De Novo Protein Design of Photochemical Reaction Centers

Nathan Ennist (✉ ennist@uw.edu)

University of Washington <https://orcid.org/0000-0002-4823-9497>

Zhenyu Zhao

University of Pennsylvania

Steven Stayrook

Yale University School of Medicine <https://orcid.org/0000-0002-1677-8293>

Bohdana Discher

University of Pennsylvania

P Leslie 'Les' Dutton

University of Pennsylvania

Christopher Moser

University of Pennsylvania

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Abstract

Natural photosynthetic protein complexes capture sunlight to power the energetic catalysis that supports life on Earth. Yet these natural protein structures carry an evolutionary legacy of complexity and fragility that encumbers protein reengineering efforts and obfuscates the underlying design rules for light-driven charge separation. De novo development of a simplified photosynthetic reaction center protein can clarify practical engineering principles needed to build new enzymes for efficient solar-to-fuel energy conversion. Here we report the rational design, X-ray crystal structure, and electron transfer activity of a multi-cofactor protein that incorporates essential elements of photosynthetic reaction centers. This highly stable, modular artificial protein framework can be reconstituted in vitro with interchangeable redox centers for nanometer-scale photochemical charge separation. Transient absorption spectroscopy demonstrates Photosystem II-like tyrosine and metal cluster oxidation, and we measure charge separation lifetimes exceeding 100 ms, ideal for light-activated catalysis. This de novo-designed reaction center builds upon engineering guidelines established for charge separation in earlier synthetic photochemical triads and modified natural proteins, and it shows how synthetic biology may lead to a new generation of genetically encoded, light-powered catalysts for solar fuel production.

Introduction

De novo construction of artificial photosynthetic reaction centers offers a means to test our understanding of biological electron transport and to re-engineer photosynthesis in ways that can be aimed directly at human needs. Natural reaction centers attain unidirectional, light-activated charge separation by manipulating electron transfer rates in chains of redox cofactors¹³⁻¹⁵. Linear electron flow in green plants and cyanobacteria begins with water as a source of electrons to produce H₂ equivalents for chemical energy, releasing O₂ in the process^{1,16}. Oxygenic phototrophs span the wide redox range between water oxidation and proton reduction by using two reaction centers, photosystems I and II (PSI and PSII), to absorb two photons for each electron abstracted from water^{1,17}. In principle, a single visible photon absorbed by natural chlorophyll pigments is sufficiently energetic to power both reactions, suggesting an avenue toward increasing the overall solar-to-fuel energy conversion efficiency^{1,10}. Our ultimate aim is to develop a one-reaction center photosynthetic system that supports water oxidation and proton reduction with minimal energy loss to heat.

Recent progress in *de novo* protein design has facilitated binding of multiple, varied small molecules and metal ions within electron tunneling distance of one another¹⁸⁻²³. The next challenge for solar energy transduction in an artificial protein is to assemble a multi-step electron transport chain that can convert photon energy to a charge separated state that persists long enough to be used for chemical reactions such as fuel production. To this end, we have designed a photosynthetic reaction center protein maquette (the RC maquette) and solved its X-ray crystal structure in multiple states of assembly. The RC maquette achieves long-lived, light-activated charge separation and reproduces many of the elements of natural reaction centers: tyrosine and metal cluster oxidation reminiscent of the water oxidizing side of PSII, as well as reduction of low potential acceptors, including Co porphyrins known to participate in proton reduction to H₂^{24,25}.

Main Text

Predicting electron tunneling rates

Natural photosynthetic reaction centers achieve photochemical charge separation by anchoring a light-activated pigment between two electron transfer chains. One chain provides an electron acceptor for the excited pigment, and the other an electron donor that restores the pigment ground state while creating a charge separated donor-acceptor pair (Figure 1a). Electron accepting or donating chains have been developed in modified natural proteins^{8,9,11,12,26-30}. However, in the RC maquette, both of the extended electron transfer chains of natural reaction centers are simplified to a core elementary donor-pigment-acceptor triad, symbolized as DPA^{6,7}. Upon light activation forming an excited pigment singlet state (D¹P*A), the pigment may undergo radiative decay to the ground state (DPA) or relax to the triplet excited state (D³P*A) via intersystem crossing. This triplet state may in turn decay to the ground state by phosphorescence (k_3 , Figure 1b).

