Structural Basis of the Drastically Increased Initial Electron Transfer Rate in the Reaction Center from a *Rhodopseudomonas viridis* Mutant Described at 2.00-Å Resolution*

C. Roy D. Lancaster‡§, Marina V. Bibikova¶, Piera Sabatino**, Dieter Oesterhelt‡, and Hartmut Michel‡

From the ‡Max-Planck-Institut für Biophysik, Abteilung Molekulare Membranbiologie, Heinrich-Hoffmann Str. 7, D-60528 Frankfurt am Main, Germany and ¶Max-Planck-Institut für Biochemie, Abteilung Membranbiochemie, Am Klopferspitz 18a, D-82152, Germany

It has previously been shown that replacement of the residue His L168 with Phe (HL168F) in the *Rhodopseudomonas viridis* reaction center (RC) leads to an unprecedented drastic acceleration of the initial electron transfer rate. Here we describe the determination of the x-ray crystal structure at 2.00-Å resolution of the HL168F RC. The electron density maps confirm that a hydrogen bond from the protein to the special pair is removed by this mutation. Compared with the wild-type RC, the acceptor of this hydrogen bond, the ring I acetyl group of the “special pair” bacteriochlorophyll, D₁, is rotated, and its acetyl oxygen is found 1.1 Å closer to the bacteriochlorophyll-Mg²⁺ of the other special pair bacteriochlorophyll, D₃M. The rotation of this acetyl group and the increased interaction between the D₃L ring I acetyl oxygen and the D₃M-Mg²⁺ provide the structural basis for the previously observed 80-mV decrease in the D⁺/D redox potential and the drastically increased rate of initial electron transfer to the accessory bacteriochlorophyll, Bₐ. The high quality of the electron density maps also allowed a reliable discussion of the mode of binding of the triazine herbicide terbutryn at the binding site of the secondary quinone, Qₐ.

Life on earth depends on the ability of photosynthetic organisms to convert solar energy into biochemically amenable energy. A central role in the photosynthetic process is played by the photosynthetic reaction center (RC), an integral membrane protein-pigment complex. The RCs from purple bacteria, which catalyze the light-induced reduction of ubiquinone to ubiquinol (or ubiquinone) involving the uptake of two protons from the cytoplasm and the oxidation of cytochrome c₂ in the periplasm, are the best characterized membrane protein complexes (see Refs. 1–4 for reviews). The RC of the non-sulfur purple bacterium *Rhodopseudomonas* (Rp.) has been reclassified as *Blastochloris* (5) *viridis*. The RC is composed of four polypeptides, namely the L, M, H, and C (a tightly bound tetra-heme cytochrome c) subunits (6) and fourteen cofactors (four heme molecules, four bacteriochlorophyll b, two bacteriopeophytin b, one carotenoid, one non-heme iron, and two quinones, as described previously (7). The four heme molecules are covalently bound by the C subunit, and all other cofactors are non-covalently bound by the L and M subunits. The complex has eleven membrane-spanning helices, five in the L, five in the M, and one in the H subunit. Large parts of the L and M subunits and their associated cofactors are related by a 2-fold rotational symmetry axis perpendicular to the plane of the membrane (7–10).

Light is absorbed by the bacteriochlorophyll of the B1015 light-harvesting antenna. Excitation energy is then transferred to a dimer of bacteriochlorophyll, the special pair D₁, thus forming the excited state D*. This decays to D⁻⁻ through electron transfer via the monomeric accessory bacteriochlorophyll Bₐ and the bacteriopeophytin d₈ (11, 12) to the primary quinone Qₐ, which is a menaquinone-9 in the *Rp. viridis* RC. The electron is then transferred to a secondary quinone, Qₐ, which is ubiquinone-9 in the *Rp. viridis* RC. Whereas Qₐ can accept only one electron, Qₐ functions as a “two-electron gate” (13), and after a second reduction and the uptake of two protons from the cytoplasm, the ubiquinol leaves its binding site (14, 15) to be reoxidized by the cytochrome bc₁ complex (16), which results in the release of protons on the periplasmic side of the membrane. This proton transport produces a transmembrane electrochemical potential that drives ATP synthesis through the ATP synthase (17). The electrons that are released upon quinol re-oxidation are cycled back to the reaction center via a small soluble protein, cytochrome c₂ (18), and ultimately re-reduce D⁻⁻ via the tetra-heme C subunit.

