 µs to several minutes. For 100 µl volume, the effective measuring pathlength was 2 mm; sample concentrations were adjusted such that A<sub>865nm</sub> was 0.45-0.55. Signal intensities were normalized to the same ground state absorbance of P’s band during data analysis. Since RCs are Q<sub>β</sub>-less (except for wild type) due to the W(M250)V mutation described below, the initial amplitude of each kinetic trace is proportional to the yield of ET to Q<sub>β</sub> solely via the B-branch, as assayed relative to control RCs on the same plate.

Further characterization of primary electron transfer events in select mutant RCs utilized a transient absorption apparatus having 130 fs excitation and white-light probe flashes operating at 10 Hz [described previously in (31,38)]. RCs were held at ~10°C in an ice-cooled reservoir and flowed through a 2 mm path length cell.

RESULTS

A system to construct and express mutants of the L and M genes of the polycistrionic, oxygen-regulated puf operon of <i>R. capsulatus</i> was developed previously (47-49). The expression host strain, U43, does not synthesize any light-harvesting antennae or RC complexes of the photosynthetic apparatus (<i>i.e.</i>, LH<sub>I</sub> LH<sub>II</sub> RC<sup>c</sup>). The original broad-host-range expression plasmid cannot be used for this type of study because its complete sequence has not been determined and its large size (32 kb) hinders its application to an approach based upon restriction enzyme cloning of
mutated cassettes. Thus, pBBR1MCS-2 (5.1 kb), whose sequence is known (43), was selected as the vector for the expression plasmid and modified as described above. Yields of RCs purified from strains carrying the newly engineered expression plasmid were found to be twofold greater than those obtained from plasmid pUHTMluBgIα.

The initial mutagenesis template was “FHV”. The “F” mutation substitutes Phe for the native Tyr at M208 near Bα and diminishes ET to the A side. The “H” mutation substitutes His for Leu at M212 near Aα and results in incorporation of a bacteriochlorophyll in the Hα site. Since this cofactor is more difficult to reduce, the A pathway is disabled further. This mutation also removes Hα absorption from the 510-550 nm spectral region, allowing quantitative assessment of Hb reduction via ultrafast (picosecond) spectroscopic measurements. The “V” mutation, which substitutes Val for the conserved Trp at M250, prevents QA binding and, thus, prevents ET to Qb via Qα. In other words, this mutation ensures that electrons can reach Qb only via the B-side cofactors.

M208Tyr is related via the C2 symmetry axis to residue L181Phe near Bβ. This conserved asymmetric pair was the target of some of the earliest mutagenesis studies of the purple bacterial RC. The swap of Tyr for the native Phe at L181 and Phe for Tyr at M208 reverses the asymmetry and enhances ET to Hb while diminishing ET to the A side. When the F(L181)Y mutation is added to the FHV template, the result is the “YFHV” RC, which serves as another control (in addition to the wild-type that results in quantitative reduction of Qb via A-branch activity) for the ms screening assay because it displays ~30% yield of P+Hb− and ~15% yield of P+Qb− (31,35,39,40,50).

A large number of residues within 5-14 amino acid segments near the cofactors have been identified as mutagenesis targets (Table 1; Figure 2). At the outset, regions proximal to Bb, Ha and Qb were targeted to further enable (compared to YFHV) the B-side pathway. For all of the mutant strains reported here, protein yields from larger-scale cultures and automated purification procedures correlated well with yields determined from the small-scale expression screening. The use of Deriphat 160C for RC solubilization and purification ensured full occupancy of Qb in the native binding site (24).

Twenty F(L181)“X”+FHV constructs comprised the initial mutant set. Residue L181 (segment D, near Bb) was chosen because of this site’s known influence on initial B-side charge separation as indicated above. Eighteen of the mutant RCs were expressed moderately or abundantly (Figure 3). None of the substitutions at L181 resulted in pronounced effects on the RC ground state absorption spectra except for the KFHV mutant, with K at L181 previously having been shown to provide a sixth ligand to Bb (11).