Light-activated electron transfer originates from the excited singlet state for short electron tunneling distances between P and A, and from the triplet state at larger distances (k_1 , forming DP*A). Charge separated states can short-circuit through charge recombination (k_4 , forming DPA) or undergo a further charge separation (k_2 , forming D*PA). In the absence of additional donors, acceptors or catalytic redox reactions the charge separated state eventually decays by direct electron transfer (k_5) or indirect electron transfer through reverse reactions of k_2 and k_1 (Figure 1b).

The expected rates of downhill intraprotein electron tunneling are estimated by a well-tested empirical expression^{13,15,31}:

$$\text{Eqn. 1) } \log(k_{\text{ET}}) = 13 - 0.6(R - 3.6) - 3.1(\Delta G + \lambda)^2 / \lambda$$

The electron transfer rate, k_{ET} , is in units of s⁻¹, the edge-to-edge distance R is in Å, the driving force between donor and acceptor ΔG (usually estimated from the difference between their redox midpoint potentials, E_m) is in eV, and the Marcus reorganization energy λ is in eV. For electron transfers slower than 20 ps, the reorganization energy ranges from 0.4 for large redox cofactors (such as tetrapyrroles) buried in non-polar protein environments, to 1.4 eV for more compact redox centers or polar environments^{13,15,31}. When λ has not been measured, a middling value around 0.8 eV will typically provide a rate estimate within an order of magnitude. Uphill reverse electron transfer rates can be estimated by using the downhill rate and applying a Boltzmann thermodynamic penalty of 1 order of magnitude of rate for every 0.06 eV uphill^{13,15,31,33}. These tunneling expressions not only allow us to understand the engineering of natural electron transfer proteins^{14,15,34} but also to consider the relative performance of artificial DPA triad designs in advance of construction (Figure 1c and d).

Light-active redox protein design

To begin reverse-engineering photosynthetic reaction centers, we use first principles of protein folding to construct a single-chain framework for positioning DPA cofactors that consists of an extended four α -helix bundle coiled-coil. Such constructs are called maquettes^{5,18,35} and they perform the same role as the scale maquettes made by architects to evaluate their designs prior to constructing the final product. Maquette amino acid sequences are not based on any specific natural protein. Instead, they employ charge complementarity and a binary pattern of polar and nonpolar residues to enforce a desired helical threading^{5,18}. Further details of RC maquette design will be described elsewhere; see Supplementary Table 1 for the selected amino acid sequences.

The RC maquette controls distances between pigments and redox centers to promote charge separation using cofactor-ligating amino acids in suitable positions. Histidine (His) residues in the hydrophobic core ligate the central metals of tetrapyrroles for site-specific cofactor binding. A single His ligates a light-activatable Zn porphyrin in the pigment site^{19-21,36}, while two His residues on opposing helices bis-His ligate an Fe porphyrin in the acceptor site^{19,23,35,37,38}. The RC maquette can bind different electron acceptors including heme B or Fe(III) 2,4-diacetyl deuteroporphyrin IX (DADPIX) tightly with $K_D \leq 10$ nM and with E_m values of -0.19 and +0.01 V vs SHE, respectively. Amphiphilic Zn tetrapyrroles show the strongest binding to our *de novo* proteins²¹. In the results reported here, we use Zn 5-phenyl, 15-(p-carboxyphenyl) porphyrin (ZnP, $K_D \leq 10$ nM, E_m 0.91 V) as our pigment, P. See Extended Data Figure 9 for spectro-electrochemical determination of E_m values of Fe and Zn porphyrins. The distance between cofactors within this 4-helix bundle can be adjusted in increments of a helical turn (~ 5.2 Å). The theoretical contour plot of Figure 1c, calculated from Eqn. 1 with the kinetic scheme of Figure 1b, shows that spacing the ligating His residues by four helical turns is likely to result in stable charge separation for various porphyrin acceptors with a wide range of redox properties. Details of the electron tunneling yield calculations are provided in Methods.

The contour plot of Figure 1d indicates that the electron donor must be placed closer to P to assure sufficiently rapid electron tunneling rates that can compete with short-circuiting DP^+A^- charge recombination, k_4 (see Figure 1b). D-P distances up to ~ 10 Å and E_m values less than 0.75 V will give the best D^+PA^- yield. To reproduce PSII-like photo-oxidation activity, we chose a Tyr-P distance of 5 Å for Tyr oxidation, and we assembled a metal cluster at a distance of 10 Å from P. This bi-nuclear metal cluster uses a two-His/four-Glu motif seen in many natural metal clusters such as bacterioferritin¹². This motif, which was expressed and characterized in an earlier *de novo* protein maquette DF3³⁹⁻⁴², forms the basis of our sequence selection in the metal cluster donor region of the RC maquette. The operation of these donors is compared with an unnatural maleimide-functionalized ferrocene donor (Fc) anchored to a Cys residue that replaces a Tyr residue at two different D-P distances (either Y168C for Fc168 or G164C/Y168L for Fc164).

