pH Modulates the Quinone Position in the Photosynthetic Reaction Center from *Rhodobacter sphaeroides* in the Neutral and Charge Separated States

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The structure of the photosynthetic reaction-center from *Rhodobacter sphaeroides* has been determined at four different pH values (6.5, 8.0, 9.0, 10.0) in the neutral and in charge separated states. At pH 8.0, in the neutral state, we obtain a resolution of 1.87 Å, which is the best ever reported for the bacterial reaction center protein. Our crystallographic data confirm the existence of two different binding positions of the secondary quinone (QB). We observe a new orientation of QB in its distal position, which shows no ring-flip compared to the orientation in the proximal position. Datasets collected for the different pH values show a pH-dependence of the population of the proximal position. The new orientation of QB in the distal position and the pH-dependence could be confirmed by continuum electrostatics calculations. Our calculations are in agreement with the experimentally observed proton uptake upon charge separation. The high resolution of our crystallographic data allows us to identify new water molecules and external residues being involved in two previously described hydrogen bond proton channels. These extended proton-transfer pathways, ending at either of the two oxo-groups of QB in its proximal position, provide additional evidence that ring-flipping is not required for complete protonation of QB upon reduction.

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Introduction

The bacterial photosynthetic reaction center (RC) is a membrane-spanning protein complex, which converts light energy into chemical free energy. The photosynthetic RC of *Rhodobacter sphaeroides* (*Rh. sphaeroides*) consists of three protein subunits L, M, and H. The L and M subunits, each contain five membrane-spanning helices. Together they bind non-covalently the ten cofactors. The H subunit has only one helical membrane anchor. The system is 2-fold pseudo-symmetric; the symmetry axis runs from a dimer of bacteriochlorophyll molecules (P), located on the periplasmic side of the complex, to a non-heme iron atom situated between the primary quinone (QA) and the secondary quinone (QB), close to the cytoplasmic side of the complex. Light energy is trapped by the primary electron donor P. The absorption of a photon generates the exited electronic singlet state of lowest energy of P, the excited dimer (P²*), which is a strongly reducing species. Transmembrane electron transfer is then initiated from P²* to the system of two quinones, QA and QB, bound at the cytoplasmic side of the RC. QA and QB differ in their redox properties. QA is never protonated and accepts only one electron. QB (the terminal electron acceptor) sequentially accepts two...
membrane. Ubiquinone from the quinone pool present in the QB position on pH. To investigate this suggested reduction recently suggested a dependence of the electron transfer to QB between the two positions being rate-limiting for positions of QB in the dark as well as under electron density both in the distal and proximal QB position relative to the non-heme iron. QB was singly reduced. Distal and proximal refer to the QB position relative to the non-heme iron. QB was proposed to undergo a light-induced shift of ~4.5 Å towards the non-heme iron, accompanied by a 180° propeller twist around the isoprenoid tail. Recent crystallographic investigations showed QB electron density both in the distal and proximal positions of QB in the dark as well as under illumination.

A direct correspondence between the two positions of QB and its redox states, as well as movement of QB between the two positions being rate-limiting for electron transfer to QB is nowadays considered as unlikely. Moreover, a recent molecular dynamics study comes to the conclusion, that the distal QB position is best fitted by an orientation of QB in the proximal position of the cryo-cooled structure of this work. Furthermore, we could confirm previous Raman and NMR spectroscopic results concerning the kinked conformation of the carotenoid molecule and indicating a 15,15'-cis bond in all our refinements. Such a conformation has also been proposed in *Rhodopseudomonas viridis* RC. Two of the three refined lipid molecules, glycosylglycerol diacylglycerol (GGD) and phosphatidylcholine (PC), had to be oriented differently compared to those of Camara-Artigas et al., to fit the available electron density (Figure 1). This re-orientation abandons the unusual conformations described previously and places the head-groups of these lipids in an orientation pointing towards the water molecules above the cytoplasmic surface (Figure 1(d)). In addition, the conflict in positioning of both the PC and the isoprenoid tail of QB in the distal position is abolished (Figure 1(c)). The density for the lipid hydrocarbon chains, that was occupied by four lauryldimethylamine N-oxide (LDAO) detergent molecules in other structures, is very distinct, but the density for the head-groups is not very pronounced, which could lead to misinterpretation.

