Is There a Conserved Interaction between Cardiolipin and the Type II Bacterial Reaction Center?

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ABSTRACT In a recent publication, the structural details of an interaction between the *Rhodobacter sphaeroides* reaction center and the anionic phospholipid diphosphatidyl glycerol (cardiolipin) were described (K. E. McAuley, P. K. Fyfe, J. P. Ridge, N. W. Isaacs, R. J. Cogdell, and M. R. Jones, 1999, *Proc. Natl. Acad. Sci. U.S.A.* 96:14706–14711). This was the first crystallographic description of an interaction between this biologically important lipid and an integral membrane protein and was also the first piece of evidence that the reaction center has a specific interaction with cardiolipin. We have examined the extent to which the residues that interact with the cardiolipin are conserved in other species of photosynthetic bacteria with this type of reaction center and discuss the possibility that this cardiolipin binding site is a conserved feature of these reaction centers. We look at how sequence variations that would affect the shape of the cardiolipin binding site might affect the protein-cardiolipin interaction, by modeling the binding of cardiolipin to the reaction center from *Rhodopseudomonas viridis*.

INTRODUCTION

Reaction centers are integral membrane protein complexes found in photosynthetic organisms, including a wide variety of bacterial species. The structures of the reaction center from two species of purple bacteria, *Rhodopseudomonas* (*Rps.*) *viridis* and *Rhodobacter* (*Rb.*) *sphaeroides* have been solved by x-ray crystallography, to resolutions approaching 2 Å (Ermler et al., 1994a,b; Deisenhofer et al., 1995; Lancaster and Michel, 1997, 1999; Stowell et al., 1997). The two structures are similar in terms of overall architecture, with a transmembrane region formed by 11 membrane-spanning α-helices contributed by three polypeptide subunits. The L- and M-subunits each have five transmembrane α-helices, and these are related by an axis of pseudo twofold symmetry that runs approximately perpendicular to the plane of the membrane. The remaining transmembrane α-helix is contributed by the H-subunit. The L-, M-, and H-subunits are encoded by the *pufL*, *pufM*, and *puhA* genes, respectively. A major difference is that the *Rps. viridis* reaction center has a fourth subunit comprising a tetra-heme cytochrome that is bound to the periplasmic faces of the L- and M-subunits (Deisenhofer et al., 1995). A combination of spectroscopy, x-ray crystallography, and mutagenesis has provided a detailed description of the mechanism of light energy transduction in the reaction center (for reviews see Parson, 1991, 1996; Fleming and van Grondelle, 1994; Woodbury and Allen, 1995; Hoff and Deisenhofer, 1997). However, relatively little is known about how the reaction center interacts with the protein/lipid environment that surrounds the complex in vivo. In *Rb. sphaeroides*, the reaction center is associated with the LH1 antenna complex and the PufX protein and so probably undergoes specific molecular interactions with both lipids and integral membrane proteins.

Recently a 2.1-Å resolution x-ray crystal structure for a *Rb. sphaeroides* reaction center carrying an Ala M260 to Trp (AM260W) mutation was determined (Protein Data Bank (PDB) entry 1QOV) (McAuley et al., 1999). A large region of continuous density was observed in the electron density map for this mutant, located on the intra-membrane surface of the reaction center (McAuley et al., 1999). This density was attributed to a single molecule of the diacidic phospholipid diphosphatidyl glycerol, or cardiolipin (Fig. 1 *A*). This electron density feature has also been seen in crystallographic data on other mutant reaction centers (McAuley et al., 2000) and therefore was not present as a consequence of the AM260W mutation.

Anionic phospholipids such as phosphatidyl glycerol and cardiolipin are found in energy-transducing membranes of both prokaryotes and eukaryotes, where they play a number of important roles. These include the formation of selective interactions with integral membrane proteins (Ioannu and Golding, 1979; Hoch, 1992; Dowhan, 1997). Cardiolipin is perhaps most widely known in this respect for its essential interaction with the respiratory protein cytochrome *c* oxidase. This protein carries a tightly bound cardiolipin even after extensive purification, and removal of this cardiolipin renders the complex inactive (Awashti et al., 1971; Robinson, 1982). Cardiolipin has also been shown to be important in the maintenance of optimal activity of a number of other major integral membrane proteins (Ioannu and Golding, 1979; Hoch, 1992). The precise details of how cardiolipin interacts with cytochrome *c* oxidase is not yet evident from the published x-ray structures for this complex (Iwata et al., 1995; Tsukihara et al., 1996; Ostermeier et al., 1997). However, a preliminary report of a new x-ray structure includes cardiolipin molecules located at the interface between the monomers in a dimer of cytochrome *c* oxidase (Mizushima et al., 1999).

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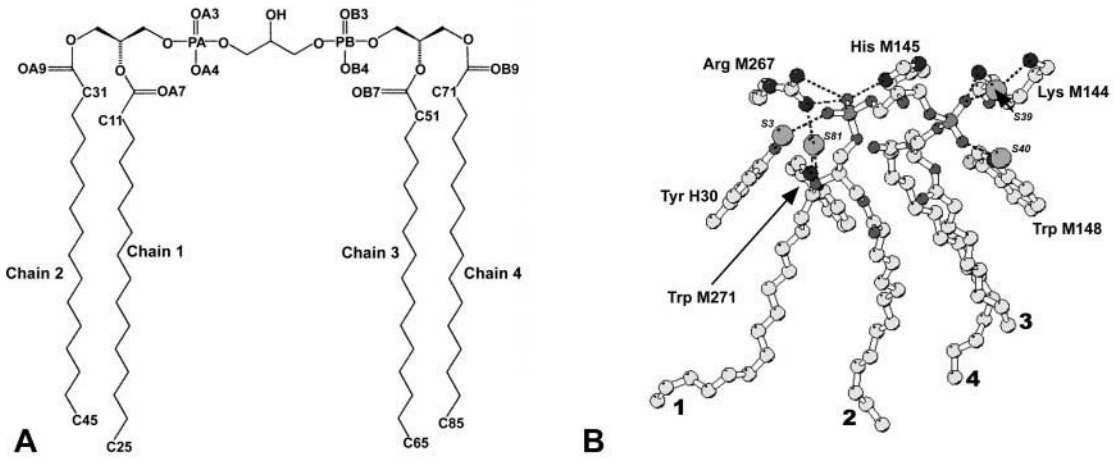

FIGURE 1 (*A*) Molecular structure of cardiolipin (diphosphatidyl glycerol), with four acyl chains each with 16 carbons. (*B*) View of the main bonding interactions between the cardiolipin headgroup and the *Rb. sphaeroides* reaction center (model 1QOV). The main-chain atoms of the amino acid residues have been omitted for clarity, with the exception of Lys M144.