Crystal structures of the RC maquette confirm the intended coiled-coil design with pigments and redox centers assembled at the designed positions (Figure 2). Despite the lack of sequence similarity with natural proteins and the absence of evolutionary natural selection, the RC maquette shows many structural features in common with natural proteins. Hydrophobic core packing, rotameric states of heme-ligating histidines, and second shell histidine-threonine hydrogen bonds are similar to the cytochrome *b* subunit of cytochromes bc_1 and b_6f (Figure 2a)⁴³. Even the orientation of the maquette heme relative to the superhelical bundle axis is conspicuously similar, suggesting that the particular conformation of heme in the natural proteins is not precisely controlled for any functional purpose, but rather follows directly from structural constraints demanded by this protein fold. The light-active pigment binding site differs from the cytochrome *b* heme site by providing only a single His for ligation. At the Tyr donor, a single Leu for His residue exchange in the bundle core immediately provides an H-bond for the Tyr hydroxyl, mimicking the proton-coupled electron transfer geometry of donor Tyr Z in PSII^{44,45} (Figure 2b). Glu/His ligation of Cd(II) in the RC maquette accurately reproduces the photochemically active geometry of the binuclear Mn(II) cluster formed in bacterioferritin (Figure 2c)^{11,12}. The successful construction of this electron donor site represents a first step in exploring the Tyr-mediated photo-oxidative assembly of a PSII-like Mn cluster for water oxidation in a designed protein⁴⁶.

Light-driven electron transfer dynamics

Transient absorption spectroscopy in the visible spectral region reveals charge separated states and electron transfer rates in the RC maquette (Figure 3a). Singular value decomposition (SVD) of the transient spectra (Figures 3b, 3e and Extended Data Figures 1-8) resolve a time and wavelength dependence that is fit to an elementary kinetic model of first order reactions between the excited, charge separated and ground states to generate rate constants (Figure 3c) and spectra of the pure intermediate states (Figure 3d). Details of data analysis are given in Methods.

When the RC maquette is assembled with only P and no donor or acceptor (Figure 3b, ZnP monad, Extended Data Figure 1a), a 2 ns 532 nm laser pulse bleaches the ground state Soret absorption of P at 424 nm, and the broad absorption from 440 to 550 nm of the excited triplet state increases, decaying to the ground state in 3 to 5 ms. This rate is similar to that observed in other artificial protein designs²¹. When an acceptor is also bound to make a PA (ZnP-heme) dyad, the same triplet signature is observed but now decaying at 60 μ s as P^+ reduces the acceptor (Figure 3b, ZnP-heme dyad and Extended Data Figure 1b). Charge recombination, which has a larger driving force more closely matching the reorganization energy, proceeds rapidly in the dyad with little accumulation of the P^+A^- intermediate. Triplet inhibition of charge recombination is not apparent.

To trap a long-lived charge separated state, the RC maquette requires a DPA triad. When an electron donor is added to the PA dyad by mutating Leu168 to Tyr, a second, rapid charge separation forms D^+PA^- (Figures 3a and 3b, Y-ZnP-heme triad and Extended Data Figure 3). As in natural PSII, Tyr oxidation is a proton coupled electron transfer (PCET) reaction⁴⁵. Depending on design and environmental conditions, electron transfer may involve Tyr deprotonation before thermodynamically favorable ET (observable at pH values near the Tyr pK_a of ~ 10)⁴⁷, endergonic ET before PT, or concerted PTET. Figures 3a-d illustrate tyrosinate ET at pH 9.5, with an E_m value of ~ 0.72 V⁴⁷. The reduced heme/oxidized Tyr charge separated spectrum is dominated by the heme redox spectrum (Figure 3d, blue) persisting for 3 ms. Known extinction coefficients for heme and ZnP provide a yield estimate of 11% from the excited triplet state, which in turn permits an estimate of Tyr to P^+ ET of ~ 30 μ s (Figure 3c and Extended Data Figure 3a). The yield of Tyr oxidation is sensitive to the engineered proton

acceptors; when Leu replaces the Asp31 adjacent to the His71 which hydrogen bonds the Tyr, redox coupled proton transfer is inhibited, lowering the yield to ~3% and slowing charge recombination to a lifetime of 30 ms (see Extended Data Figure 5a).

