Light-induced structural changes in the bacterial reaction center were studied by a time-resolved crystallographic experiment. Crystals of protein from Blastochloris viridis (formerly Rhodopseudomonas viridis) were reconstituted with ubiquinone and analyzed by monochromatic and Laue diffraction, in the dark and 3 ms after illumination of the crystal with a pulsed laser (630 nm, 3 mJ/pulse, 7 ns duration). Refinement of monochromatic data shows that ubiquinone binds only in the “proximal” QA binding site. No significant structural difference was observed between the light and dark datasets; in particular, no quinone motion was detected. This result may be reconciled with previous studies by postulating equilibration of the “distal” and “proximal” binding sites upon extended dark adaption, and in which movement of ubiquinone is not the conformational gate for the first electron transfer between QA and QB.

The mechanism of secondary electron transfer has been investigated using crystallographic freeze-trapping experiments in reaction centers from Rhodobacter sphaeroides. Several reviews describe the structure of the bacterial reaction center in detail (8, 9). The terminal acceptor of electrons within the reaction center is a ubiquinone-entailed Qb, which binds to an active site near the cytoplasmic side of the photosynthetic membrane. While bound to the reaction center, Qb accepts two electrons, which are coupled to the uptake of two protons. The transfer of one electron leads to the state P’Qb. At room temperature and pH values ranging from 6–9, this state decays with a half-time of ~100 ms (10, 11). Should the special pair be reduced within this time frame, the state Qb decays with a half-time of 65 s (10).

Upon transfer of a second electron, Qb is released from the reaction center as dihydroquinol (12).

The mechanism of secondary electron transfer has been proposed to involve a structural change at or near the Qb binding site (13–15). Recently, several crystallographic freeze-trapping experiments in reaction centers from Rhodobacter sphaeroides have suggested a significant rearrangement within the Qb binding site in response to electron transfer (16–18). The ubiquinone binds in a “distal” binding site in the dark, and moves approximately 4.5 Å to a “proximal” binding site upon illumination (Fig. 1), with an accompanying 180° rotation of the aromatic ring about the isoprene tail. In these experiments, the occupancy of the Qb site was increased by reconstitution with synthetic ubiquinone UQ2, which has a significantly shorter isoprenoid tail than native ubiquinone (UQ9 in Blastochloris viridis, UQ10 in R. sphaeroides).

Separately, crystals of the reaction center of B. viridis were prepared in which endogenous ubiquinone was specifically depleted and reconstituted with UQ2. In this case, the refinement of a dark structure showed UQ2 to occupy only the proximal binding site (19). However, concurrent reexamination of the original structure of the B. viridis reaction center (20), which contained a minor fraction of endogenous Qb, suggested partial occupancy of ubiquinone UQ9 in both the distal and proximal binding sites (21).

Two recent molecular dynamics simulations probed the position of Qb in R. sphaeroides and B. viridis. In R. sphaeroides, the ubiquinone was found to occupy the distal or proximal binding sites in both neutral and semianionic states. The site occupied depended on the protonation state assigned to Glu-L212 and Asp-L213 (22). In B. viridis, spontaneous movement from the distal site to the proximal site depended on the initial orientation of the quinone in the distal site in both the PO4Qb and P’Qb states (23). Neither simulation reproduced the 180° rotation of the aromatic ring between dark and light structures observed in the freeze-trapping experiments (16–18).

Elucidation of the structural mechanism accompanying electron transfer from QA to Qb is hampered by several factors: low occupancy of or heterogeneity within the Qb binding site; influence of solvent on occupancy of the proximal or distal binding site; influence of the conditions of freezing; and sensitivity to the conditions of illumination, in which the timing, intensity, and duration of illumination may lead to incomplete photoactivation or significant charge recombination between Qb and the special pair.

The advent of third generation synchrotrons as sources of brilliant, pulsed, polychromatic x-ray radiation has fostered the technique of time-resolved crystallography, utilizing the Laue diffraction method (24). A laser pulse and subsequent train of synchrotron x-ray pulses represent a pump–probe experiment, in which the temporal resolution can be as short as 150 ps (25).