Additional rounds of mutagenesis focused on residues near Qb in attempts to increase the low (~40%) yield of ET from Hα to Qb (24,35,39). Saturation mutagenesis was performed at conserved residue L216Phe (segment J) since its C2-symmetry-related counterpart, M250Trp, is essential for rapid ET from Aα to Qα (33) and also is important for Qα binding. The F(L216)“X” mutations were coupled with YFHV. Additionally, since previous work showed that replacement of the native Glu and Asp at L212 and L213, respectively, with alanines stabilizes P+Qb− (51), the F(L216)X variants were coupled with the E(L212)A-D(L213)A+YFHV (“AA+YFHV”) substitutions in another set of mutants. These two rounds of mutagenesis yielded 40 additional strains with 36 expressing RCs at abundant or moderate levels – nineteen F(L216)X+YFHV mutants and seventeen AA+FL216)X+YFHV mutants (Table S4). Only the D, H, and K substitutions failed to produce RCs in the AA+F(L216)X+YFHV background.

All 54 of the mutant RCs studied here gave light-induced formation of P’Qb− via the B-side cofactors in the ms transient absorption assay. The relative amounts of P’Qb− formed are plotted in Figure 4. Of particular note are the F(L181)H+FHV (“HHFHV”), F(L181)N+FHV (“NFHV”) and F(L181)D+FHV (“DFHV”) mutant RCs, which produce P’Qb− signals that are 20-30% larger than that of the YFHV control. The A, G, K and W mutations produce RCs with the poorest yields of P’Qb− in the F(L181)X+FHV series. Figure 5A shows kinetic traces derived from the HFHV, NFHV, DFHV and WFHV RCs along with the YFHV and W(M250)V-only control RCs from the ms screening assay. The profiles, displaying decay time constants ≥10 s, are characteristic of P’Qb− charge recombination when this state is formed via activity of the B-branch cofactors; in these RCs, decay of this state via routes involving Qα is prohibited by the W(M250)V mutation (24). Thus, the initial amplitudes of the kinetic traces in Figure 5A are measures of the (relative) yields of P’Qb− formed.
solely via B-branch activity; this value is plotted for all of the mutant RCs in Figure 4.

The results for the HFHV, NFHV, DFHV and WFHV mutant RCs were confirmed by ultrafast transient absorption measurements (Figure 5B). These spectra show the magnitudes of bleaching of H_b at ~528 nm, reflecting the relative yields of ET from P^+ to H_b and confirm an ordering of H-N>D-Y>>W, the same ordering as determined in the P^A-Q_b^- yield assay. Additional ultrafast measurements are underway and will be reported elsewhere.

Expression yields for the F(L216)X+YFHV and AA+F(L216)X+YFHV sets of mutant RCs were quite high, especially for the latter set given the number of mutations introduced into the RC. However, yields of P^A-Q_b^- in these mutant RCs did not exceed that of the template YFHV. In general, there is less ET to Q_b in RCs containing the E(L212)A-D(L213)A substitutions than in mutant RCs carrying the native residues at these sites. The relative rank order of the residues in each set is different (see, e.g., the effect of a Val substitution in each set), suggesting complex interplay of effects introduced by substitution of the small, non-polar alanines for the larger, ionizable Glu and Asp.

**DISCUSSION**

Directionality of ET in type II RCs is a conundrum that has existed ever since the 1985 structure of the bacterial RC (3) revealed two branches of cofactors related by an axis of approximate C_2 symmetry while spectroscopic measurements demonstrated activity of only one of them. The free energies the charge-separated states on the A pathway (P^B^-_A, P^H_A^- and P^Q_A^-) are successively downhill from each other and from P^+ facilitating rapid forward ET. In contrast, B-branch inactivity presumably stems in large measure from P^B^-_B being somewhat higher in free energy than P^+, resulting in much slower, non-competitive, B-side charge separation. Differences between the electronic couplings of P^+ with B_A and B_B may also contribute to unidirectional A-side ET. The rate constant for P^+ → P^B^-_B on the native B pathway has been measured to be ~(100 ps)^{-1} (17,31), about 30-fold slower than charge separation on the A side (3-4 ps for P^+ → P^H_A^-), suggesting that yields of B-side ET in wild-type RCs should be ~3%.