Addition of metal ions to the binuclear His/Glu site introduces secondary electron donors. Mn(II) binding stabilizes the site, increasing RC maquette thermal stability (monitored by circular dichroism) from 81°C to > 100°C. Spectroscopic monitoring of Co(II) binding yields K_D values of 0.6 and 15 μ M for the first and second metal bound. The Irving-Williams series⁴⁸ predicts the trend of metal complex stability as Mn(II)<Fe(II)<Co(II); thus the Co(II) affinity represents an approximate upper limit for Mn(II) and Fe(II) affinities. (Details of metal binding thermodynamics will be reported elsewhere). Inserting Fe(II) into the binuclear metal site results in a charge separation lifetime of 250 ms (Figure 3e, orange and Extended Data Figure 7a), consistent with an E_m of ~0.53 V, similar to ferrocene. Mutation of Tyr168 to Leu causes the yield of charge separation to fall to an undetectable level, demonstrating that Tyr promotes electron transfer from Fe(II) (Extended Data Figure 7b). Substitution of Fe(II) with Zn(II), Cd(II) or Mn(II) suppresses Tyr oxidation at neutral pH, presumably because the metals inhibit proton transfer from Tyr to protonatable residues in the metal cluster site.

Mn oxidation is not simple. During photo-assembly in natural PSII⁴⁶, initial Mn(II) photo-oxidation steps are coupled to a dark rearrangement that takes ~150 ms, while in modified purple bacterial reaction centers somewhat similar to our system, Mn(II) oxidation has a lifetime of 12 ms^{26,28}. These rates are much slower than the rate of direct electron tunneling at these distances. We expect Mn(II) photo-oxidation to be slowed by such site rearrangements on a timescale longer than the 2.7 ms charge recombination of the tyrosine-ZnP-heme triad (see Extended Data Figures 5b and 6). Slower charge recombination is required for higher yields of Mn oxidation.

Like Fe(II), ferrocene (Fc) electron donors permit long charge separation lifetimes in the RC maquette (Figure 1d), because their relatively low E_m values inhibit the uphill reverse electron transfer from P to D⁺. Maleimide-functionalized Fc (E_m 0.56 V in DMF) is coupled to a Cys residue to make Fc168 or Fc164. Fc E_m values are modulated by the protein environment; Fc-modified azurin ranges from 0.51 to 0.55 V depending on surrounding amino acids⁴⁹. Figure 3e and Extended Data Figure 2a show the lifetime of the charge separated state of the Fc168-ZnP-heme B triad is 350 ms (cyan).

At the electron accepting end of the RC maquette, we confirm the prediction (Figure 1c) that the electron transport chain remains functional over a wide range of acceptor midpoint potentials. Replacement of heme B in the Fc168-ZnP-heme B triad with the Fe porphyrin DADPIX, which is 200 mV more oxidizing, changes the lifetime of charge separation from 350 ms to 20 ms and the yield of D⁺PA⁻ from 4% to 31% (see Extended Data Figure 2).

As an acceptor, Co(III)PPIX achieves highly desirable electrochemical and photochemical proton reduction to H₂ in natural proteins²⁵. It binds tighter to the RC maquette (K_D 250 nM) than to the natural 4-helix bundle cytochrome *b*₅₆₂²⁵. While bound CoPPIX is indeed photoreduced by the Zn porphyrin in the RC maquette under steady state illumination in the presence of ascorbate and EDTA as sacrificial electron donors, the quantum yield of long-lived charge separation using CoPPIX as the direct acceptor is low (see Extended Data Figure 8). Work with CoPPIX in myoglobin (E_m +0.1 V) is orders of magnitude slower than heme reduction⁵⁰, a sign that additional reorganization barriers, such as ligand exchange, accompany CoPPIX reduction. Just as slow Mn photo-oxidation is likely to be assisted by extending the donor redox chain, designs using CoPPIX as an effective terminal reductive catalytic center must pair it with a rapid initial electron acceptor in a reducing electron transfer chain.

Platform for photo-catalytic engineering

By exploiting the multi-cofactor adaptability of maquette design with permutations of donors and acceptors, we show how electron tunneling theory can guide the engineering of artificial proteins to harness light for directed electron transfer reactions. Observed charge separated states persist for hundreds of milliseconds, making the RC maquette a viable framework for photosynthetic redox catalysis. In contrast, chemically synthesized photochemical triads usually have charge separation lifetimes too short for chemistry; for example, the D⁺PA⁻ lifetime of a THF-solubilized Zn porphyrin-freebase porphyrin-C60 compound was reported to be just 34 μ s, a relatively long lifetime for a molecular triad⁶. Unlike synthetic constructs, artificial reaction center proteins co-opt cellular machinery to economically construct a nanometer scale scaffold. This synthetic biology approach takes advantage of the chemical functionality of natural amino acids while building on our understanding of the distance and energetic requirements for biological electron transport.