For the investigation of the mechanism of the primary electron transfer reactions, RC mutants where the energetics of D⁺ and D⁻ are changed by the removal or addition of hydrogen bonds between the special pair and amino acid side chains have been extensively studied (see Ref. 19 for a review). In both

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The atomic coordinates and structure factors (code 1DXR) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

§ To whom correspondence should be addressed. Tel.: 49-69-96769-419; Fax: 49-69-96769-423; E-mail: Roy.Lancaster@mpib-frankfurt.mpg.de.

‡ Present address: Dept. of Biochemistry, University of Utah School of Medicine, 50 N. Medical Dr., Salt Lake City, UT 84132.

** Present address: Dipartimento di Chimica G. Ciancian, Università di Bologna, via Selmi 2, I-40126 Bologna, Italy.

The abbreviations used are: RC, reaction center; Bₐ and Bₘ, monomeric bacteriochlorophyll on the active and inactive branch, respectively; D₁ and D₃M, special pair bacteriochlorophyll bound to the L and M subunits, respectively; Fₐ and Fₘ, observed and calculated structure factors, respectively; HL168F, mutant with His L168 → Phe RC; PDB, Protein Data Bank; Qₐ and Qₘ primary and secondary acceptor quinones, respectively.

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Rhodobacter sphaeroides and *R. viridis* RCs, histidine L168 donates a hydrogen bond to the ring I acetyl group of D_5 (see Ref. 20 for a review). In a mutant RC from *R. viridis*, where His L168 is replaced by phenylalanine (HL168P), the D/D' redox potential is 80 mV lower than that of the wild-type RC (21). The decay of the excited electronic state D' is accelerated by more than a factor of three. This leads to a remarkable increase in the population of D_A, thus providing strong support for a stepwise electron transfer mechanism from D' to Φ_A with the accessory bacteriopheophytin B_5 as a short-lived real electron carrier (21, 22).

Here we report the structural characterization of the His L168 → Phe *R. viridis* RC by crystallographic refinement to a resolution of 2.00 Å. The data collected to resolve the nature of the changes introduced by this mutation turned out to be of sufficient quality to also reliably describe the mode of binding of the triazine inhibitor terbutryn (2-t-butylamino-4-ethylamino-6-methylthio-s-triazine) to the Q_B site. Although the terbutryn binding site had been localized by x-ray crystallographic analysis of the RC-terbutryn complex, based on 38,663 unique reflections up to a resolution of 2.9 Å (R-factor = 23.8% (23)), description of the exact nature of protein-terbutryn interactions has had to await the refinement of this higher resolution structure. In addition, possibly biologically relevant interactions has had to await the refinement of this higher resolution structure. The crystallographic assignment of the atomic model also include the crystallographic assignment of a large number of additional tightly bound water molecules, which are of particular relevance to the discussion of proton transfer pathways to the Q_B site.

**EXPERIMENTAL PROCEDURES**

**Protein Preparation and Crystallization**—The *R. viridis* HL168F mutant was grown, and chromatophores were isolated as described (21). Reaction centers were purified as reported in Refs. 24 and 25, with modifications reported in Ref. 26, and a Q_{B0} removal step, which is described in Ref. 27. Crystallization of the RCs was performed as reported (24), except that the pH value was 7.0, and the RCs were crystallized in the presence of 50 μM terbutryn (Riedel-de-Haen, Seelze, Germany). Crystals were carefully removed from the depression wells and rinsed with soak buffer (as reported in Ref. 8, except that the pH value was 7.0, and 50 μM terbutryn were present). Crystals belong to the tetragonal space group P4_2_2_2 and were isomorphous to the original crystals with unit cell dimensions a = b = 223.5 Å, and c = 113.6 Å (8).

**Data Acquisition and Processing**—Data collection was performed in the dark with a temperature of −10 °C at the crystal. Diffraction data were collected using monochromatic synchrotron radiation at a wavelength of 1.07 Å at the GMB/MPG-BWS beamline (HASYLAB/DESY, Hamburg, Germany) with a MARCCD detector in frames of 0.2° through a continuous angular range of 45°. Diffraction data from just one crystal were processed and scaled using the HKL programs DENZO and SCALEPACK (28) and TRUNCATE from the CCP4 program suite (29) as summarized in Table I.

**Initial Model Building and Refinement**—Because the crystal used in this study was isomorphous to those of the original (8) and previous work (27, 30), difference Fourier and simulated annealing omit map techniques (31) with refined co-ordinates of the structure of the RC complex with a chiral aziridine derivative, PDB entry code 6PRC (30), as a starting structure could be used for the approximation of initial phases and the construction of an initial model.