Although significant yields of P^B^-_B formation have been reported in some *R. capsulatus* mutants (17,31,32,35,38), the largest rate constant for P^+ → P^B^-_B is ~40 ps)^{-1}, obtained in the YFH mutant RC (31). The best efforts by several laboratories over many years have thus achieved only about a two-fold increase in the rate of ET to H_b compared to the wild-type value. This enhanced rate is still a factor of ten slower than ET to the A side. Interestingly, rapid initial charge separation to the B-side has been reported in a mutant where a bacteriochlorophyll substitutes for B_B. While ET from P^+ to this new cofactor takes place in ~10 ps, ET onward to H_b does not occur (18), creating another, different, bottleneck in the quest for efficient use of the B-side cofactors for transmembrane charge separation. Similar issues occur at the final step of ET from H_b to Q_b, which has a yield of only ~40% as mentioned above. The rate of this ET reaction is ~4 (ns)^{-1}, while the rate of competing charge recombination of P^H^-_B is ~3 (ns)^{-1} (39). For comparison, the rates of the corresponding reactions on the A side are (200 ps)^{-1} and ~20 (ns)^{-1}.

To sample a larger number of variants and remove much of the prejudice inherent in rational design schemes, a directed molecular evolution approach has been adopted for engineering of the RC to enable efficient B-branch ET. Directed molecular evolution has been applied to the discovery of new functions, increased expression, increased stability, and enhanced activity for many proteins [reviewed in (52,53)] but has had relatively limited application to membrane proteins [e.g., (54-57)]. The present study merges principles of both rational design and directed molecular evolution to generate versions of the heterotrimeric, protein/cofactor RC complex that have acquired increased ability to utilize a vestigial ET pathway for transmembrane charge separation.

The mutagenesis employed here is best termed “semi-random” and relies on oligonucleotide cassettes rather than error-prone PCR or fragment shuffling. The rationale for this approach is based on knowledge of the structure, awareness of the substitutions that have been tried before, the concern that massively random mutagenesis will produce RCs with assembly and/or stability problems, the difficulties imposed by the high degree of sequence similarity between the *pufL* and *pufM* genes (especially in highly-conserved regions that surround the cofactors), and the fact that their coding regions overlap (Figure 2). Since this approach has worked well here for these genes embedded in the polycistronic *puf* operon, it is likely that the chosen mutagenesis strategy could be applied to
many gene clusters encoding transmembrane complexes where components share a high degree of sequence similarity – a common occurrence in sequence data emerging from representatives of all kingdoms.

Directional cloning of cassettes required significant downsizing and engineering of the broad-host-range expression vector and was enabled by synthetic biology. Surprisingly, the yield of RCs expressed from the new, partially-synthetic platform vector was twofold greater than the yield obtained from the original expression plasmid. This result may be due to differences in plasmid copy number and/or unexpected changes in (i) transcript stability, (ii) codon preferences, or (iii) modification of regulatory elements that occurred as a result of the engineering that was required.

In the initial round of saturation mutagenesis at the L181 site, surprises were encountered when yields of B-side ET were found to be highest in RCs carrying His, Asn, Asp and Gln mutations. Numerous RCs carrying single mutations at this site have been reported previously [reviewed in (9)], but none of these studies had ever suggested that any of the above residues would match or exceed a Tyr at L181 in enhancing B-branch activity, or that the functional consequences of a Glu substitution would be similar to those conferred by a Tyr substitution (Figure 4). In fact, considering that placement of an acidic residue close to BA in the G(M201)D RC disfavors ET to the A side (12), it was surprising that an Asp substitution at L181 near BA had the opposite effect, enabling more B-branch activity. Similar effects observed for the polarizable His, Asn, Asp and Gln residues at L181 cannot all be attributable to a common fundamental mechanism, but all of these substitutions will influence, rather unpredictably, the dielectric environment of the cofactors involved in the initial electron transfer reactions. The close parallel effects on B-side ET for the Asn/Asp and Gln/Glu pairs – with the smaller Asn/Asp substitutions producing RCs with larger yields of B-side ET – are noteworthy.