With the achievement of adequate charge separation lifetimes, the stage is now set for the challenging task of engineering catalytic sites, first by clarifying the principles for photo-oxidative assembly of Mn clusters in a framework that is simple, stable, and adaptable compared to PSII. The maquette method offers the promise of integrating artificial reaction centers with cellular light-harvesting systems in photosynthetic microbes or plants to divert energy into biofuel production, to couple water oxidation to fuel production in a single reaction center with high thermodynamic efficiency, or to create new arrangements and combinations of photosystems to improve solar input for photosynthesis.

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Methods

Protein expression and purification. A gene encoding the RC maquette including an N-terminal His₆-tag and TEV protease cleavage sequence of MGKGGHHHHHGGDGENLYFQ was purchased from DNA2.0 in a pJexpress414 vector with codons optimized for expression in *E. coli*. The TEV protease cleavage site is between Q21 and G22; G22 becomes G1 in the protein used for experimental studies discussed here. Primers for mutagenesis were purchased from Invitrogen, and mutant plasmids were PCR amplified using AccuPrime™ Pfx SuperMix (Invitrogen). Mutations including Y168L, Y168C, G164C, L71H, and L31D were prepared in this way. The final amino acid sequence of the RC maquette protein expressed for experimental studies (not including the N-terminal tag cleaved using TEV protease) was:

GSPELRQEHHQLAQEFQQLLQEIQLGRELKLGELQGIKQLREASEKARNPEKKSVLQKILEDEEKHIELLETLQQTGQEAQQLLQELQQTGQELWQLGGSGGPRLRQKHQQLA

Full sequences of all mutants are given in Supplementary Table 1. The plasmids were transformed into BL21-(DE3) competent *E. coli* cells (New England Biolabs), and bacteria were grown to 0.6 OD at 600 nm. Expression was induced with between 40 μ M and 1 mM IPTG for 4 hours at 37°C. Bacteria were pelleted by centrifugation, resuspended in 20 mM sodium phosphate buffer at pH 7.4 with 500 mM NaCl, 40 mM imidazole, 1% w/v octylthioglucoside, ~0.1 mg/mL DNase, and lysed by homogenization or sonication. Lysates were centrifuged at 25,000 g for 30 minutes, and supernatants were applied to a 5 mL Ni-NTA HisTrap FF prepacked column (GE Healthcare Life Sciences) on an Akta FPLC. Protein was eluted in 250 mM imidazole, 500 mM NaCl, 20 mM sodium phosphate at pH 7.4. The protein was treated with TEV protease and incubated at 4°C for up to 2 weeks, applied to an Ni-NTA column again, and finally purified by size exclusion chromatography (SEC) using an XK 16/70 column (GE Healthcare Life Sciences) packed with 110 mL of Superdex 75 prep grade gel filtration medium (GE Healthcare Life Sciences). Product molecular weight was verified by Matrix Assisted Laser Desorption Ionization-Time of Flight mass spectrometry (MALDI-TOF) with a synapic acid matrix.

Redox cofactors. Zn 5-phenyl 15-(p-carboxyphenyl) porphyrin (ZnP) was kindly provided by Dr. Tatiana Esipova from the research group of Sergei Vinogradov at the University of Pennsylvania. The synthesis details are described in Kodali *et al.*, 2017²¹. N-ferrocenyl maleimide lyophilized powder was purchased from Sigma-Aldrich. The two cysteine-containing RC maquette mutants (Y168C and Y168L/G164C) were subjected to the following standard maleimide-cysteine reaction protocol: N-ferrocenyl maleimide was dissolved in DMF at 10 mM concentration. Appropriate dilution was made so that 50 μ M of the maquette in 6 M Guanidine hydrochloride and 5 mM TCEP reacted with the N-ferrocenyl-maleimide at 1:10 ratio. The reaction was allowed to proceed with constant rotation overnight with protection from light. Labeled and unlabeled protein were separated by high-performance liquid chromatography (HPLC) using a Vydac 218TP54 C18 column with a gradient of water/acetonitrile (ACN) mixture going from 35% ACN:65% water to 65% ACN:35% water over 40 minutes. The unlabeled protein eluted at 51% ACN:49% water while the labeled protein eluted later at 55% ACN:45% water. The purity of the labeled protein fraction was examined and verified using MALDI-TOF.