Time-resolved crystallography with nanosecond time resolution has been successfully employed to observe the photolysis, protein relaxation, and rebinding of carbon monoxide from myoglobin (26) and the photocycle of photoactive yellow protein (27).

We are motivated by the following questions. Are the quinone motion, rotation, and accompanying structural changes reported in R. sphaeroides also observed in B. viridis? If so, with what time course do they occur? To identify the position of the ubiquinone during secondary electron transfer, we performed two experiments. First, we repeated the crystallographic refinement against
in 24-well plates (Hampton Research, Riverside, CA) with 20μL/H9262

Experimental Procedures

observe a homogeneous charge-separated state within the crystallization process to minimize the possibility of spectroscopic heterogeneity and a shorter delay between excitation and monitoring, we performed experiments at room temperature and thus retain the possibility of observing tertiary structural changes upon electron transfer and eliminate none (UQ10) similar to the endogenous UQ9. Second, we reacted center complexed with a naturally occurring ubiquinone binding. The distal position is shown in purple, the proximal in orange. Shown are coordinates from 1AIG and 1AIJ (16).

Figs. 1, 3 and 4 were prepared with Pymol.

Protein samples were exchanged into a buffer containing 50 mM sodium phosphate at pH 6.8, 0.1% lauryldimethylamine oxide (LDAO) (Fluka), and 10 μM EDTA. UV/Visible absorption spectroscopy was used to judge the purity (A280/A30 < 2.1) and oxidation state (cytochrome c553) of the purified protein. Protein samples were exchanged into a buffer containing 50 μM UQ2 or UQ10 prior to crystallization, and the same concentration was present in all solutions prepared thereafter. Crystals were grown in the dark at 18°C, by sitting drop vapor diffusion in 24-well plates (Hampton Research, Riverside, CA) with 20 μL of sample and 1 ml of reservoir. Conditions were as originally described (30), with the presence of triethylenediammonium phosphate as an additional variable (31). Diffraction quality crystals grew in 1–3 weeks in 1.8 M ammonium sulfate, 0.1% LDAO, 3% 1,2,3-heptanetriol.

X-Ray Data Collection. Prior to mounting, crystals were soaked in a buffer containing 80/20 μM potassium ferri-/ferrocyanide to oxidize the high-potential hemes in the cytochrome subunit. Crystals were then transferred to an identical soak buffer without iron to remove the oxidant, which is known to interfere with secondary electron transfer (32). Crystals were mounted in 0.7- to 1.0-mm capillaries (glass no. 50, Hampton Research), with the longer dimension of the crystals (c axis) aligned along the capillary axis. Monochromatic diffraction data were collected at BioCARS beamline 14-BM-C, Advanced Photon Source, Argonne National Laboratory, at room temperature in darkness, with an x-ray wavelength of 0.9 Å. Oscillation images (180) spaced 0.5° apart were collected on an Area Detector Systems Corporation (Poway, CA) Quantum-4 charge-coupled device detector positioned 290 mm from the crystal ensure full coverage of reciprocal space.