In general, large residues at the L216 site result in lower yields of reduced quinone. RCs in which ionizable residues are substituted at the L216 site – when in combination with the Ala substitutions at L212-213 – either fail to assemble or perform poorly in the functional assay. It is possible that substitutions within this region of the QB binding site affect its occupancy. A disappointment is that the Trp substitution at this site does not result in increased ET from HB to QB whereas the conserved Trp residue at the symmetry-related M250 site is critical for efficient ET from HA to QA in the wild-type RC (33). These results suggest that targeting of residues based on their relationship to the C2 symmetry axis in this region of the RC is less productive and emphasize that a more random, evolutionary approach will be required to enhance secondary electron transfer reactions of the B pathway.

Overall, the results reported herein validate the experimental strategy and underscore the plasticity of the RC in accommodating substitutions that result in a range of B-branch activity. A subset of the new mutant RCs displays increased quinone reduction by the exclusive use of B-branch cofactors. Unexpected, productive combinations of amino acid substitutions that previously have not been discovered by rational design were revealed by the approach described herein. Realization of these mutant RCs was enabled by miniaturization at two screening steps (expression testing and spectroscopic assays) and automation of protein purification and time-resolved spectroscopy. A system is now in place whereby mutant RCs with altered ET functionalities can be identified rapidly as candidates for more exhaustive functional characterization and iterative engineering to produce next-generation RCs with desired ET capabilities, even those that are unimagined by Nature.

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REFERENCES

1. Allen, J. P., Feher, G., Yeates, T. O., Komiya, H., and Rees, D. C. (1987) Proc. Natl. Acad. Sci. USA 84, 5730-5734
2. Chang, C.-H., El-Kabbani, O., Tiede, D. M., Norris, J. R., and Schiffer, M. (1991) Biochemistry 30, 5352-5360
3. Deisenhofer, J., Epp, O., Miki, K., Huber, R., and Michel, H. (1985) Nature 318, 618-624
4. Ermel, U., Fritsch, G., Buchanan, S. K., and Michel, H. (1994) Structure 2, 925-936
5. Dashdorj, N., Xu, W., Cohen, R. O., Golbeck, J. H., and Savikhin, S. (2005) Biochim. Biophys. Acta 1607, 53-63
6. Coleman, W. J., Bylina, E. J., Aumeier, W., Siegl, J., Eberl, U., Heckmann, R., Ogrodnik, A., Michel-Beyerle, M. E., and Youvan, D. C. (1990) Influence of mutagenic replacements of tryptophan M250 on
Evolution of an electron transfer pathway in reaction centers of *Rhodobacter capsulatus*. in *Structure and Function of Bacterial Photosynthetic Reaction Centers* (Michel-Beyerle, M. E. ed.), Springer-Verlag, New York. pp 273-281