The function of the molecule of cardiolipin that is observed to bind to the intra-membrane surface of the reaction center is unclear. As discussed by McAuley and co-workers (1999), it is known that cardiolipin is a minor component of the cytoplasmic membrane in many species of purple bacteria (Russell and Harwood, 1979; Imhoff and Bias-Imhoff, 1995), and it is also known that bacteriochlorophyll-containing proteins from these bacteria preferentially interact with anionic lipids (Birrell et al., 1978; Welte and Kreutz, 1982). However, to our knowledge there has been no specific study of the influence of cardiolipin on the properties of the bacterial reaction center. In the structure described by McAuley and co-workers (1999), the cardiolipin molecule occupies a depression in the intra-membrane surface of the reaction center. This depression is located between the N-terminal transmembrane α-helix of the H-subunit and the C transmembrane α-helix of the M-subunit. At the periplasmic side of the membrane the depression is bounded by amino acids 59–65 of the H-subunit. The lipid makes specific interactions with several amino acids from both the H- and M-subunit (Fig. 1 *B*). These contacts are either direct bonding interactions between the protein and the polar headgroup of the lipid or indirect bonding interactions between the two mediated by crystallographically defined water molecules. In addition, the interaction between the lipid and the protein is augmented by extensive van der Waals contacts between hydrophobic surface of the transmembrane region of protein and the lipid acyl chains (McAuley et al., 1999).

For many years following the first descriptions of the structures of reaction centers of the type found in *Rps. viridis* and *Rb. sphaeroides*, sequence information for the L- and M-subunits of the protein was limited to just four species of photosynthetic bacteria, namely, *Rps. viridis* (Michel et al., 1986), *Rb. sphaeroides* (Williams et al., 1986), *Rb. capsulatus* (Youvan et al., 1984), and *Rhodospirillum rubrum* (Belanger et al., 1988). Alignments of the sequences for the L- and M-subunits indicated a high degree of conservation of structurally and functionally important residues (Komiya et al., 1988). In recent years, a great deal of new sequence information for the L-, M-, and H-subunits has become available, enabling more extensive alignments to be constructed. In this report, we have compiled the sequence information available for reaction centers of the type found in *Rps. viridis* and *Rb. sphaeroides*. These complexes have (bacterio)pheophytin/ubiquinone electron acceptors and are classified as type II reaction centers, to distinguish them from the type I reaction centers of green sulfur bacteria and heliobacteria that have iron-sulfur complex acceptors and a more complex overall architecture. We use the compiled sequence information to examine whether the residues located at the site of interaction between cardiolipin and the *Rb. sphaeroides* reaction center are conserved in other species of photosynthetic bacteria with this type of reaction center. We also examine how sequence variations might affect the binding of cardiolipin.

MATERIALS AND METHODS

Sequence retrieval and alignment

Searches were made of the SWISS-PROT (Bairoch and Apweiler, 2000) and GenBank (Benson et al., 1999) sequence data bases, using PufL, PufM, and PuhA as the search subjects. Where multiple entries were found for a particular species only one of these was included in the alignment unless there were differences in the deposited sequences, in which case both sequences were used. The sequences were aligned with the program Clustal W (1.8) (Thompson et al., 1994). The reference sequences used in the alignments were those for *Rhodobacter sphaeroides*, and amino acid numbering is as for these sequences unless specified otherwise.

Molecular modeling and energy minimization

The coordinates of the structural model of the *Rps. viridis* reaction center (PDB entry 3PRC) (Lancaster and Michel, 1997) were superimposed on those of the model of the *Rb. sphaeroides* AM260W reaction center (PDB entry 1QOV) (McAuley et al., 1999) using the program LSQKAB (Collaborative Computational Project No. 4., 1994). The α-carbon positions of the amino acids of the L-, M-, and H-subunits were fitted, giving an average root mean square deviation of 0.29 Å. The cardiolipin molecule was then selected from the AM260W model and added to the superimposed *Rps. viridis* coordinates. The cardiolipin was translated such that the position of the phosphorus atom of P_A phosphate group overlapped with the central sulfur atom of the modeled sulfate ion (SO_4 802) in the 3PRC coordinate set, which was subsequently removed from the model. The resulting model was assigned the code VMOD1.

The coordinates of VMOD1 were prepared for energy minimization in the following manner. The addition of hydrogens and force-field parameterization was performed using the program Insight II 2.3.0. (Molecular Simulations, San Diego, CA), and the complex was soaked in a 4-Å shell of water. Energy minimization was then performed using the program Discover 2.9.7. (Molecular Simulations), with the protein backbone tethered to its crystallographic coordinates. Initial energy minimization was carried out for 100 iterations of steepest descents with a tethering force constant of 100 kcal/Å^2. This was followed by 2500 cycles of conjugate gradient minimization with a force constant of 50 kcal/Å^2, to give a final average absolute derivative of energy with respect to coordinates of 0.02 kcal/Å. This allowed the amino acid side chains and cofactors to relax while maintaining the backbone geometry of the polypeptides. The resultant minimized model was assigned the code VMOD2.

The figures were produced using the programs Molscript (Kraulis, 1991), SPOCK (J. A. Christopher, Texas A & M University, College Station, TX), Raster3D (Merritt and Bacon, 1997), and GRASP (Nicholls et al., 1991).