Laeu data were collected on BioCARS beamline 14-ID-B at 15°C in darkness and 3 ms after illumination by a 7-ns laser pulse from a frequency-doubled Nd:YAG pumped dye laser at 630 nm, 3 ml/pulse. This is the minimum optical density of the sample in the visible region, which is approximately 1 per 140 μm. The pulse was delivered to the crystal by two oppositely aligned optical fibers, focused to a spot of an ~1-mm diameter at the sample. According to calculations, the resulting fluence should achieve ≥95% photoactivation within the crystal (Supporting Methods, which is published as supporting information on the PNAS web site). The fibers were inclined at 45° to the x-ray beam, the crystal rotation axis, and the horizontal plane. The laser pulse was synchronized to the x-ray pulse by means of a millisecond x-ray shutter, such that the laser pulse arrived at the sample 3 ms before the center of a 2-μs x-ray pulse. The x-ray pulse was derived from an undulator source that produced a 1st order maximum at 1.05 Å, with significant intensity from 0.95 to 1.4 Å, delivered into a cross-sectional area of 100 × 100 μm². Diffraction data were collected on a MAR CCD detector positioned 200 mm from the crystal, in two sets of 11 static images spaced by 9°. The second set of images was offset from the first by crystal translation along, and rotation by 4.5° about, the goniometer axis. Depending on the size of the crystal, this method resulted in exposure of 20–30% of the crystal to the x-ray beam during collection of a complete dataset whereas the entire crystal was illuminated by the laser in every light image. Dark and light data were collected at each angular setting, with ~3 s between each light image and the subsequent dark image to allow for recovery of the dark state.

Data Processing and Refinement. Monochromatic data were processed to 2.9 Å resolution with DENZO and SCALEPACK. Refinement was performed with CNS (33), with visualization and model manipulation performed in XTLVIEW (34). An initial model was obtained from the structure of Q9-depleted RC from B. viridis, PDB code 3PRC (19). Detergent, sulfate ions, and all multiple side-chain conformations were removed. All occupancies were set to either 1 or 0, and all B-factors to 30 Å². Following initial rigid-body refinement, several rounds of simulated annealing, minimization (maximum-likelihood function with implicit solvent mask), and model building led to a model with R (Rfree) of 0.250 (0.253). At this stage, positioning of detergent, sulfate, and Q9 was justified on the basis of density in the Fobs – Fc map. The final stage involved restrained individual B-factor refinement followed by a final round of minimization.

Laeu data was processed using LAUEVIEW, which provides for indexing, integration, and scaling of Laue data sets including harmonic deconvolution of energy overlaps (35, 36). A total of 80 images from five crystals were used to obtain a sufficiently

Fig. 1. The Qb binding site and its immediate environment, showing the two positions observed for ubiquinone binding. The distal position is shown in purple, the proximal in orange. Shown are coordinates from 1AIG and 1AIJ (16).
Results

Characterization of the Sample. Oxidation of the cytochrome subunit is required for generation of the $P^+\text{Q}_0$ state, which decays with a rate constant between 1 s$^{-1}$ and 10 s$^{-1}$ (11). Fig. 2 shows the absorption spectrum of reaction centers in solution under ambient and oxidizing conditions, and after dissolution of the crystals used in the Laue diffraction experiment. The Inset shows the expanded wavelength range around the absorption band of the bacteriohopophytin, in which the absorption of reduced hemes appears as a shoulder at 558 nm. Evidently, the conditions used in the initial crystal soak buffer were sufficient to fully oxidize the bound cytochrome subunit of the $B.\ viridis$ RC, and the cytochrome subunit remained oxidized throughout the course of the Laue experiment. Furthermore, all three spectra show equivalent magnitudes of the absorption bands below 600 nm and a negligible extent of bacteriochlorophyll oxidation as judged by absorbance at 680 nm. This result demonstrates that the special pair was not oxidized by the addition of ferricyanide to the sample and that the cofactors remained intact throughout the experiment. The increased background absorption in the spectrum of dissolved crystals is due to the fact that the spectra were normalized to the absorption maxima at 600, 830, and 970 nm.

Intense synchrotron radiation is capable of photoreduction of metal centers such as those in cytochrome P450cam (38), and photoreduction of $Q_b$ within the crystal may occur during crystallographic studies of the reaction center. This possibility may be discounted in our experiment for the following reasons. The high-potential hemes in the cytochrome subunit are more easily reduced than $Q_b$ by reductants in solution; hence, photoreduction of the ubiquinone by radicals in solution would be preceded by photoreduction of cyt $c$-558, which is not observed (Fig. 2). Even if the ubiquinone were directly photoreduced within the reaction center, the electron would slowly recombine with oxidized $c$-558 leading to reduction of the cytochrome subunit, which again is not observed.