Ultraviolet/visible spectroscopy. Ultraviolet/visible spectroscopy (UV/vis) was performed using a Varian Cary-50 spectrophotometer at room temperature. RC maquette concentration was determined using an extinction coefficient of $\epsilon_{280\text{nm}}=12490 \text{ M}^{-1}\text{cm}^{-1}$ attributed to Tyr and Trp absorbance. Heme B stock concentrations were measured by hemochrome assay⁵¹. Stock concentration of ZnP was estimated by mass measurement of dry powder. Tetrapyrrole stock solutions were prepared in dimethyl sulfoxide (DMSO).

Spectro-electrochemistry. UV-Vis light from an Ocean Optics light source was guided through fiber optic through a fine gold-honeycomb working electrode (Pine Instruments) surface-modified with cystamine and passed via fiber optics to an ocean optics spectrometer. The gold working and counter electrodes relative to an Ag/AgCl reference electrode, were set by a CH Instruments Electrochemical Analyzer. The following redox mediators⁵² were used to speed redox equilibration to a few minutes at each potential: for DADPIX and B-heme, the redox mediator mix was anthra-quinone-2-sulfonate (100 μ M), benzyl viologen (50 μ M), methyl viologen (50 μ M), indigo trisulfonate (50 μ M), phenazine (50 μ M). ZnP titration used a mixture of high potential mediators K₂[IrCl₆] (20 μ M), 2,6-p-quinone (20 μ M), K₄[Mo(CN)₈] (20 μ M), dicyano bis(1,10-phenanthroline) iron(II) dehydrate (10 μ M), K₃[Fe(CN)₆] (20 μ M).

Crystallography. All RC maquette crystals were grown in hanging drops at 4°C. Protein stock solutions contained 20-40 mM NaCl and 10-20 mM piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES) buffer at pH 6.5 and were stored at 4°C. Small amounts of porphyrin cofactors were added at room temperature from dimethyl sulfoxide stock solutions until a 1.0:1.0 equimolar ratio of porphyrin to protein was reached. Several minutes of equilibration time were given between additions of porphyrin in order to prevent aggregation and precipitation of cofactor. (Heme B was added prior to ZnP for crystal structure in PDB ID: 5VJS). Sample buffers were exchanged to the low salt PIPES buffer using 5 kDa MWCO Vivaspin Turbo 15 concentrators (Sartorius AG). Supplementary Table 2 gives for each crystal the RC maquette stock concentration used, cofactors included in the stock solution, well solution composition, drop volume, and cryoprotectant for each crystal structure. Crystal structure 5VJU used streak seeding with a cat whisker.

All X-ray crystallographic data sets were collected from single crystals at 100 K. X-ray diffraction data for crystal structures with PDB IDs 5VJS and 5VJT were collected at the National Synchrotron Light Source, beamline X6A using an ADSC Q270 CCD x-ray area detector. X-ray diffraction data for the crystal structure of L71H mutant (PDB ID: 5VJU) was collected using a Rigaku Micromax-007 HF rotating copper anode X-ray generator and VariMax HF optics with a Rigaku Saturn 944 HG CCD detector. Multiwavelength anomalous dispersion (MAD) data was collected to solve crystal structure 5VJS. Data were integrated with XDS⁵³ and initial phases were calculated using SOLVE⁵⁴. All other crystals used the 5VJS crystal structure as a model for molecular replacement using Phaser⁵⁵. Software packages CCP4i⁵⁶ and PHENIX^{57,58} were used throughout the structure solution and refinement process. Intensities were scaled using SCALA⁵⁹. Refinement was done using REFMAC5⁶⁰, phenix.refine⁶¹, and PDB_REDO⁶². Real space refinement was done by manually fitting models into electron density maps in Coot⁶³. Density modification was done using RESOLVE⁶⁴. Composite omit maps were created with simulated annealing in PHENIX to reduce model bias^{61,65,66}. Omit maps of cofactor binding sites are presented in Supplementary Figure 1. Statistics for the three data sets collected for MAD solution of crystal structure 5VJS are given in Supplementary Table 3. Data collection and refinement statistics for all three crystal structures, 5VJS, 5VJT, and 5VJU, are given in Supplementary Table 4.