**Results and Discussions**

**Structural basis and general features of the high resolution structure**

Here, we have investigated the structure of the photosynthetic RC from *Rh. sphaeroides* at liquid nitrogen temperature. Since the resolution especially for the data collected in the dark at a pH of 8 could be considerably improved compared to the structure determined at 277 K (PDB data base entry 1pcr), the number of modeled water molecules that could be included in to the model increased from 160 to 430. For the cryo-cooled structure, several new water molecules are visible in the membrane spanning part of the RC. These water molecules constitute two hydrogen-bonded networks between QB and the bulk solvent at the cytoplasmic side of the RC, including several charged residues and the ligands of the non-heme iron. These two water chains confirmed by our work have been described before. Two water molecules of the 277 K structure at the QB side of the water chains (Wat65 and 66 of 1pcr) are replaced by QB in the proximal position of the cryo-cooled structure of this work. Furthermore, we could confirm previous Raman and NMR spectroscopic results concerning the kinked conformation of the carotenoid molecule and indicating a 15,15'-cis bond in all our refinements. Such a conformation has also been proposed in *Rhodopseudomonas viridis* RC. Two of the three refined lipid molecules, glycosylglycerol diacylglycerol (GGD) and phosphatidylcholine (PC), had to be oriented differently compared to those of Camara-Artigas et al., to fit the available electron density (Figure 1). This re-orientation abandons the unusual conformations described previously and places the head-groups of these lipids in an orientation pointing towards the water molecules above the cytoplasmic surface (Figure 1(d)). In addition, the conflict in positioning of both the PC and the isoprenoid tail of QB in the distal position is abolished (Figure 1(c)). The density for the lipid hydrocarbon chains, that was occupied by four lauryldimethylamine N-oxide (LDAO) detergent molecules in other structures, is very distinct, but the density for the head-groups is not very pronounced, which could lead to misinterpretation.

**Binding of QB**

The orientation of QB in the proximal and distal position cannot be determined unambiguously from the electron density. For the proximal position, only the positioning of the two oxo-groups of QB between the hydrogen bond partners His L190 N and Gly L225 N and the position of one methoxy-group, which accepts a weaker hydrogen bond from Thr L226 N, seems to be secure. These restraints still allow two possible orientations of QB in the proximal position, which are related to each other by an approximately 180° rotation around the longitudinal pseudo-symmetry axis of the head-group ring of QB.
leading to two different orientations of the isoprenoid tail. A fit to the shorter electron density rod extending from the head group density was chosen for our model (Figure 2(a)). The resulting orientation is roughly in agreement with that in the illuminated structure of Q$_B$ (PDB entry 1aij) found by Stowell et al.$^1$ as may be seen in Figure 2(b). The distal position of Q$_B$ is restrained by only a single hydrogen bond between one oxo-group of Q$_B$ and the N of Ile L224. The electron density again gives no clear information about the orientation of Q$_B$ in this position. Two weak (about 0.2 $\sigma$) electron density rods extend from the head-group density; each of them can accommodate the isoprenoid tail leading again to two possible Q$_B$ orientations (Figure 2(a)). The longer of these density rods was used to model the distal isoprenoid tail, leading to a Q$_B$ orientation that differs from previous models.$^1$ The corresponding density here was interpreted as a PC molecule. The new orientation of Q$_B$ in the distal position allows a movement to the proximal position without the so-called propeller twist around the longitudinal pseudo-symmetry axis of the head-group ring. To analyze the relative energetics of the two orientations in the distal position, we performed quantum chemical and electrostatic calculations. The quantum chemical calculations using density functional theory show that the two possible orientations of isolated Q$_B$ are energetically close: the non-ring-flipped orientation of the isolated Q$_B$ is favored by 0.4 kcal/mol. Also the electrostatic interaction of the protein with Q$_B$ allows both orientations with approximately equal energy.

From our Monte Carlo simulations, we estimate that electrostatic contributions to the difference between the ring-flipped and the non-ring-flipped orientation range from 0.3 kcal/mol at pH 6 to 0.0 kcal/mol at pH 10, favoring the non-flipped orientation.

**Movement of Q$_B$**

The present, high resolved crystallographic information allows us to propose a mechanism for the movement of Q$_B$ between its two putative positions.