Charge-Neutral (Dark) Structure of the RC. Table 1 provides the parameters and statistics for the monochromatic and Laue x-ray data collection, processing, and refinement. The final structure obtained from refinement of monochromatic data, with $R (R_{free})$ of 0.201 (0.228), varies little from the starting model. Several detergent molecules and sulfate ions could not be well refined as illustrated by uneven density or high B-factors, presumably due to the lower resolution of the dataset. Water molecules were omitted from the model.

Density for ubiquinone was clearly identified in initial omit maps as a shovel-shaped contour at 1$\sigma$ (Fig. 3A). This density could not be adequately represented by an alternative model of a detergent molecule and several tightly bound water molecules. Following refinement, the quinone head-group, with the exception of the methoxy atoms O2 and CM2, is contained within the 1$\sigma$ contour of electron density. The quinone is clearly bound in the proximal binding site, as seen in the previous structure of the RC-$UQ2$ complex (19).

The ubiquinone in the $Q_b$ site was modeled as a UQ2 molecule even though the crystal contains UQ10, with an extended isoprenyl tail. A detergent molecule occupies the $Q_b$ site in the absence of quinone (19), and the disordered tail of one of the four bacteriochlorophyll molecules also intrudes into this region.

Hence, the observed density is presumably a composite of these three different molecules, which we are unable to adequately model at the present resolution. Based upon a comparison of the average B-factor for the UQ2 model with the B-factors of surrounding residues (Ile-L189, His-L190, Glu-L212, Asn-L213, Phe-L216, Tyr-L222, Ser-L223, and Ile-L224), we estimate the $Q_b$ site occupancy to be 0.6 ± 0.1.

Processing of Laue Datasets. The time-resolved experiment was performed on five crystals of the RC center complexed with UQ2. For each crystal, the initial dark images showed good diffraction to 2.9-Å resolution. The effect of the laser pulse was evident in the streaking and splitting of reflections in the diffraction pattern. This commonly observed phenomenon is believed to be physical rather than chemical or structural in origin. Nonuniform absorption of light due to the appreciable optical density of the crystal leads to a small, transient temperature gradient across it that dissipates in the millisecond time range (39). This effect was fully reversible; the subsequent dark pattern regained excellent reflection profiles. However, significant overall degradation of the diffraction pattern was apparent over the course of the two data collection passes, most likely arising from secondary radiation damage.

In all cases, the first set of 11 images was integrated to 2.9-Å resolution, and the second set was integrated to 3.1-3.5 Å. Data were of similar quality and redundancy for the different crystals, and the parameters of the scaling such as the wavelength.
normalization curve were very similar. Hence, the integrated images from all crystals were scaled simultaneously. Within the redundant subset of data from a single crystal, harmonic deconvolution of energy overlaps recovered up to 10% of the total data, but, when all five datasets were scaled together, the increase in completeness after harmonic deconvolution was insignificant, indicating that the majority of deconvoluted reflections were successfully merged with otherwise nonredundant single reflections.

The presence of ubiquinone in the Laue crystals was confirmed for the dark dataset by calculation of a $2F_o - F_c$ map omitting Qb from the model (Fig. 3B).

**Discussion**

We report here a time-resolved crystallographic structural study of the photosynthetic RC, the largest protein to be studied in this manner, and an integral membrane protein. Our monochromatic structure of the RC-UQ10 complex in B. viridis exhibits proximal binding of ubiquinone. This result is in agreement with published models 1PRC (20) and 2PRC (19). Because our monochromatic model is not significantly overdetermined, we are unable to perform the grouped occupancy refinement used in reporting partial occupancy of the distal site in the original dataset (21).

The experimental difference Fourier map (Fig. 4A) comparing the light and dark state contains no significant features. The time-resolved experiment is very sensitive to changes in electron density by virtue of the light and dark datasets being collected on the same crystals at very nearly the same time. We would readily observe motion of $\sim 4.5$ Å postulated by wholesale displacement of ubiquinone from the distal to the proximal position, with as little as 10% change in occupancy (Fig. 4C).