34. Coleman, W. J., and Youvan, D. C. (1993) *Nature* **366**, 517-518
35. Kirmaier, C., Laible, P. D., Hanson, D. K., and Holten, D. (2003) *Biochemistry* **42**, 2016-2024
36. Li, J., Coleman, W. J., Youvan, D. C., and Gunner, M. R. (2000) *Photosynth. Res.* **64**, 41-52
37. Paddock, M. L., Chang, C., Xu, Q., Abresch, E. C., Axelrod, H. L., Feher, G., and Okamura, M. Y. (2005) *Biochemistry* **44**, 6920-6928
38. Chuang, J. I., Boxer, S. G., Holten, D., and Kirmaier, C. (2006) *Biochemistry* **45**, 3845-3851
39. Kee, H. L., Laible, P. D., Bautista, J. A., Hanson, D. K., Holten, D., and Kirmaier, C. (2006) *Biochemistry* **45**, 7314-7322
40. Laible, P. D., Kirmaier, C. K., Holten, D., Tiede, D. M., Schiffer, M., and Hanson, D. K. (1998) Formation of P+QH via B-branch electron transfer in mutant reaction centers. in *Photosynthesis: Mechanisms and Effects* (Garab, G. ed.), Kluwer, Dordrecht. pp 849-852
41. Wakeham, M. C., Goodwin, M. G., McKibbin, C., and Jones, M. R. (2003) *FEBS Lett.* **540**, 234-240
42. Sambrook, J., Fritsch, E., and Maniatis, T. (1989) *Molecular cloning: A laboratory manual*, 2nd ed., Cold Spring Harbor Press, New York
43. Kovach, M. E., Elzer, P. H., Hill, D. S., Robertson, G. T., Farris, M. A., Roop II, R. M., and Peterson, K. M. (1995) *Gene* **166**, 175-176
44. Bylina, E. J., Robles, S. J., and Youvan, D. C. (1988) *Isr. J. Chem.* **28**, 73-78
45. Weaver, P. F., Wall, J. D., and Gest, H. (1975) *Arch. Microbiol.* **105**, 207-216
46. Kirmaier, C., Bautista, J. A., Laible, P. D., Hanson, D. K., and Holten, D. (2005) *J. Phys. Chem. B* **109**, 24160-24172
47. Bylina, E. J., Ismail, S., and Youvan, D. C. (1986) *Plasmid* **16**, 175-181
48. Bylina, E. J., Jovine, R. V. M., and Youvan, D. C. (1989) *Bio/Technology* **7**, 69-74
49. Youvan, D. C., Ismail, S., and Bylina, E. J. (1985) *Gene* **33**, 19-30
50. Kirmaier, C., Laible, P. D., Hindin, E., Hanson, D. K., and Holten, D. (2003) *Chem. Phys.* **294**, 305-318
51. Hanson, D. K., Baciou, L., Tiede, D. M., Nance, S. L., Schiffer, M., and Sebban, P. (1992) *Biochim. Biophys. Acta* **1102**, 260-265
52. Yuan, L., Kurek, I., English, J., and Keenan, R. (2005) *Microbiol. Mol. Biol. Rev.* **69**, 373-392
53. Jackel, C., Kast, P., and Hilvert, D. (2008) *Annu. Rev. Biophys.* **37**, 153-173
54. Bokma, E., Koronakis, E., Lobedanz, S., Hughes, C., and Koronakis, V. (2006) *FEBS Lett.* **580**, 5339-5343
55. Dong, S., Rogan, S. C., and Roth, B. L. (2010) *Nat. Protocols* **5**, 561-573
56. Sarkar, C. A., Dodevski, I., Kenig, M., Dudli, S., Mohr, A., Hermans, E., and Pluckthun, A. (2008) *Proc. Natl. Acad. Sci. USA* **105**, 14808-14813
57. Wise, K. J., Gillespie, N. B., Stuart, J. A., Krebs, M. P., and Birge, R. R. (2002) *Trends Biotechnol.* **20**, 387-394
Evolution of an electron transfer pathway

FOOTNOTES

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The nucleotide sequence for pBBRKW2HTsLsM has been deposited in the Genbank database under GenBank Accession Number (GenBank = GenBank Accession Number JN565028).

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The abbreviations used are: RC, reaction center; ET, electron transfer; P, primary donor; B, bacteriochlorophyll; H, bacteriopheophytin; Q, quinone; R, Rhodobacter; LH, light-harvesting antennae; YFHV, F(L181)Y-Y(M208)H-M212)H-W(M250)V; KFHV, F(L181)K+FHV; AA+YFHV, E(L212)A-D(L213)A+YFHV; HFHV, F(L181)H+FHV; NFHV, F(L181)N+FHV; DFHV, F(L181)D+FHV; WFHV, F(L181)W+FHV; RT, room temperature; Ni-NTA, nickel-nitrilotriacetic acid

5 Strains and plasmids used in this study are listed in Table S1.

FIGURE LEGENDS

FIGURE 1. Electron transfer pathways and positions of substituted amino acids in bacterial RCs. Despite equivalent sets of symmetry-related cofactors, only the A branch is active in native RCs. For visual clarity, all isoprenyl anchoring groups and bulky porphyrin-ring substituents were removed from cofactors.