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**Figure 1.** Refined lipid molecules and overall solvent structure of the reaction center. (a) cardiolipin (CDL), (b) glucosylgalactosyl diacylglycerol (GGD), and (c) phosphatidylcholine (PC) with their respective electron densities at 0.2 and 1 $\sigma$ in wireframe representation, color-coded blue and magenta, respectively. The lipid molecules from the PDB entries 1qov (cyan) and 1m3x (light red) are superimposed in (a)–(c). In the center (c) shows the distal orientation of Q$_B$ (orange), which is clearly in conflict with the PC molecule of entry 1m3x. (d) Overview of the photosynthetic reaction center with the protein subunits shown in ribbon representation, with the L subunit color-coded in green, M in blue, and H in yellow. Pigment and solvent molecules are shown in licorice representation. The carbon atoms of pigment molecules are shown in cyan, while the carbon atoms of solvent molecules are colored in orange. The CDL is visible in the middle, the GGD on the right, and the PC molecule on the left side. The Figure was generated using Xtalview,$^{40}$ VMD,$^{59}$ and Raster3D.$^{60}$
The distal position is only weakly stabilized by a single hydrogen bond between QB and Ile L224 N (Figure 2(c)). An intermediate position with two hydrogen bonds of the protein towards one oxo and one methoxy-group on the same side of QB, might be conceivable when QB moves from the distal to the proximal position (Figure 2(c)). QB could glide along the backbone from Ile L224 towards Gly L225, keeping thereby the contact to Ile L224 N and forming a new one to Gly L225 N. Unfortunately, is it not possible to unambiguously determine such an intermediate position from the electron density. The distance of the final movement from this hypothetical intermediate with its two hydrogen bonds
towards the proximal position is then about 3.8 Å, corresponding to the Cα–Cα distance between two residues. Not only does the oxo-group of QB bind to the new hydrogen bond partner Gly L225 N on its way deeper into the pocket, but also the QB is able to move further until the methoxy-group on the same side of QB is in hydrogen bond distance to the adjacent backbone nitrogen Thr L226 N. In this position, the other oxo-group of QB is in hydrogen bond distance to His L190 Nδ3 and three H-bonds are formed (Figure 2(c)) between the protein and QB with a geometry symmetric to QA (Figure 2(d)). The total movement of QB is therefore with 5.2 Å longer than the Cα–Cα distance and the overall movement thus gradually increases the number of H-bonds accepted by QB from one to three.

Proton delivery to QB

Two proton translocating pathways from the cytoplasm to QB and ending either close to Glu L212 or Asp L213, respectively, have been previously identified1,9,15 (defined below as E- and D-pathways according to these two residues). Glu L212 and Asp L213 have been suggested to be the ultimate carriers of the first16 and the second proton, respectively,17 to QB. The improved resolution of our crystallographic data allows us to observe extensions of these pathways including the participation of new water molecules and new external residues (see below).

Glu L212 itself is in hydrogen bond distance to Wat O89 of the E-pathway water chain (Figure 3(a) and (c)) and in close distance to one methoxy-oxygen of QB in the proximal position (3.5 Å). Water molecule N100 connects the E-pathway via two H-bonds to Glu M234, coordinating the none-heme iron. The D-pathway water chain (Figure 3(b) and (d)) extends directly to the vicinity of the second methoxy-group, with the water molecules O90, N40, and N39 hydrogen-bonded to Asp L213. Both pathways have no direct connection to the bulk solvent in our structure, but start at Arg H118 (E-pathway) or Arg M13 (D-pathway). Arg H118 was found in two orientations, indicating the flexibility of these two arginine residues, which could allow proton uptake from the bulk solvent to the water chains by a spatial movement. At the entry of the E-pathway, also Asp M240 might be responsible for proton translocation, but the Arg H118 seems more likely to be involved, due to its obvious flexibility. These two pathways probably each deliver a proton to either side of QB after its reduction. We do not see a direct connection of the two pathways to the two QB oxo-groups, therefore two additional mobile water molecules, not visible to the X-ray experiment, might be required for the protonation of the QB oxo-groups (indicated in Figure 3(c) and (d) by a hypothetical, flexible water molecule). Due to the existence of the two separate proton pathways, proton uptake by the two oxo-groups of QB does not require rotation of QB.

Both the new and the old QB orientations seem to differ energetically only slightly from structural and theoretical considerations, but only in the new QB orientation a movement between the proximal and the distal QB positions seems to be possible without large conformational rearrangements.

Cd²⁺ binding studies suggested that His H126, His H128 and Asp H124 could function as a cluster constituting the unique entry point for protons from the bulk phase of the protein.18,19 It has been proposed that protons taken up at this entry point would then be transferred to QB through a bifurcated pathway involving Asp M17 and Asp L210.