Assuming the Qb site to be 60% occupied on the basis of the quinone density, the monochromatic model, and that our method of illumination was

Table 1. X-ray data collection and refinement statistics

<table>
<thead>
<tr>
<th>Data collection*</th>
<th>PQb (Bragg dark)</th>
<th>PQb (Laue dark)</th>
<th>P+Qb (Laue light)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wavelength, Å†</td>
<td>0.9</td>
<td>0.9–1.4</td>
<td>0.9–1.4</td>
</tr>
<tr>
<td>Data range, Å</td>
<td>42–2.9</td>
<td>42–2.9</td>
<td>42–2.9</td>
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<tr>
<td>Observations</td>
<td>445,487</td>
<td>373,404</td>
<td>366,170</td>
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<tr>
<td>Unique reflections</td>
<td>62,939</td>
<td>52,845</td>
<td>49,977</td>
</tr>
<tr>
<td>Redundancy‡</td>
<td>7.1</td>
<td>7.1</td>
<td>6.5</td>
</tr>
<tr>
<td>$R_{	ext{sym}}$ (%)</td>
<td>8.7 (44.7)</td>
<td>8.1 (12.1)</td>
<td>9.8 (12.8)</td>
</tr>
<tr>
<td>Resolution, Å</td>
<td>2.9</td>
<td>2.9</td>
<td>2.7</td>
</tr>
<tr>
<td>Completeness§ (%)</td>
<td>99.5 (95)</td>
<td>88.2 (62.3)</td>
<td>83.1 (50.8)</td>
</tr>
<tr>
<td>$1/\sigma_i$</td>
<td>23.0 (3.8)</td>
<td>16.0 (1.9)</td>
<td>12.4 (1.5)</td>
</tr>
<tr>
<td>Refinementβ</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of reflections used</td>
<td>61,245</td>
<td>45,590</td>
<td>41,648</td>
</tr>
<tr>
<td>No. of atoms in model</td>
<td>10,136</td>
<td>10,113</td>
<td>10,113</td>
</tr>
<tr>
<td>$R_{	ext{cryst}}$ (%)</td>
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<td>25.2</td>
<td>27.1</td>
</tr>
<tr>
<td>$R_{	ext{free}}$* (%)</td>
<td>22.8</td>
<td>27.1</td>
<td>29.6</td>
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<td>rmsd bond length, Å</td>
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<td>0.009</td>
<td>0.010</td>
</tr>
<tr>
<td>rmsd bond angles, °</td>
<td>1.5</td>
<td>1.6</td>
<td>1.6</td>
</tr>
<tr>
<td>$Q_{	ext{RQO}}$</td>
<td>0.55</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>$Q_{	ext{RQO}}$</td>
<td>0.35</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

*Crystal dimensions (μm): monochromatic, 500 × 500 × 800; Laue, 150 × 300 × 600; 250 × 350 × 800; 250 × 350 × 700; 100 × 250 × 600; 150 × 150 × 300. Space group: P4$_2_1_2_1$. Unit cell dimensions: 223.5 × 223.5 × 112.5 Å.
†Wavelength for Laue data collection corresponds to that used during scaling.
‡Laue data redundancy is calculated from single reflections used in scaling whereas completeness is calculated following harmonic deconvolution.
§Completeness for the Laue datasets reported by for the restricted resolution range 42–3.7 (3.4–3.27); for the resolution range 42–2.9 (3.0–2.9) the completeness is 71.6% (19.5%) for the dark dataset and 65.3% (13.1%) for the light dataset.
¶for the Laue datasets estimated from $F$ and $\sigma(F)$, using the relations $I = F_i^2, \sigma(I) = 2\sigma(F)$. At the resolution limit (3.4–3.27 Å), the values of $I/\sigma_i$ are 4.2 and 3.2 for the dark and light datasets, respectively.
††Monochromatic refinement statistics are for the final model including Qb. Laue refinement statistics are from a single round of simulated annealing and minimization starting from the refined monochromatic model, omitting Qb.
**$R_{	ext{free}}$ calculated from 10% of dataset for monochromatic refinement, from 5% of dataset for Laue refinement.
†††Relative Quinone Quality factor, see ref. 21.
Fourier transform IR spectroscopy (41) density from a simulated-annealing omit map omitting QB (green) and density for the re
sult does not support a large-scale motion of QB between the proximal and distal positions. However, several other effects may also explain the discrepancy between our result obtained from RC of B. viridis and those obtained from RC of R. sphaeroides, such as sequence differences between the two species (for example residues L209 and L213) and the differing pH and ionic strength of the mother liquor in the crystallographic studies.