RESULTS AND DISCUSSION

Sequence alignment

The cardiolipin molecule described by McAuley and coworkers (1999) makes contacts with amino acids in the C and E transmembrane α-helices of the M-subunit, amino acids in a short loop that connect the D and DE helices of the L-subunit, and several residues in the transmembrane α-helix of the H-subunit. These amino acids are listed in Tables 1 and 2. To look at the cross-species conservation of these amino acids, the sequences for the PufL (L-subunit), PufM (M-subunit), and PuhA (H-subunit) polypeptides available in the SWISS-PROT (Bairoch and Apweiler, 2000) and GenBank (Benson et al., 1999) databases were collated and aligned, as described in Materials and Methods. Tables 1 and 2 contain a summary of the relevant information. Full details of these extensive alignments will be published elsewhere (manuscript in preparation). The relevant parts of the alignments, together with the database accession codes and supporting references, are available as supporting information in the form of a text file. The file can be accessed from the web page of the authors at http://www.bch.bris.ac.uk/staff/mrj.html or can be requested by E-mail to p.k.fyfe@bristol.ac.uk or m.r.jones@bristol.ac.uk.

A total of 49 full or partial sequences were obtained for the PufL protein, 45 full or partial sequences for the PufM protein, and 7 sequences for the PuhA protein. Sequences for PufL and PufM were obtained for several genera of the family *Rhodospirillaceae,* including three species of *Rhodobacter*, three species of *Rhodospirillum*, and either one or two species from the genera *Rhodopseudomonas*, *Rhodocyclus*, *Rhodoferax*, *Rhodomicrobium*, *Rhodoplanes*, *Rubrivivax, Blastochloris*, and *Rhodovulum*. Sequences were also obtained for two species of *Chromatiaceae*, namely, *Chromatium vinosum* and *Thiocystis gelatinosa*, one species of *Ectothiorhodospiraceae* (*Ectothiorhodospira shaposhnikovii*), two *Sphingomonas* species, and a number of aerobic anoxygenic phototrophs. The latter included several species of *Erythrobacter* and several *Acidiphilium* species. Sequences for the PuhA protein were obtained for *Rb. sphaeroides*, *Rb. capsulatus*, *Rps. viridis*, *Rhodospirillum rubrum*, *Rubrivivax gelatinosus*, and *Roseobacter denitrificans*.

For the L-subunit, it was possible to align all 49 sequences with only two gaps. The first of these was between amino acids L57 and L58 (*Rb. sphaeroides* numbering), to

TABLE 1 Conservation of residues in the region of the protein surface opposing the cardiolipin headgroup

Rb. sphaeroides residue	Identity in alignment (number of occurrences)	Percentage conservation*	Residue and position in *Rps. viridis*†
Leu L195	Leu (47), Val (2)	96	Leu L195
Asn L199	Asn (49)	100	Asn L199
Pro L200	Pro (44), Val (4), Thr (1)	90	Pro L200
Gly M143	Gly (42), Ser (3)	93	Gly M141
Lys M144	Thr (23), Met (11), Lys (7), Gln (3), Tyr (1)	16	Thr M142
His M145	His (45)	100	His M143
Trp M148	Trp (45)	100	Trp M146
Arg M267	Arg (14)	100	Arg M265
Trp M271	Trp (13), Phe (1)	93	Phe M269
Tyr H30	Tyr (7)	100	Tyr H31

The residues shown are within 6 Å of the modeled cardiolipin in the 1QOV structure. Data were collated from 49 full or partial sequences for the L-subunit, 45 full or partial sequences for the M-subunit, and 7 sequences for the H-subunit (see text).

*Percentage conservation relative to the *Rb. sphaeroides* sequence.

†Identity and position of the corresponding residue in the *Rps. viridis* reaction center.

TABLE 2 Conservation of residues in the region of the protein surface opposing the cardiolipin acyl chains

Rb. sphaeroides residue	Identity in alignment (number of occurrences)	Percentage conservation*	Residue and position in *Rps. viridis*†
Leu M151	Ala (40), Leu (5)	11	Ala M149
Ser M152	Ser (33), Ala (11), Met (1)	73	Ala M150
Trp M155	Trp (31), Phe (13), Leu (1)	69	Phe M153
Ile M270	Trp (11), Ile (2), Tyr (1)	14	Trp M268
Trp M271	Trp (13), Phe (1)	93	Phe M269
Val M274	Val (13), Leu (1)	93	Leu M272
Leu M278	Leu (9), Ile (3), Phe (1), Val (1)	64	Val M276
Ile M282	Ile (12), Val (1)	92	Val M280
Ser H19	Ser (3), Ala (3), Leu (1)	43	Ala H19
Phe H20	Phe (6), Gln (1)	86	Gln H20
Ile H22	Ala (2), Ile (4), Leu (1)	57	Leu H22
Phe H23	Phe (6), Val (1)	86	Val H23
Ala H25	Ala (6), Trp (1)	86	Trp H25
Gly H26	Gly (5), Tyr (1), Thr (1)	71	Thr H26
Leu H27	Leu (6), Val (1)	86	Val H27

The residues shown are within 6 Å of the modeled cardiolipin in the 1QOV structure. Data were collated from 49 full or partial sequences for the L-subunit, 45 full or partial sequences for the M-subunit, and 7 sequences for the H-subunit (see text).

*Percentage conservation relative to the *Rb. sphaeroides* sequence.

†Identity and position of the corresponding residue in the *Rps. viridis* reaction center.

accommodate an extra five amino acids in the three sequences for *Chromatiaceae* species. The second was either between amino acids L257 and L258 or between amino acids L258 and L259, to accommodate an extra two amino acids in the *Rps. palustris* sequence. All of the sequences appeared to be complete at the C-terminal end, but a number of sequences were lacking amino acids at the N-terminal end, such that amino acid L39 was the first to be present in all 49 sequences. Full sequence information was available for the central 200 amino acids of the L-subunit between positions L58 and L257, and the alignment in this region had no gaps.

For the M-subunit, it was again possible to align all 45 sequences with very few gaps. A one- or two-amino-acid gap had to be inserted into 41 of the 45 sequences in the M31-M35 region and a one-amino-acid gap in approximately two-thirds of the sequences in the M105-M108 region. Three of the sequences also required a one-amino-acid gap near the N-terminus. All of the sequences appeared to be complete at the N-terminal end, but many of the sequences were truncated before the C-terminus, at varying points between amino acids M242 and M278, such that only 16 of the 47 sequences used in the alignment were of the full PufM protein (the longest PufM sequence used had 330 amino acids). Full sequence information was available between amino acids M109 and M242, and the alignment in this region had no gaps.