This hypothesis was based on experiments combining Cd²⁺ binding effects on variants carrying respective replacements of Asp M17 and Asp L210 by Asn residues.20 However, a different view can be derived from other results. We have performed the same kind of functional analysis on the L209PY RC variant in the presence of Cd²⁺.21 Identical effects on the electron and proton transfer kinetics were observed in this variant showing that the proton pathway from the bulk to QB does not only involve M17DN and L210DN per se but is likely to be more delocalized. In support of this hypothesis, we have previously shown that in the L212E/L213DA double RC mutant, the pH dependencies of H+/QB⁺ proton uptake that collapse above pH 8 are remarkably restored to a WT level where an additional mutation (M249AY) is introduced in the close vicinity of QA.22 The participation of a large

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**Figure 3.** Proton translocating pathways. (a) the E-pathway is translocating protons from the bulk solvent, via Arg H118, and Asp M240 towards Glu L212 and the vicinity of one methoxy-group of QB in the proximal position. (b) the D-pathway is translocating protons from the bulk solvent via Arg M13 towards Asp L213 and the other methoxy-group of the proximal position of QB (italicised residues were used to name the pathways). An additional, flexible water molecule, invisible to the X-ray diffraction method, is probably necessary to translocate the proton to its actual destination, the oxo-group of QB. Molecules are drawn in licorice representation and possible hydrogen bonds are indicated by green broken lines, with their length given in Å. (c) Schematic drawing of the E-pathway and (d) of the D-pathway. Hydrogen atoms were added and oriented according to the acceptor and donor capabilities of neighboring hydrogen-bonded residues. The vertical water chain in the schematic drawings corresponds to the shortest connection through the water network of the pathways between QB in its proximal position and the bulk water. A flexible water molecule in red was added to either drawing to demonstrate a possible completion of the pathways at its QB end. To facilitate the identification of the water molecules forming the vertical water chain in (c) and (d), the possible H-bridges in (a) and (b) joining these water molecules of the chain, are indicated by orange, broken lines and their respective distance measures color-coded in red. All solvent molecules were given chain identifiers (id) in the respective PDB entry 2j8c, to indicate the protein chain to which they are most closely associated with: H subunit, solvent id S; M subunit, solvent id N; and L subunit, solvent id O. Xtalview60 was used for visualization, Raster3D60 for rendering, and ChemDraw for the schematic drawing.
Figure 3 (legend on previous page)
portion of the inter-quinone cytoplasmic surface of the protein for taking up protons therefore seems to be more likely.

The present structural data demonstrating the existence of an extended proton transfer network starting at Arg H118 and Arg M13, which has never been reported before, support multiple entry points for protons as well as an extended proton transfer network within the protein to reach their target, the QB species.

**Population of the two QB positions**

The relative populations of the two QB positions in the dark-adapted and the illuminated state were calculated from the electron densities as described in Materials and Methods and are shown in Table 1. Due to a high correlation between temperature factors and occupancy of an atom in a medium resolution structure,23 the occupancy could not be refined independently for the different QB positions (distal and proximal). However, assuming that the temperature factors are equal for the two positions, it is possible to estimate the relative populations for the two QB positions. The mean QB head-group temperature factors at the estimated populations are listed in Table 1. Test calculations indicate that only a small deviation in occupancy (0.1) can be compensated by an appropriate change in temperature factors at medium resolution.24 Therefore we expect the error to be in the same range.

The computed population curves are shown in Figure 4. The population curve for QB in the dark-adapted state (Figure 4(a)) is in agreement with the experimentally determined data. There is only a slight pH dependence of the population. In order to track the possible origin of the variation of the QB population in the dark-adapted state, we have calculated the correlation function, $c_{ij}$, between the protonation of all titratable residues ($i$) and the proximal QB position (see Materials and Methods). By far the highest $c_{ip}$ value arises from the interaction between Glu L212 (whose calculated protonation curve is presented in Figure 5(a)) and the QB proximal population. The $c_{ip}$ correlation curve between the protonated Glu L212 and the proximal position of QB is presented in Figure 5(c). Obviously, the deprotonation of Glu L212 above pH 9 is correlated to the decrease of the proximal population (positive value of $c_{ip}$).

For the illuminated state, the computed populations (Figure 4(b)) coincide with the experimental data except for the experimental value measured at pH 6.5. However, the three evaluations of the proximal population for the illuminated state, respectively, 50, 50 and 60% arise from three independent measurements on three different crystals. Therefore these experimental points seem reliable.

Our model is satisfactory in fitting four dark (pH 6.5, 8.0, 9.0 and 10.0) and three illuminated states (pH 8.0, 9.0 and 10.0) based on the same structural information. The discrepancy between the calculated and measured populations for QB in the proximal position at pH 6.5 in the illuminated state may suggest that subtle structural changes may specifically occur at this pH that we cannot identify.