FIGURE 2. Engineered expression plasmid for RC mutagenesis. Segments of the L and M genes targeted for mutagenesis (striped regions; Table 1) are flanked by unique restriction sites that facilitate the insertions of oligonucleotide cassettes encoding multiple substitutions. Fifteen restriction sites (parentheses) in the plasmid were removed to enable the cassette-based mutagenesis approach.

FIGURE 3. Expression yields of mutant RCs. UV-vis spectra of RCs purified from the F(L181)”X”+FHV set of mutant RCs indicate three expression categories. The residue present at the L181 site is designated with the single letter code.

FIGURE 4. B-branch quinone reduction in mutant RCs. Yields of B-side P+QB− formation from time-resolved assays of mutant RCs relative to signals from wild-type RCs. Signals from the YFHV (yellow) and W(M250)V (white) RCs serve as benchmark controls. Error bars reflect variability in replicate spectroscopic screening measurements.

FIGURE 5. Transient light-induced optical signals indicative of B-branch activity in mutant RCs. A, P-bleaching amplitudes and decay profiles for state P’Q_B− observed in the ms screening assay. The gray trace is for the W(M250)V mutant RC; the other traces are as indicated in the legend in panel B. Each trace represents the average of seven data acquisitions (single excitation flash events). Signal amplitudes were corrected by factors of ≤1.2 to normalize the sample concentrations. B, Comparison of transient absorption spectra acquired using 130 fs excitation flashes at 850 nm in ultrafast studies of the primary photochemistry. RC concentrations and laser excitation conditions were matched. The spectra shown were acquired at 1 ns after the flash in order to minimize the contribution of shorter-lived states. The relative yields of P’H_B− (determined by the magnitude of bleaching of the band of H_B at 525-528 nm) agree well with those measured for the P’Q_B− yields in the ms screening assay (panel A).
| Cofactor | Amino Acid Segment (Region) | 5'-Flanking Enzyme | 3'-Flanking Enzyme |
|----------|-----------------------------|--------------------|--------------------|
| B_B      | M150-160 (A)                | AflII              | PpuMI              |
| B_B      | M173-184 (B)                | PpuMI              | XmnI               |
| B_B      | L168-178 (C)                | NdeI               | HindIII            |
| B_B / H_B| L179-190 (D)                | HindIII            | BbvCI              |
| H_B      | L215-220 (E)                | AccIII             | AcI                |
| H_B      | M124-131 (F)                | EcoRV              | Xmal               |
| H_B      | M144-151 (G)                | XmnI               | AflII              |
| H_B      | M271-275 (H)                | NeoI               | BamHI              |
| Q_B      | L186-194 (I)                | HindIII            | BbvCI              |
| Q_B      | L212-216 (J)                | AccIII             | AcI                |
| Q_B      | L220-232 (K)                | AcI                | DraI               |
| B_A      | L127-131 (L)                | Ball               | MluI               |
| B_A      | L146-157 (M)                | MluI               | NdeI               |
| B_A      | M195-207 (N)                | XmnI               | BlpI               |

Table 1. Regions targeted for mutagenesis.
Figure 1
Figure 2
Figure 3

Evolution of an electron transfer pathway

Absorption (a.u.)

Wavelength (nm)

Abundant

Moderate

Poor

650 700 750 800 850 900 950

YWML

ECINKVDGT

PR
Figure 5

A

B

ΔA

0.00

-0.04

-0.08

0

2

4

6

8

Time (s)

ΔA

0.00

0.02

0.04

0.06

0.08

500

550

600

650

700

Wavelength (nm)

WFHV

YFHV

DFHV

NFHV

HFHV
High-throughput engineering to revitalize a vestigial electron transfer pathway in bacterial photosynthetic reaction centers.
Kaitlyn M. Faries, Lucas L. Kressel, Marc J. Wander, Dewey Holten, Philip D. Laible, Christine Kirmaier and Deborah K. Hanson

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