Seven sequences were found for the H-subunit, including two sequences for *Rb. sphaeroides* that differed by three amino acids (H38, H81, and H107). The sequences could be aligned if a total of eight gaps were introduced, the largest of which was of five amino acids. One of these gaps was to accommodate a single additional amino acid in the N-terminal α-helix of the H-subunit in *Rps. viridis*. As it was not obvious from the aligned sequences, the decision as to where to place the single amino acid gap in the remaining six sequences was taken using the x-ray crystal structures of the *Rps. viridis* and *Rb. sphaeroides* reaction centers as a guide.

Overall conservation

For the alignments of the available sequence information to be useful in the present study, it had to be assumed that the overall backbone fold of the protein is essentially the same across the different bacterial species. The fact that the collated sequences could be aligned with only a few small gaps suggests that this assumption is valid. Those gaps that had to be introduced to achieve successful alignment of the PufL and PufM sequences were located outside regions expected to correspond to major structural elements such as the transmembrane α-helices or connecting amphipathic α-helices. Using the x-ray structure of the *Rb. sphaeroides* reaction center as a guide, all of the gaps in the alignment represented short (one- to five-amino-acid) insertions or deletions in surface-exposed loops. In addition, analysis of the aligned sequences showed absolute conservation of many of the amino acids that are important for the structure of the reaction center. These absolutely conserved residues include the axial His ligands to the four reaction center bacteriochlorophylls, the ligands to the non-heme iron, and key residues in the binding pockets of the Q_A and Q_B ubiquinones. The heavily studied residue pair Tyr M210 and Phe L181 was also absolutely conserved. There was also strong conservation of residues likely to be important

for the structure and assembly of the reaction center, including Pro residues that interrupt helical regions and Trp residues at the ends of transmembrane helices. The overall conclusion, therefore, is that it is highly likely that the overall backbone fold of the protein is essentially the same across the different bacterial species, and the remainder of the analysis in this report is based on this premise.

Bonding interactions between cardiolipin and the reaction center protein

In Table 1 we show the degree of cross-species conservation of each of the residues identified in the x-ray structure of the *Rb. sphaeroides* AM260W reaction center as contributing to that part of the protein surface that interacts with the cardiolipin headgroup. As shown in Fig. 1 *B*, the modeled cardiolipin makes possible bonding interactions with the side chains of residues His M145 and Arg M267 and with the backbone amide of Lys M144. Both Arg M267 and His M145 are absolutely conserved among the available sequences (Table 1). Lys M144 is not conserved (Table 1), but a backbone amide will be conserved at this position regardless of the identity of the M144 residue.

The modeled cardiolipin also makes a number of possible indirect bonding interactions with this region of the protein, mediated by water molecules (Fig. 1 *B*). Contacts can be traced to the hydroxyl of Tyr H30 (via water S3), to the indole nitrogen of Trp M271 (via water S81), to the side chain of Arg M267 (via water S81), to the terminal nitrogen of Lys M144 (via water S39), and to the indole nitrogen of Trp M148 (via water S40). The sequence information shows that the potential for these interactions is retained across the range of bacterial species considered, with both Trp M148 and Arg M267 being absolutely conserved. Tyr H30 is also conserved in the seven available sequences for the H-subunit. Trp M271 is conserved in 13 of the 14 available sequences for this region of the M-subunit, but is a Phe in the *Rps. viridis* reaction center. In the 45 sequences collated the M144 residue appears as, in descending order of frequency, a Thr, Met, Lys, Gln, and Tyr. Conservation of a Lys residue is therefore poor, but with the exception of 10 sequences that contain a Met, the M144 residue is conserved as a polar amino acid that could form a hydrogen bond interaction with an adjacent polar group (as illustrated below).

Interactions between the intra-membrane surface of the protein and the lipid acyl chains

The strength of the interaction between the protein and the cardiolipin is not due to ionic interactions alone. A large contribution is made through van der Waals interactions between the intra-membrane hydrophobic surface of the reaction center and the lipid acyl chains. This hydrophobic surface is contributed to by backbone and side-chain atoms of ~15 apolar amino acids, from the C and E transmembrane helices of the M-subunit and the single transmembrane helix of the H-subunit. The depth of the depression into which the cardiolipin binds is contributed to by a cleft between the C and E helices of the M-subunit. The area of the protein surface that engages in interactions with cardiolipin is ~543 $Å^2$, with the lipid tails bedding into grooves in the irregular surface of the protein. The identity and conservation of the amino acids that interact with the lipid acyl chains is summarized in Table 2.

Although the amount of conservation was not as high among these residues as those involved in bonding interactions with the lipid headgroup, with one exception where these amino acids were not conserved they were replaced by a hydrophobic amino acid of a similar size, or smaller. This suggests that the depression at this position on the surface of the *Rb. sphaeroides* reaction center will be retained in the reaction centers of the other species of photosynthetic bacteria, although the precise contours of the depression will vary according to the precise sequence.

The exception to this size conservation was residue Ile M270, which is located at the bottom of the depression that accommodates the cardiolipin, at the position where the acyl chains of the lipid join the headgroup. This residue is replaced by a Trp in all but one of the other available sequences, including that of the *Rps. viridis* reaction center (Table 2). In the remaining sequence, Ile M270 was replaced by a Tyr. In the next section, we look at the possible consequences of this substitution for the binding of cardiolipin.

Modeling the binding of cardiolipin to the intra-membrane surface of the *Rps. viridis* reaction center

In addition to the published data on the *Rb. sphaeroides* reaction center, high-resolution structural information is also available for the reaction center from *Rps. viridis* (Deisenhofer and Michel, 1989; Deisenhofer et al., 1995; Lancaster and Michel, 1997, 1999). This presents the opportunity to look at how differences in the protein sequence will affect the contours of that part of the surface of the reaction center that has been shown to bind cardiolipin in *Rb. sphaeroides*. In addition, it is interesting to examine whether the substitution of Trp for Ile at residue M270 would be catastrophic for the binding of cardiolipin to the reaction center in other species of photosynthetic bacteria.