In a similar way as for the dark-adapted state we have probed the possible origin of the variation of the QB population in the illuminated state. We have also calculated the correlation function, $c_{ip}$, between the protonation of all titratable residues ($i$) and the proximal QB position (see Materials and Methods). Two protonable residues show high $c_{ip}$ values. These are Asp L213 and QB itself, which calculated protonation curves are presented in Figure 5(b). The $c_{ip}$ correlation curves between the protonated Asp L213 and the proximal position of QB, and between the protonated semiquinone and its proximal position are presented in Figure 5(d). Therefore, in the illuminated state, both the concomitant deprotonations of Asp L213 and the semiquinone lead to a decreased proximal population of QB.

An interesting feature displayed in Figure 5(d) is the very negative $c_{ip}$ (see Materials and Methods) correlation curve between the protonated Asp L213 and the protonated semiquinone. The high calculated correlation $c_{ip}$ reveals the strong destabilization of the proximal position for the deprotonated semiquinone when Asp L213 is deprotonated. Thus, according to our calculations the electrostatic repulsion between deprotonated Asp L213 and deprotonated QB depopulates the proximal position. When QB gets protonated, the proximal position can get more populated.

**Proton uptake by QB and the complete RC**

The proton uptake of the RC upon illumination was calculated as described in Materials and Methods. The computed proton uptake data in Figure 6 are similar to the experimental values,25,26 although the shape of the experimental curve is not reproduced exactly. Between pH 4 and 8, Asp L213 and the ubiquinone (see below) almost exclusively account for the shape of the proton uptake curve. In contrast, in the pH range from 8 to 10, no individual residue could be identified that has a major influence on the proton uptake curve. Instead, many titratable groups contribute to the proton uptake in this pH range.

At low pH we find that the semiquinone is protonated predominantly (see Figure 5(b)). The protonation probability decreases slowly from a value of about 0.8 at pH 4.0 down to 0 at pH 9. In RCs isolated in detergent, the formation of QB leads to substoichiometric proton uptake in the pH range from 5 to 10.25 At variance, it has been reported27 that in chromatophores from Rb. capsulatus and Rb. sphaeroides proton uptake occurs directly to the QB species to form a hydroquinone ($Q_B^-$) and leads to a measured value of $H^+/[RC]=1$ up a pH value of 7.29 The $Q_B^-$ species deprotonates to $Q_B^-$ in the pH range from 5 to 7. The titration curve observed by Lavergne et al.29 has a much smaller slope than a standard Henderson-Hasselbalch curve would have. The calculated titration curve for the
### Table 1. X-ray data collection statistics

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a. $R_{sym} = \frac{\Sigma hkl |I_i - \langle I\rangle|}{\Sigma hkl \langle I\rangle}$, where $I_i$ is the intensity of the $i$th measurement of reflection $hkl$ and $\langle I\rangle$ is the average intensity of a reflection.

b. $R_{free}$ is calculated from 5% of the measured unique data that were not used during refinement.
semiquinone shown in Figure 6 is reminiscent of the report by Lavergne et al., but with a more shallow shape. These authors have suggested that the apparent pK (∼6) of the semiquinone form in chromatophores, would not reflect electrostatic interactions but would rather be due to a conformational change affecting the quinone binding and occurring between pH 5 and 7.

We do observe here an unexpected change in the quinone population at pH 6.5 in the illuminated state, which we cannot explain by our calculations. Our model is successful in fitting seven (four dark adapted state and three illuminated state pH points) out of eight quinone populations versus pH as determined by X-ray diffraction crystallography. However, we are unable to fit the point measured at pH 6.5 in the illuminated state. This point has been repeated three times (50, 50 and 60% proximal occupation) on three different samples. It is therefore quite firm. We are facing an apparent discrepancy between the measured position of the quinone in the illuminated state by crystallography and evaluated by electrostatic calculations.

The reason for this discrepancy will be further investigated.

Improvements to earlier calculations

Several theoretical studies on protonation probabilities and conformational changes of the Q₈b binding site have been performed previously. Apart from Taly et al., none of these studies considered the pH dependence of the population of the distal and proximal position.

The results of the calculations by Taly et al. propose that with increasing pH the probability of the proximal conformation increases independently of whether the quinone or the semiquinone is bound. This finding was not confirmed by experimental data or by the calculations reported here.

An explanation for this discrepancy is that by Taly et al., the semiquinone was not considered as protonatable in the illuminated state. However, as shown in Figure 5(d), the protonation state of the semiquinone Q₈b influences its own binding position. Neglecting this influence will therefore modify the calculated proximal population.

Moreover in our previous study, we used two independently refined crystal structures as models for the proximal and the distal positions of Q₈b. These two structures also differ in residues outside of the binding pocket. Here we used one structure for each state (dark-adapted and illuminated) to analyze the position of the quinone in which Q₈b was modeled as a disordered residue. A detailed analysis of the correlation of our previous calculation shows that residues outside of the Q₈b binding pocket trigger the transition between the distal position found in the dark-adapted state and the proximal position found in the illuminated state. This behavior was an artefact of the approach. In the present study, this problem is avoided and the structural transition depends only on the protonation of residues in the vicinity of Q₈b and of Q₈b itself.