Crystallographic refinement was performed as described previously (27, 30) using iterative cycles of simulated annealing, conventional positional refinement, isotropic overall B-factor refinement, and restrained refinement of individual B-factors with the program X-PLOR (version 3.1; see Ref. 32). Manual inspection and refitting were done with the molecular graphics program O (33). In particular, the assignments of the oxygen and methyl positions in the acetyl groups of the bacteriopheophytin and bacteriopheophytin molecules were checked as described previously (27), and the same criteria for the assignment of water molecules as described earlier (27, 30) were applied. Individual B-factors were refined but restrained to target standard deviations of 1.5 Å² for the difference of B-factor values of bonded main-chain atoms (2.0 Å² for side-chain atoms) and 2.0 Å² for main-chain atoms related by bond angles (2.5 Å² for side-chain atoms). The refinement statistics and the quality of the final model are summarized in Table I. Unless stated otherwise, comparisons to the wild-type structure are with respect to the starting structure coordinates 6PRC (30), because these had been refined with the same protocol and parameters as were used here. Figures were prepared with a version of Molscript (34) modified for color rendering (35) and map drawing (36) capabilities.

### RESULTS

The structure of the HL168F RC in complex with terbutryn is summarized in Table I. This is the best-defined *R. viridis* reaction center structure to date, and the crystallographic R-factor and R_free are 19.4% and 21.8%, respectively, for all data between 10.0 and 2.00 Å (Table I). The position error is estimated to be 0.15 Å. Simulated annealing omit electron density maps (31) were calculated for the site of mutation (Fig. 1) and the terbutryn binding site (Fig. 2). All residues of interest can be detected clearly in the electron density.

As can be seen in Fig. 1A, the Phe side chain of the exchanged residue L168 adopts a conformation very similar to the wild-type His. In the wild-type RC, the His Nε atom donates a hydrogen bond to the ring I acetyl oxygen of bacteriopheophytin D_5 (23). The position of this oxygen atom in the wild-type RC structure is not covered by the mutant RC electron density (Fig. 1, A and B). In the refined structure of the HL168F RC, this acetyl group has undergone a 20° rotation relative to the wild-type RC, thus shortening the distance between the acetyl oxygen atom and the Mg²⁺ center of bacteriopheophytin D_5.

| Table I | Data collection and refinement statistics for the Rp. viridis His L168 → Phe RC crystal (PDB entry 1DXR) |
|---------|-------------------------------------------------------------------------------------------------|
| Data                                            |                                                                                               |
| Resolution range (Å)                           | 15.0–2.00                                                                                     |
| No. of measured reflections                    | 864,524 (4.6)                                                                                  |
| Completeness (%)                               | 87.2                                                                                            |
| No. of unique reflections                      | 187,940                                                                                        |
| Rfree (%)                                      | 29.6                                                                                            |
| Rfree (%)                                      | 0.15                                                                                            |
| Refinement                                     |                                                                                               |
| Resolution range (Å)                           | 10.0–2.00                                                                                     |
| Rfree (%)                                      | 29.4                                                                                            |
| Data collection and refinement statistics for the Rp. viridis His L168 → Phe RC crystal (PDB entry 1DXR) |

### Data

- **Protein atoms**: 9385
- **Heterogen atoms**: 807
- **Solvent atoms**: 585
- **B from Wilson plot (Å²)**: 24.2
- **Average B-factor (Å²)**: 30.2
- **n_free/R_free²**: 4.3
- **r.m.s. Deviations from ideal values**: 0.011 Å
- **Bond lengths (Å)**: 1.4 Å
- **Dihedral angles (°)**: 2.1 Å
- **Improper angles (°)**: 23.3 Å

### References

1. Rp. *viridis* His L168 → Phe Mutant RC at 2.00-Å Resolution

2. Data collection and refinement statistics for the Rp. viridis His L168 → Phe RC crystal (PDB entry 1DXR)
HL168F mutant RCs.

**DISCUSSION**

**Primary Electron Transfer**—The mechanism of the initial electron transfer reactions in the RC has been a subject of intense studies (3, 37, 38). A useful tool in this context are intense studies (3, 37, 38). A useful tool in this context are mutant RCs where the energetics of D* and D+ are changed by removal or addition of hydrogen bonds between the special pair and amino acid side chains. A hydrogen bond to the bacteriochlorophyll groups of the special pair stabilizes the neutral

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state of D relative to the oxidized state D\(^{\text{Ox}}\) and thus increases the D/D\(^{\text{Ox}}\) redox potential (39). In the \(\text{Rp. viridis}\) HL168F mutant RC studied here, the D/D\(^{\text{Ox}}\) redox potential is 440 mV, 80 mV lower than that of the wild-type RC (21). The decay of the excited electronic state D\(^*\) is accelerated, with the associated time constant being reduced from 3.5 to 1.1 ps. The effects of the His L168\(^{\text{Phe}}\) exchange on the structure described above, involving the loss of the hydrogen bond to the ring I acetyl group of D\(_{L}\) and its 20° rotation, resulting in an increased interaction between the acetyl group oxygen and the Mg\(^{2+}\) of DM, may contribute to the stabilization of the oxidized state D\(^{\text{Ox}}\) relative to the neutral special pair and thus at least partially explain the observed lowering of the redox potential and the accelerated rate of initial electron transfer from D\(^*\) to D\(^{\text{Ox}}\) BA.