The published x-ray structures of the *Rps. viridis* reaction center do not include a modeled cardiolipin. However, several of these structures do include a modeled sulfate ion in a position that is approximately equivalent to that of the P_A phosphate group of cardiolipin in the 1QOV structure of the *Rb. sphaeroides* AM260W mutant. This sulfate is in a position where it could interact with the absolutely conserved residues Arg M267 and His M145. In addition, in

some models of the *Rps. viridis* reaction center several water molecules have been modeled in positions that closely follow the expected position of the cardiolipin headgroup, when the P_A phosphate group of the latter is matched to the position of the sulfate ion (Deisenhofer and Michel., 1989; Lancaster and Michel, 1997, 1999). However, no detergents have been modeled in the region where the acyl chains of the cardiolipin might be expected to be located.

It is known that cytoplasmic membranes from *Rps. viridis* contain cardiolipin as a minor component (Imhoff and Bias-Imhoff, 1995). As a result, it is conceivable that the modeled sulfate marks a position where the headgroup of cardiolipin would bind if this lipid were attached to the *Rps. viridis* reaction center. It is even possible that the density modeled as a sulfate is in fact well defined partial density attributable an otherwise poorly defined bound cardiolipin. Studies of the *Rb. sphaeroides* reaction center have shown that the extent to which the cardiolipin is resolved varies between data sets for different mutant reaction centers, in a way that is not yet understood (unpublished data). Furthermore, a number of the more recent, higher-resolution x-ray structures for the *Rb. sphaeroides* reaction center include a single lauryl dimethylamine-N-oxide molecule modeled in the deep groove between the C and E helices of the M-subunit and/or an ion (phosphate or chloride) (Ermler et al., 1994a,b; Stowell et al., 1997; Axelrod et al., 2000). These are in positions that correspond to the most deeply buried acyl chain 4 of the cardiolipin and the P_A phosphate group, respectively. Again, it is an open question as to whether these features are genuinely as modeled or whether they are well resolved parts of an otherwise poorly resolved cardiolipin molecule. Clearly, this is a difficult issue to address, as it is difficult to distinguish between a partially resolved lipid and a mixture of bound ions and detergents, unless features such as the glycerol bridges that connect the phosphate groups to each other and to the lipid acyl chains are clearly resolved in the electron density.

To look at how the relevant region on the surface of the *Rps. viridis* reaction center would interact with a cardiolipin, the 3PRC structural model of this reaction center (Lancaster and Michel, 1997) was superimposed on the 1QOV model of the *Rb. sphaeroides* AM260W mutant, as described in Materials and Methods. The main part of the structure of the AM260W reaction center was removed, leaving the cardiolipin molecule and the *Rps. viridis* reaction center. The cardiolipin was then translated such that the phosphate group overlapped with the modeled sulfate in the 3PRC coordinate set, and this sulfate was then removed. Fig. 2 compares the resulting model of cardiolipin bound to the surface of the *Rps. viridis* reaction center (VMOD1) with the 1QOV structure of the AM260W mutant. As can be seen from Fig. 2 *A*, in the VMOD1 model there is good preservation of the direct interactions between the phosphate oxygens of the cardiolipin and residues Arg M267,

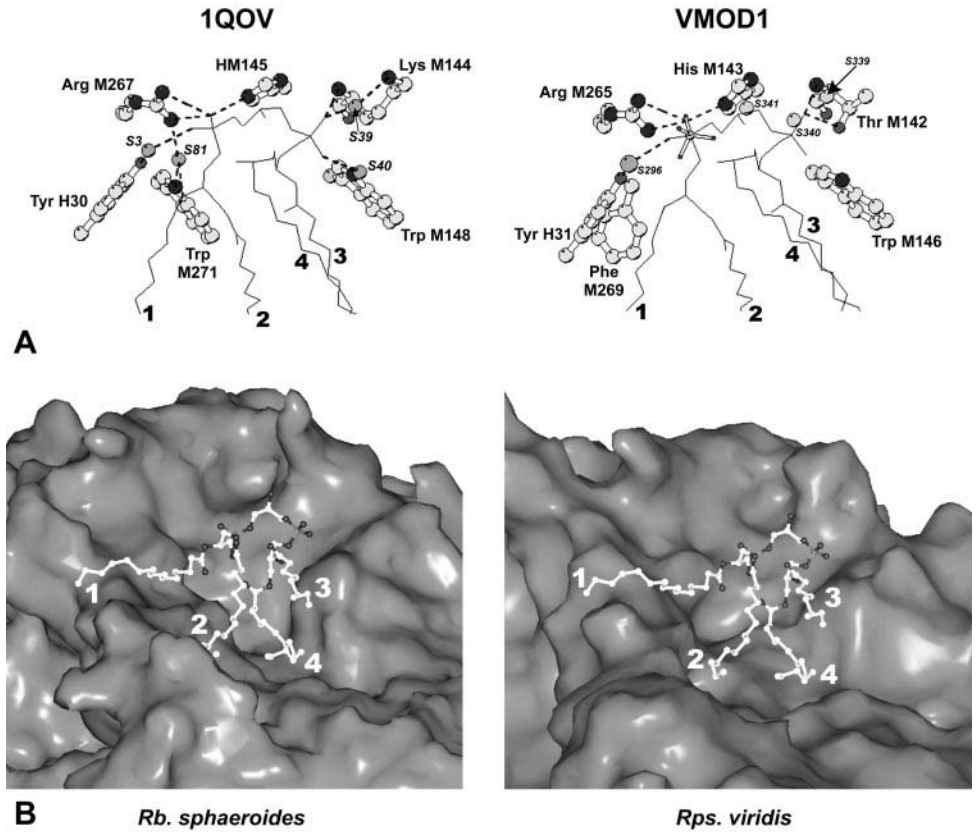

FIGURE 2 Comparison of the crystallographically determined interaction of cardiolipin with the reaction center of *Rb. sphaeroides* (1QOV) and a model of cardiolipin bound to the reaction center from *Rps. viridis* before energy minimization (VMOD1). (*A*) Main bonding interactions between cardiolipin (wire-frame format) and the surrounding protein. In the VMOD1 structure, the position of sulfate SO_4 802 used to position the P_A phosphate of the cardiolipin is also shown (stick format). (*B*) Surface representation model of the reaction center proteins with cardiolipin shown in stick format. The view is along the intra-membrane surface of the protein, from the periplasmic side of the membrane. In both *A* and *B* the acyl chains of the cardiolipin are numbered.