Conclusion

By X-ray diffraction crystallography, we have analyzed the binding position of the secondary quinone as a function of pH in the reaction center from Rb. sphaeroides. The different structures of the protein have been obtained at pH 6.5, 8.0, 9.0 and 10.0, in the dark adapted and illuminated states.

The electron density in the Q₈b binding pocket, obtained with good quality data, suggests a new orientation of Q₈b in the distal position, which has never been described before. In this conformation, no rotation of the head-group of Q₈b is needed for its movement between the distal and the proximal positions. Most likely a putative movement between the distal and proximal binding of Q₈b is not a rate-limiting factor for the QA– to Q₈ electron transfer.

We confirm the existence of two potential proton delivery pathways and further extend their content to new water molecules and residues close to the protein surface. These pathways can be involved in the proton delivery to the two oxo-groups of Q₈b in the proximal position. It is interesting to note that
these proton pathways start at Arg H118 and Arg M13, respectively. These entry points for protons are distinct from the previously proposed “unique” entry point formed by Asp H124, His H126, and His H128. Our present finding further supports the previously suggested idea\textsuperscript{21,22} that neither the proton entry points nor their pathways to the Q\textsubscript{B} site are unique. Instead, the RC works more like a proton “trap” as previously described\textsuperscript{37}. The higher the obtained resolution of the crystal structure, the more water molecules are detected and the more diverse potential proton transfer pathways are identified.

It is certainly a physiological advantage for the RC to transfer protons through several pathways. The alternative activation of these proton transfer pathways depends on the relative \( pK_a \) values of their participating groups. Multiple proton transfer pathways have the advantage that a single mutation does not lead to the loss of function of the protein. In this regard, multiple pathways within the protein delivering protons certainly provide a higher functional flexibility and a higher evolutionary fitness.

As we show here, pH is important for positioning the quinone in its site and this positioning is correlated to the proton uptake. We were able to fit the pH dependencies of the quinone position and the proton uptake upon charge separation at the same time. Our results indicate that pH should be taken into account as a parameter regulating cofactor activities in proteins, for example by influencing their binding positions and/or affinities.
Materials and Methods

Crystallographic data collection and refinement

The RC from *Rh. sphaeroides* was purified and crystallized as reported earlier. Reconstitution to obtain a fully occupied QB binding site, illumination conditions to achieve a complete charge separation, and stepwise equilibration with the cryo-buffer have been described previously. The RCs crystallized in the space group *P*321, but occasionally in the same crystallization batch, tetragonal crystals of space group *P*422 were formed. We measured 14 datasets at four different pH values, 6.5, 8, 9, and 10 using 11 different crystals (Table 1). The first two pH 6.5 datasets and both pH 10 datasets with space group *P*422 were measured like both pH 8 datasets from the same crystal (crystals 1, 5, and 9 in Table 1). In other cases, the crystal did not survive the thawing and illumination after measuring the dark dataset. For some pH values, additional datasets were taken as control. In particular, illuminated datasets were repeated to ensure the illumination experiment had failed. Except for one dataset, all data were collected at the beamlines X11 and BW7B of the EMBL-Outstation in Hamburg. The second dark dataset at pH 10 (crystal 10 in Table 1) was collected at the protein crystallographic (PX) beamline of the Swiss Light Source (SLS) in Villigen/CH.

When we refer here to a pH value in connection with an investigated dataset or a refined structure, we actually mean that the pH was adjusted by the potassium-hydrogen-phosphate buffer in each crystallization attempt. Since the pH value is defined as the negative logarithm of the chemical potential of the hydronium ions, which is constant in the whole system (solution and crystal) in thermodynamic equilibrium, this assignment is justified. Measured datasets were processed with the HKL program suite and structure factors were calculated from the measured intensities employing TRUNCATE of the CCP4 (Collaborative Computing Project, 1994). Refinement was carried out using Refmac5. The QB site is expected to be fully occupied, i.e. occupancies of the distal and the proximal positions add up to 100%. The populations of the two positions are thus equal to the occupancies, which are characterized by identical temperature factors for QB in the two positions.