Another reason for the increased electron transfer rate may be an increased electronic coupling between D and BA (21). However, the reduction in the distance between D and BA in the HL168F RC discussed above is only on the order of the estimated position error of the structure.

Another previously described spectroscopic feature of the HL168F RC is that the Q\(_{Y}\) transition of the special pair is shifted by 35 nm to shorter wavelengths (930 nm), indicating perturbations of the electronic structure and/or a changed excitonic coupling of the two bacteriochlorophyll molecules of the special pair (21). In addition to the structural changes of the D\(_{I}\) ring I acetyl group, the slight rotation of D\(_{L}\) and the reduced Mg-Mg distance between D\(_{L}\) and D\(_{M}\) may also contribute to this feature. It is expected that the high quality of the determined structure will provide a useful basis for computational studies for a more quantitative description of the observed effects of the His L168 \(\rightarrow\) Phe mutation.

Terbutryn Binding—The Q\(_{B}\) site is a well established site of herbicide action, both in the purple bacterial RC and in the reaction center core of photosystem II, for which the former serves as a model (40–44). Over 50% of commercially available herbicides function by inhibition of higher plants at the Q\(_{B}\) site of the D1 polypeptide of the photosystem II reaction center (45). A commercially very important class of herbicides are the triazines, which were introduced by J. R. Geigy S. A. in the 1950s (46). Prominent examples are atrazine and terbutryn. Interestingly, the binding affinity for the \(\text{Rp. viridis}\) wild-type RC of terbutryn is 14-fold higher than that of atrazine (47). The reduction in the interplanar angle between the triazine ring and Phe L216 from 24° in the case of atrazine to 17° in the case of terbutryn may not be sufficient to explain the higher affinity by increased \(\pi-\pi\) interactions. The 6-methylthio group of terbutryn, as opposed to the 2-chloro group of atrazine, apparently fits more snugly to the Q\(_{B}\) pocket with the methyl group within van der Waals distance to the C\(_{b}\) atom and a C\(_{d}\) atom of Ile L229, thus offering a second explanation for the increased affinity.

Water Molecules—Generally in proteins, internal water not only fills structural cavities, but it is also necessary to stabilize three-dimensional folding (48, 49). As has been discussed earlier for the structures 2PRC, 3PRC, and 4PRC (27), some of the additionally determined water molecules make important contributions to crystal packing and are also possibly relevant to the pathways and kinetics of proton transfer to the Q\(_{B}\) site (27, FIG. 2 .

**FIG. 2.** Terbutryn binding to the Q\(_{B}\) site. A and B (orthogonal views). Simulated annealing omit 2\(\text{Fo}\) – 2\(\text{Fc}\) electron density maps contoured at a level of 1.2 \(\sigma\) are shown. The terbutryn S atom is shown in yellow, and other atom colors are as for Fig. 1. Hydrogen bonds are indicated by dashed lines. C and D, orthogonal views. Comparison of terbutryn and atrazine binding to the Q\(_{B}\) site is shown. The RC complex with atrazine (Ref. 30; PDB entry 5PRC) is drawn in black, and the complex with terbutryn (see this work and PDB entry 1DXR) in green.
50) and quinol release from the Qb site (27, 51). In the specific case of triazine binding, they are also important for optimal inhibitor binding (see Ref. 30 and this work). In particular, we expect that the large number of additionally assigned water molecules will enrich computational simulations of coupled electron-proton transfer to the Qb site.

Concluding Remarks—The present results demonstrate that the structural changes underlying the drastic functional changes associated with replacing His L168 with Phe are limited. In principle, it would appear advantageous for the RC to have a Phe at position L168, because the increased initial electron transfer rate improves the quantum efficiency of photosynthesis (21). However, the associated “blue shift” of the Qy absorption band of the primary donor D also makes energy transfer from the B1015 light harvesting antennae less efficient, whose absorption characteristics are optimized for the natural environment of the R. viridis RC. In summary, the presence of His at position L168 demonstrates how nature tunes the RC to function more efficiently with its surrounding antennae, rather than optimize the isolated function of the RC.

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