His M145, and Trp M148 (numbered M265, M143, and M146, respectively, in *Rps. viridis*) and the backbone amide of residue M144, which is Thr (M142) in *Rps. viridis* rather than Lys. In Table 3 the details of these putative bonding interactions in the different models are compared. An indirect contact with residue Tyr H30 (H31 in *Rps. viridis*) is also preserved, via water S296 present in the 3PRC *Rps. viridis* structure. Finally, it is apparent that the water-mediated contact between phosphate P_B and the side chain of Lys M144 in the 1QOV structure could be replaced by a direct contact between phosphate P_B and the side chain of the equivalent residue, Thr M142.

Fig. 2 *B* shows that the depression into which the cardiolipin fits in the *Rb. sphaeroides* reaction center is broader and shallower in the *Rps. viridis* reaction center, particularly at the point where the acyl chains join the headgroup of the lipid. The increased breadth is mainly due to the replacement of Trp M271 by Phe (M269), whereas the decreased depth is due to the replacement of Ile M270 by Trp (M268). As detailed in Fig. 3, this latter change results in part of the Trp M268 residue in the *Rps. viridis* VMOD1 model occupying the same volume as part of acyl chain 4 of the cardiolipin (Fig. 3, *B* compared with *A*), which is the chain most deeply embedded in the protein surface in *Rb. sphaeroides* (see Fig. 2 *B*). This clash shows clearly that a Trp at this position, as found in all available sequences other than that from *Rb. sphaeroides*, would not allow cardiolipin to adopt the conformation modeled in the VMOD1 structure.

To look at the question of whether this steric problem could be overcome, the VMOD1 model was subjected to energy minimization analysis, as described in Materials and Methods. Energy minimization of the VMOD1 model was continued until the point at which the average absolute derivative value had fallen to 0.02 kcal/Å. This point was reached after 2500 iterations of conjugate gradient minimization. Fig. 4 *A* shows a stereo view of the cardiolipin binding site in the *Rb.* sphaeroides reaction center, whereas Fig. 4 *B* shows the corresponding region in the energy-minimized VMOD2 *Rps. viridis* reaction center. The conformation of the cardiolipin headgroup region remains largely unaffected by the minimization. Electrostatic and hydrogen bonding interactions between the headgroup and the surrounding protein all show reasonable bonding distances (Table 3). In particular, the interactions of both Arg M267 and His M145 with the OA3 oxygen of the P_A phosphate group are well maintained.

More movement of the cardiolipin was observed in the acyl chain region following energy minimization. The largest shifts occurred near residue Trp M268, which in *Rps. viridis* partially blocks the deep cleft that is found in the *Rb. sphaeroides* structure. The acyl chain that occupies this cleft in the *Rb. sphaeroides* reaction center (chain 4) was lifted away from the protein by up to 3.7 Å in the VMOD2 model, flexing in order to pass over the Trp M270 (Figs. 3 *C* and 4 *B*). Unlike in the crystal structure of the *Rb. sphaeroides* structure where this acyl chain resides in a deep U-shaped cleft, the acyl chain in the VMOD2 model rests on a shelf formed by transmembrane helix E of the M subunit (Fig. 4 *B*). The movement of this acyl chain also causes some alteration of the packing of the adjacent chain 2. The top part of this chain is again pushed away from the protein surface by up to 2.5 Å and toward the end falls back to lie along a shallow groove on the protein surface. Acyl chains 1 and 3 showed little alteration in conformation. The shorter chain (chain 3) lies along the top of the more deeply buried acyl chains and showed a maximal displacement of ~1.5 Å. The remaining acyl chain (chain 1), which interacts most closely with the transmembrane helix of the H-subunit, shows very little change in the overall conformation. However, some alteration was observed near the end of the acyl

TABLE 3 Distances (in Å) calculated for bonding interactions between the protein and the cardiolipin headgroup

Atom 1	Atom 2	1QOV	VMOD1	VMOD2
PA OA3	Arg M267 NH1	3.2	2.9	2.6
PA OA3	Arg M267 NH2	2.8	2.7	2.9
PA OA3	His M145 NE	2.8	2.8	2.9
PA OA4	WAT 3 (WAT 296)	2.9	2.6	2.8
WAT 3 (WAT 296)	Tyr H30 OH	2.7	3.2	2.8
WAT81	*Arg M267 NH2*	3.1		
CDL O3	*WAT 81*	2.5		
WAT 81	*Trp M271 NE*	3.1		
PB OB3	Lys M144 N (Thr M144 N)	2.7	2.8	2.3
PB OB3	WAT 39 (WAT 339)	2.8	2.5	3.0
PB OB4	*WAT 40*	2.6		
WAT 40	*Trp M148 NE*	2.9		
WAT 39	Lys M144 NZ (Thr M144 NZ)	2.9		
CDL OB3	*Thr M142*		3.0	2.6

Discrepancies in the number and position of crystal waters built into the *Rb. sphaeroides* and *Rps. viridis* reaction center models preclude a complete comparison of all possible interactions. Text in plain type indicates interactions that are common to both *Rb. sphaeroides* and *Rps. viridis.* Text in parentheses indicates data for the *Rps. viridis* reaction center where the protein differs from that of *Rb. sphaeroides.* Text in italics indicates interactions that are unique to either *Rb. sphaeroides* or *Rps. viridis.*