Between each refinement round 2Fo−Fo and Fo−Fo electron density maps were inspected using the graphics program XtalView. Firmly bound water molecules were added by detecting peaks >3σ in the Fo−Fo difference density map with a geometry suitable for hydrogen bonding. The quality of the electron density can be judged from Figure 1, where the electron density of 1.87 Å resolution around a refined cardiolipin (CDL), a GGD, and a PC molecule is shown. The molecules were found roughly at the same position as by Jones et al. for the AM260W mutant (PDB entry 1qv0) and by Camara-Artigas et al. (PDB entry 1m3x). However, the orientation of the GGD molecule is pointing in the opposite direction (Figure 1(b)), while the PC molecule is rotated by about 70° (Figure 1(c)), thus placing them roughly parallel to the CDL molecule (Figure 1(d)). In this orientation, the PC is no longer in steric conflict with the isoprenoid tail of QB in the distal position (Figure 1(c)). Another example for the map quality can be inspected in Figure 2(a), where the electron density of the two QB positions (distal and proximal) in the QB binding pocket at pH 8 in the dark-adapted state is shown again with a resolution of 1.87 Å. In preliminary data collections, we were not able to detect any useful density in the QB binding pocket, indicating that the native QB must have lost during the purification procedure. However, we might not exclude an amount of native ubiquinone-10 remaining in the binding pocket unverifiable to X-ray diffraction methods. To replenish this loss, crystals were soaked with ubiquinone-2. The density found in the QB binding site after soaking was consequently modeled by a quinone with an isoprenoid tail consisting of only two units. Therefore a similar density observed in the *Rps. viridis* reaction center and modeled by a longer isoprenoid tail was refined in this work against a LDAO detergent molecule and three water molecules, in hydrogen bond distance to two LDAO head-group oxygen atoms in the periplasmic solvent region.

Structure preparation for theoretical calculations

The two crystal structures obtained for the dark-adapted and the illuminated state at pH 8 were used for the calculations. The electron density of the N and C termini of the H subunit (ten residues each) and the C terminus of the M subunit (five residues) was lacking in both structures. Since all of these three terminal regions have helical folds, the missing amino acids were used to elongate the respective helices. In a subsequent refinement round, very weak electron density showed up for some of the added amino acids. In each of the structures, the two possible binding positions of ubiquinone in the QB binding site are resolved. The hydrogen atoms were placed with the HBUILD module of CHARMM followed by an energy optimization of the hydrogen atom positions while the heavy-atom positions were kept fixed. The same partial charges were used as in previous calculations. The detergent molecules seen in the crystal structures were taken into account explicitly.

The structures of the dark-adapted and illuminated RC were modified in loop regions (L, 149–165; M, 230–250; H, 137–165) in order to remove minor structural differences that cause a different titration behavior but were structurally insignificant. Therefore the coordinates of the named residues were taken from the higher resolved dark structure to replace the coordinates of the corresponding residues in the illuminated structure.

Protonation probability and population calculations

The pH-dependent protonation state energy *G*\textsubscript{int}(pH) of a certain protein conformation and of a certain protonation state *n* is determined by the intrinsic pK (pK\textsubscript{int}) of each titratable group, the interaction energy *W*\textsubscript{int} between the charged forms of each possible pair of titratable groups and the conformational energy of the reference protonation state Δ*G*\textsubscript{conf}.

\[
G_{\text{int}}(pH) = \sum_{i=1}^{N} \left( x_i^+ - x_i^- \right) RT \ln \left( pH - pK_{\text{int}}^i \right) + \frac{1}{2} \sum_{i=1}^{N} \sum_{j=1}^{N} W_{ij} \left( x_i^+ - x_j^- \right) \left( x_j^+ - x_i^- \right) + \Delta G_{\text{conf}}
\]

where \( x_i^+ \) is the reference protonation form and \( x_i^- \) the actual protonation form of the titratable group *i*. Both \( x_i^+ \) and \( x_i^- \) are 1 or 0 depending on whether the group is protonated or deprotonated. In the performed calculations, the reference protonation form of a titratable group is its neutral form (acidic groups 1; basic groups 0). The intrinsic pK is given by the experimentally determined pK (pK\textsubscript{int}) of the titratable group alone in aqueous solution.
solution\textsuperscript{46} and the shift in the pK\textsubscript{a} (ΔpK\textsubscript{a}\textsuperscript{prot}) due to a different solvation environment inside the protein (pK\textsubscript{a(i)} = pK\textsubscript{a(i)}\textsuperscript{model} + ΔpK\textsubscript{a(i)}\textsuperscript{prot}). The terms ΔpK\textsubscript{a(i)}\textsuperscript{prot} and W\textsubscript{ij} were calculated by solving the linearized Poisson-Boltzmann equation (LPBE) with the program package MEAD.\textsuperscript{46} In the LPBE calculations, the effect of the solvent is modeled by a dielectric constant of 80 and an ionic strength of 0.1 mol/l. A dielectric constant of ε = 4 was assigned to the protein. A temperature of 300 K and the following atomic radii were used: 1.00 Å for hydrogen, 1.50 Å for oxygen, 1.55 Å for nitrogen, 1.70 Å for carbon and 1.80 Å for sulfur. The LPBE was solved by the finite difference method using three focusing steps for the protein and two for each titratable group: for the protein, the grid spacing was decreased from 2 Å to 1 Å and finally to 0.25 Å. For each titratable group, a grid spacing of 1 Å was used followed by a grid spacing of 0.25 Å. The boundary between the protein and solvent is defined by rolling a solvent probe sphere (radius 1.4 Å) over the van der Waals surface of the protein.\textsuperscript{46}