Transient absorption. Samples contained ~3.5 μ M protein and 50 mM NaCl. For samples at pH 7.5 the buffer was 10 mM 3-(N-morpholino)propanesulfonic acid (MOPS), and at pH 9.5 the buffer was 10 mM N-cyclohexyl-2-aminoethanesulfonic acid (CHES). Where the electron acceptors heme B or DADPIX were used, the acceptor was added to the protein solution prior to ZnP from a DMSO stock solution. We used 1.0 molar equivalent of acceptor and ~0.8 equivalents of ZnP per protein. Solutions containing FeCl₂, MnCl₂, CdCl₂, or ZnSO₄ used a 10 μ M concentration of the metal salt. Protein solutions were maintained anaerobic under argon scrubbed by a reducing VSO₄ solution⁶⁷.

Transient absorption experiments were performed at 20°C with a 10 Hz Q-switched frequency-doubled Nd:YAG laser (532 nm, DCR-11 Spectra Physics). A Xe flash lamp emitted probe beams of white light into split fiber optic cables with a gate width of 600 ns to 1 μ s at delay times after the laser flash to measure the change in absorbance spectra as a function of time after the laser flash. The split fiber optic cables were used to direct one probe beam through the laser-pumped (experimental) region and the other probe beam through the dark (reference) region of the sample in the cuvette. Each probe beam was collected by a fiber optic bundle and flattened into a line of fibers at the entrance slit of an Acton SP-2156 spectrograph. The images from the exit slit of the monochromator were focused onto a Princeton Instruments PiMax-3 ICCD camera operated with WinSpec32 proprietary software. Delay times were set by a Stanford Research System DG 535 digital delay generator. A Thorlabs beam shutter allowed the 10-Hz laser repetition rate to be lowered to seconds for measurements at times longer than 100 ms.

Difference spectra were collected for delay times of 1 μ s to ~1 s in intervals of half log time units. The same sample was measured at different delay times, and at least 50 spectra were collected for each sample at each delay time. Comparison of difference spectra at 1 μ s that were collected at the beginnings and ends of transient absorption experiments revealed no significant photodegradation over the course of the experiments.

SVD analysis. Singular Value Decomposition (SVD) is applied to the data matrix obtained from preprocessing of the raw transient absorption data. The analysis in Mathematica (Wolfram) calls the built-in “svd” function on the raw data. The raw data is represented by matrix A, with dimensions l by t, where l is the number of wavelengths and t is the number of time-resolved snapshots taken during the experiment. Calling the “svd” function using A as the input variable returns a left matrix U with dimensions l by l, a central matrix S with dimensions l by t, and a right matrix V with dimensions t by t.

The right matrix V resulting from the SVD is used to fit a kinetics model described by solutions of ODEs depending on the configuration of the photosystem examined. The model fitting follows a modified version of the method described by Henry and Hofrichter⁶⁸ where a linear transformation matrix C is applied to the expected population matrix P computed from the model. The L₂ norm of the difference between the right t by t matrix and the product CP is minimized by the standard minimization function, in which the rates of various electron transfers in the kinetics model along with the elements of the C matrix are allowed to vary. The minimization function is instructed to utilize the quasi-newton minimization procedure. Various initial conditions are tried in order to obtain the best fitting of the data. The results of the minimization is examined by comparing the residual of the minimization to other values obtained from different sets of initial conditions to ensure that the minimum discovered by the algorithm is, as far as the information reveals, the global minimum or a satisfying local minimum. The values of the C matrix as well as the rates of the electron transfer between the states are then used to compute the reconstructed elemental spectra that make up the observed raw data as well as the electron transfer kinetics that best explains the observed data.

Electron-tunneling yield calculations. The contour plot of Figures 1c and d are calculated by solving the set of differential equations for the kinetic scheme of Figure 1b, beginning with the triplet state D³P*A at time zero, and evolving to the states DP*A, D⁺PA⁻ and DPA using the electron tunneling expression of equation 1 for exergonic electron transfer reactions; for endergonic reactions, Equation 1 is used to calculate the electron tunneling rate for the reverse, exergonic reaction and then a rate penalty of 1 order or magnitude is applied for every 0.06 eV of endergonic free energy. The non-electron-tunneling rate of phosphorescence decay of the triplet state is 250 s⁻¹. The set of differential equations are solved in Mathematica for the fractional yield of the charge separated D⁺PA⁻ state 100 μ s after time zero for plotting. For Figure 1c, the edge-to-edge distance between P and A is varied, as is the E_m of the acceptor A. Other tunneling parameters are held constant: D-P distance 4.6 Å, D-A distance 23.0 Å, E_m D 0.72 V corresponding to tyrosinate donor, E_m P 0.91 V, triplet

photon energy 1.6 eV, reorganization energy 0.8 eV. For Figure 1d, the edge-to-edge distance between P and D is varied, as is the E_m of the donor D, while the P-A distance is held constant at 13.2 Å and the E_m of A set at -0.19 V, corresponding to heme B acceptor.