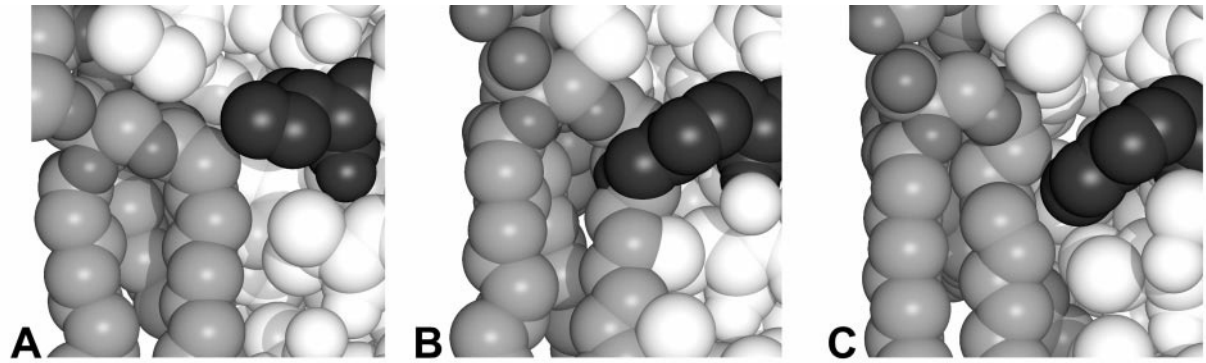

FIGURE 3 Cut away views of space-filling models of cardiolipin (mid-gray) interacting with the surface of the reaction center (white). (*A*) Cardiolipin interacting with the *Rb. sphaeroides* reaction center (1QOV structure). Acyl chain 4 passes over residue Ile M270 (black). (*B*) Same view of the VMOD1 model of cardiolipin interacting with the *Rps. viridis* reaction center. There is a steric clash between acyl chain 4 of the cardiolipin and residue Trp M269 (black). (*C*) Same view of the energy-minimized VMOD2 model of cardiolipin interacting with the *Rps. viridis* reaction center. Acyl chain 4 flexes by as much as 3.5 Å, to pass over residue Trp M269 (black).

chain where it rises by ~3.5 Å to accommodate the substitution of Thr for Gly at residue H26.

In Fig. 4 *C* the cardiolipin molecules from the 1QOV and VMOD2 models have been overlaid by matching their headgroup regions between phosphates P_A and P_B, as this region was hardly affected by the energy minimization procedure. As can be seen, the main effect of the minimization was to introduce a kink at the top of acyl chain 4, to accommodate residue Trp M268 in the *Rps. viridis* reaction center. This change in conformation of acyl chain 4 also pushes the top part of acyl chain 2 away from the protein surface, which is on the right in the view shown in Fig. 4 *C*.

Function of cardiolipin

Turning to the possible function of the bound cardiolipin in *Rb. sphaeroides*, the simplest explanation for its presence at this location on the surface of the reaction center may be that it is the most suitable lipid, in terms of size and shape, to interact with this part of the protein. Cardiolipin is a non-bilayer-forming lipid, with a headgroup that is small in relation to the size of the acyl chain region. A lipid with a larger headgroup might have more difficulty binding in this region, due to the overhang presented by residues H59–H65 and L200–L202 (see Fig. 4). However, it is tempting to speculate that this cardiolipin also has a more specific role. In cytochrome *c* oxidase a bound cardiolipin is essential for activity, and a preliminary report of an x-ray crystal structure of this protein places cardiolipin molecules at the interface between monomers in a cytochrome oxidase dimer (Mizushima et al., 1999). Recent high-resolution x-ray structures of bacteriorhodopsin have also shown very clearly the role of lipids in mediating protein-protein contacts between monomers in a trimeric structure (Essen et al., 1998; Luecke et al., 1999). These observations raise an obvious question: could the molecule of cardiolipin that is attached to the surface of the reaction center be located at the point of contact between reaction centers in a dimeric structure? When purified and crystallized, the *Rb. sphaeroides* reaction center is clearly not a dimer. However, there is both spectroscopic and biochemical evidence that the membrane-bound *Rb. sphaeroides* reaction center is dimeric (Morrison et al., 1977; Vermeglio et al., 1993; Francia et al., 1999; Loach, 2000), and this is clearly a point that warrants further investigation. That part of the surface of the *Rb. sphaeroides* reaction center to which cardiolipin binds would provide a good contact point between monomers in a dimer. Examination of space-filling models of the reaction center show that it is by far the flattest part of the intramembrane surface of the reaction center, particularly when the cardiolipin molecule is included in the structure.

General conclusions

The protein sequence data analyzed in this report reveals that the depression in the intra-membrane surface of the *Rb. sphaeroides* reaction center, which provides a site of interaction with a molecule of cardiolipin, is well preserved in terms of size, shape, and charge distribution over a wide range of photosynthetic bacteria. It is also known that cardiolipin is a component of the cytoplasmic membrane in a variety of photosynthetic bacteria, including many species of the *Rhodospirillaceae*, *Chromatiaceae*, and *Ectothiorhodospiraceae* (Imhoff and Bias-Imhoff, 1995), and it is probable that it is present in the membranes of most of the species of photosynthetic bacteria surveyed in this work. It is not ubiquitous, however, and, for example, it does not appear to be present in either *Rb. capsulatus* or *Rhodomicrobium vanielli* (Imhoff and Bias-Imhoff, 1995). Given this, it is not our purpose in this report to attempt to prove that the reaction centers from all of these bacteria bind cardiolipin at the position indicated by the *Rb sphaeroides*