We propose a model for the first electron transfer to QB that accounts for the observation of a single binding site for ubiquinone in Fourier transform IR difference studies (41, 42), the dependence of ubiquinone position on the protonation or position of ionizable residues within the QB pocket, as suggested by simulations (22, 23, 44–46), and the observed distal binding of ubiquinone in the dark (16–18, 21). The distal binding site is a metastable binding site for neutral ubiquinone, but it does not correspond to a conformational gate in the reaction. Rather, it is a site that becomes populated during longer periods of dark adaptation, such as those that occur during x-ray data collection of the charge-neutral state. Equilibration between the distal and proximal position occurs through dissociation to the solvent, or perhaps by means of an intermediate binding position, such as that observed in simulations in B. viridis (23) in which the quinone headgroup is free to rotate with respect to the isoprenyl tail.

Upon illumination, an electron arrives at QA. The proximal position is the only active position for electron transfer, as originally suggested (16). The actual conformational gate involves the response of ionizable residues near the QB pocket to the charge at QA, or to the movement of the electron to QB, and may be coupled to proton uptake as concluded in a recent Fourier transform IR study (43). Once QB becomes a semiquinone, it is tightly bound in the proximal position, in agreement with the light structures from freeze-trapping studies (16–18), where it is stabilized by additional electrostatic or hydrogen-bonding interactions due to the proton uptake by surrounding residues. Meanwhile, ubiquinone is still free to exchange with the solvent or intermediate site, and so the equilibrium shifts to

**Fig. 3.** Electron density for the QB binding pocket. 2Fo – Fo maps show density from a simulated-annealing omit map omitting QB (green) and density for the refined model (cyan). (A) Monochromatic dataset, 2.9-Å resolution, contour level 0.5σ. (B) Dark Laue dataset, 3.3-Å resolution, contoured at 0.3σ.

**Fig. 4.** (Fo−Fc)w maps of the QB-Fe-QA region. (A) Experimental structure factors. (B) Fobs = ω(Fo) + ω(Fc). (C) Simulated map: dark, 10% distal and proximal; light, 20% proximal. Positive density is shown in blue, negative density in red. Maps contoured at ±3σ.
complete proximal binding, as described by the pull-transition suggested previously (44). This equilibration is slower than the time delay in our experiment (3 ms), but shorter than the timescale of freezing in freeze-trapping studies (≈150 ms). After charge recombination, the time for reequilibration of the distal position is slower than the time allowed for relaxation in our time-resolved crystallographic experiment (3–6 s), and also slower than the acquisition time in previous Fourier transform IR studies (23 s) (41).

Future time-resolved crystallographic studies may test this hypothesis by varying the time-delay between the laser pulse and acquisition of the light dataset, and by extending our investigation to *R. sphaeroides*. Crystallographic studies should also be conducted at varying pH as suggested (46), and at the same pH when comparing results from different species. Further improvement in the time-resolved technique should focus on obtaining better resolution and determining the conditions for optimal photoactivation in the crystal, and independent estimation of quinone content in the crystal. Time-resolved crystallography can thus be used to gain fresh insights into the mechanism of quinone oxidation and reduction in bioenergetic proteins.

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