Data availability

X-ray crystallographic coordinates and data files were deposited at the Protein Data Bank (PDB) with accession codes 5VJS (RC maquette with heme B, ZnP, and Zn²⁺), 5VJT (RC maquette with heme B and Zn²⁺), and 5VJU (RC maquette L71H mutant with heme B and Cd²⁺). All data reported here are available from authors upon request.

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Declarations

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Author contributions

N.M.E., Z.Z., P.L.D., and C.C.M. conceived the project. B.M.D. contributed to the conception and design of experiments. N.M.E. designed and crystallized the RC maquette protein. S.E.S. and N.M.E. collected and processed X-ray diffraction data and solved crystal structures. N.M.E. and Z.Z. purified proteins. Z.Z. crosslinked N-ferrocenyl maleimide to cysteine-containing RC maquette mutants. N.M.E., Z.Z., and C.C.M. conducted transient absorption experiments and analyzed data. N.M.E. and C.C.M. wrote the manuscript with contributions from Z.Z., S.E.S., B.M.D. and P.L.D.

Competing Interests

The authors declare no competing interests.

Additional Information

Extended data is available for this paper.

Supplementary Information is available for this paper.

Correspondence and requests for materials should be addressed to N.M.E.

Figures

Figure 1

Design of natural and artificial photosynthetic reaction centers. a The light-activated donor-pigment-acceptor (DPA) electron transfer triad core of photosynthesis and X-ray crystal structure at 2.0 Å resolution of the RC maquette with a metal cluster/tyrosine donor, Zn porphyrin pigment, and heme B acceptor (PDB ID: 5VJS). b Kinetic schemes of a light activated system show energies plotted relative to ground state before light activation. An RC maquette DPA triad is compared with representative PSII charge separated states (orange)³². c Contour plots of expected relative charge separated D+PA- yield after 100 μs for a range of P-to-A edge-to-edge distances vs. acceptor E_m (using E_m of 0.91 V for P/P+ and E_m of 0.72 V for tyrosinate donor). Dashed lines show cofactor anchoring residues adjusted in increments of ~1 helical turn (~5.2 Å). Fe porphyrins heme B and DADPIX shown as acceptor alternatives. d Corresponding contour plot for D-to-P distances and driving forces; acceptor is heme B; tyrosinate, Mn(II), Fe(II) and cysteine-coupled ferrocene (Fc164 and Fc168) shown as possible donors.

Figure 2

Structural similarities between cofactor binding sites of designed RC maquette and natural proteins are significant despite lack of sequence identity. RC maquette crystal structures are shown in white with heme electron acceptor and ligating His residues shown in green, ZnP pigment and His in red, and electron donating Tyr, metal ion spheres and first shell ligands in blue. Natural protein structures are shown in grey with cofactors colored as in RC maquette. Metal-bridging oxygens are shown as red spheres. Blue dotted lines represent hydrogen bonds. a RC maquette (PDB ID: 5VJS) and cytochrome b_6 (PDB ID: 6RQF43). b RC maquette-L71H mutant with Cd(II) (PDB ID: 5VJU) and PSII (PDB ID: 6DHE44). c RC maquette-L71H mutant with Cd(II) (PDB ID: 5VJU) and bacterioferritin with Cd(II) (PDB ID: 4CVS12).

Figure 3

Transient spectroscopy of RC maquette reveals the dynamics of light-activated charge separation and recombination. **a** Difference spectra of the tyrosinate-ZnP-heme triad in L31D/L71H mutant at pH 9.5 are shown for delay times from 1 μs to 3 seconds after the laser flash at log time intervals shown in **B**. **b** The first and second principal time varying SVD components of the difference spectra (circles and squares, respectively) fit to a simple kinetic model connecting DP^*A , DP^*A^- , D^+PA^- , and DPA states with single exponential first order reactions (solid lines): ZnP monad (red), ZnP-Heme dyad (green), tyrosinate-ZnP-heme triad (blue), (see also Extended Data Figures 1, 3). **c** Fitted log rates for the tyrosinate-ZnP-heme triad. **d** Fitted DP^*A and D^+PA^- difference spectra for the tyrosinate-ZnP-heme triad dominated by ZnP bleach and heme redox difference spectra, respectively. **e** Fitted time varying SVD components for the ferrocene-ZnP-Heme triad (cyan) and iron-Tyr-ZnP-heme tetrad (orange) show charge separation lifetimes up to 300 ms.

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