In addition to the titratable groups of the amino acids of the protein, the semiquinone was considered as a titratable group. The model pK\textsubscript{a} for all titratable residues are those reported earlier.\textsuperscript{47} For the semiquinone a model pK\textsubscript{a} of 4.9 was used.\textsuperscript{47} For each titratable residue, the average protonation probability was calculated by a Monte Carlo procedure\textsuperscript{50} using the program GMCT. All titratable residues were treated as a single tautomer (representing the average over all possible tautomers) except for histidine residues and semiquinone, for which two tautomers were included in the calculations. This treatment led to good agreement with experimental data in previous studies.\textsuperscript{45,51–53}

In order to obtain the energy difference between the two possible orientations of QB in the distal position, we performed gas phase calculations on isolated QB using the Amsterdam Density Functional program suite\textsuperscript{54} functionals VWN\textsuperscript{55} and PW91.\textsuperscript{56} Input coordinates of the two possible orientations of the distal QB positions were taken from our crystal structures at pH 8 and were subsequently minimized. We used the obtained relative gas phase energies together with results from electrostatic calculations to determine the probabilities of the two orientations of QB in the protein. The electrostatic calculations allow the protein to change its protonation states and were performed with MEAD (same parameters as above). GMCT was then used to perform the Monte Carlo titrations.

Analogously to the calculation of protonation probabilities, populations of the two QB positions were calculated using GMCT. Due to difficulties in determining ΔC\textsubscript{cond} for the two positions by theoretical approaches,\textsuperscript{47} this energy was adjusted to fit the experimental data points at pH 18.0 for the dark-adapted and illuminated states, respectively. This procedure is analogous to what we used previously.\textsuperscript{5,52}

To analyze the mutual influence between the protonation forms of two given residues, we introduce\textsuperscript{57,58} a correlation function, defined as: \( c_i = \langle x_{i,j} \rangle - \langle x_i \rangle \langle x_j \rangle \), where \( \langle x_{i,j} \rangle \) is the probability of having residues \( i \) and \( j \), both protonated at the same time and \( \langle x_i \rangle \) and \( \langle x_j \rangle \) are the respective probabilities of having residues \( i \) and \( j \) protonated independently.

To analyze the mutual influence between the protonation form of a given residue and the position of the QB, we also introduce a correlation function \( c_p = \langle x_{i,p} \rangle - \langle x_i \rangle \langle x_p \rangle \), where \( \langle x_{i,p} \rangle \) is the probability that residue \( i \) is protonated when the QB is in the proximal position; \( \langle x_i \rangle \) is the probability for QB to occupy the proximal position. \( \langle x_p \rangle \) is equivalent to the proximal population.

\( c_i \) and \( c_p \) can vary in the range between −0.25 and +0.25. Positive values of the correlation functions correspond to mutual stabilisation of both events (protonation and/or proximal position). Negative values reflect destabilisation of one event (protonation or proximal position) by the other one.

The protonation probabilities of all titratable groups were computed for the dark-adapted and the illuminated state as described above. The resulting protonation probability difference between these two states corresponds to the number of protons taken up upon the first reduction of QB, which were compared with experimental data.\textsuperscript{22,25}

### Protein Data Bank accession codes

The continuum electrostatics calculations were based on the coordinates of the dark-adapted and illuminated 3D structures obtained at pH 8 and deposited together with their respective structure factors in the RCSB Protein Data Bank (PDB) with accession codes 28ic and 28id, respectively. The different refined protein models and their respective structural amplitudes at the remaining pH values were deposited in the PDB as well. The corresponding accession codes in the dark-adapted and illuminated states are at pH 6.5: 2uwv to 2uwv, at pH 9.0: 2ux3 to 2ux5, and at pH 10.0: 2uxj to 2uxm. In order to facilitate the assignment, the accession codes are also listed in Table 1.

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### References


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