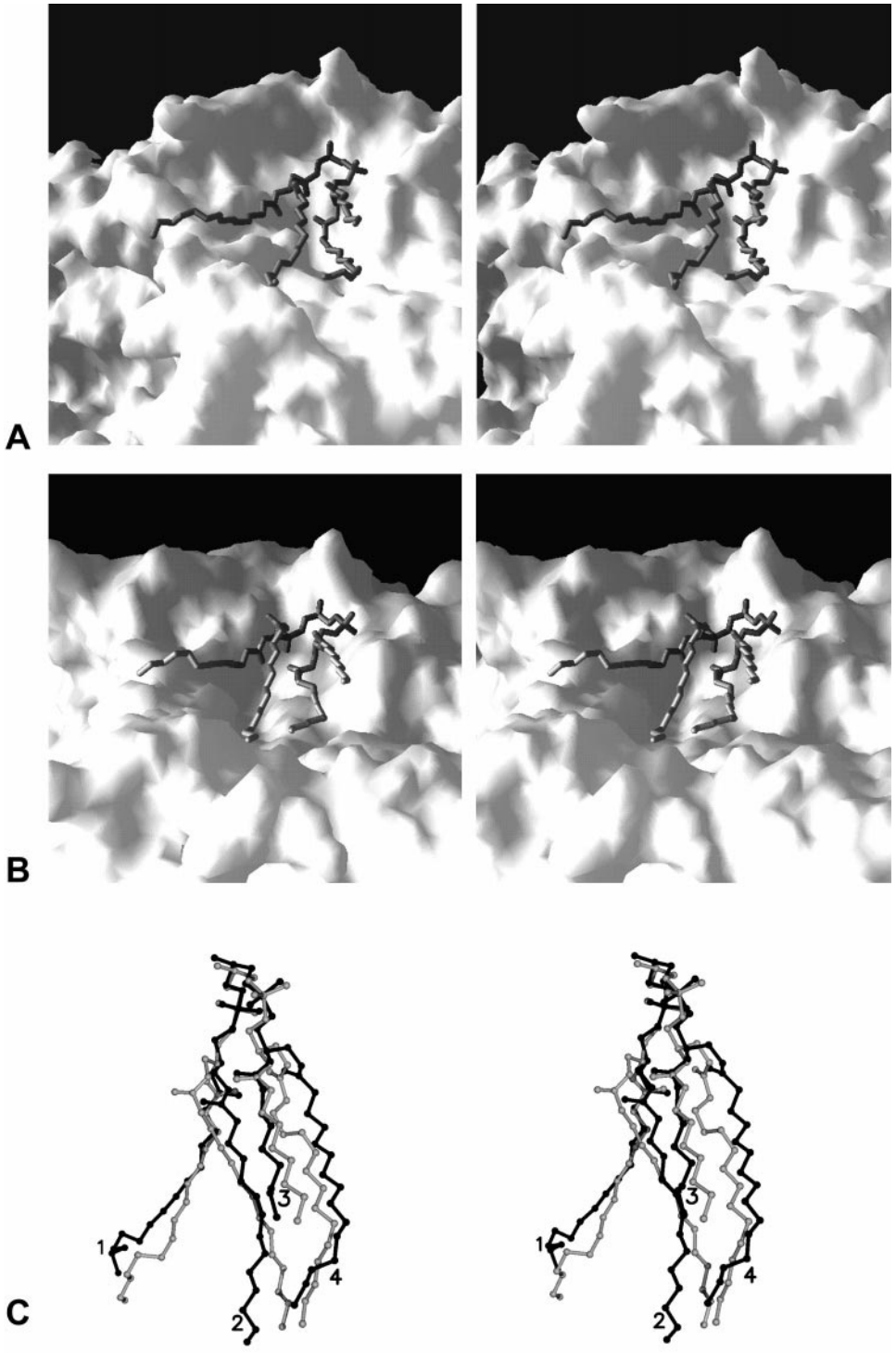

FIGURE 4 Cardiolipin in the VMOD2 model of the *Rps. viridis* reaction center. (*A*) Stereo image of a surface representation of the *Rb. sphaeroides* reaction center, with the crystallographically resolved cardiolipin molecule shown in stick format. The view is along the intra-membrane surface of the protein, from the periplasmic side of the membrane. (*B*) Stereo image showing a surface representation of the *Rps. viridis* reaction center, with the cardiolipin shown in stick format. (*C*) Stereo image of the cardiolipin molecules from the 1QOV (dark gray) and VMOD2 (light gray) models.

1QOV structure. Rather, our aim is to point out the sequence conservation in this region of the protein and to draw attention to the possibility that cardiolipin may bind to the protein surface at this position in those photosynthetic bacteria that contain this lipid. The modeling studies with the *Rps. viridis* reaction center illustrate how modest changes in the detailed conformation of the cardiolipin could overcome variations in the precise architecture of the surface of the reaction center caused by variation in the amino acids that contribute to this surface.

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REFERENCES

Awashti, Y. C., T. F. Chuang, T. W. Keenan, and F. L. Crane. 1971. Tightly bound cardiolipin in cytochrome oxidase. *Biochim. Biophys. Acta.* 226:45–52.

Axelrod, H. L., E. C. Abresch, M. L. Paddock, M. Y. Okamura, and G. Feher. 2000. Determination of the binding sites of the proton transfer inhibitors Cd^{2+} and Zn^{2+} in bacterial reaction centers. *Proc. Natl. Acad. Sci. U.S.A.* 97:1542–1547.

Bairoch, A., and R. Apweiler. 2000. The SWISS-PROT protein sequence database and its supplement TrEMBL in 2000. *Nucleic Acids Res.* 28:45–48

Belanger, G., J. Berard, P. Corriveau, and G. Gingras. 1988. The structural genes coding for the L-subunit and M-subunit of *Rhodospirillum rubrum* photoreaction center. *J. Biol. Chem.* 263:7632–7638.

Benson, D. A., M. S. Boguski, D. J. Lipman, J. Ostell, B. F. F. Ouellette, B. A. Rapp, and D. L. Wheeler. 1999. GenBank. *Nucleic Acids Res.* 7:21–17.

Birrell, G. B., W. R. Sistrom, and O. H. Griffith. 1978. Lipid-protein associations in chromatophores from the photosynthetic bacterium *Rhodopsuedomonas sphaeroides. Biochemistry.* 17:3768–3773.

Collaborative Computational Project No. 4. 1994. *Acta Crystallogr. D.* 50:760–763

Deisenhofer, J., O. Epp, I. Sinning, and H. Michel. 1995. Crystallographic refinement at 2.3-Å resolution and refined model of the photosynthetic reaction center from *Rhodopseudomonas viridis. J. Mol. Biol.* 246: 429–457.

Deisenhofer, J., and H. Michel. 1989. The photosynthetic reaction center from the purple bacterium *Rhodopseudomonas viridis. EMBO J.* 8:2149–2170.

Dowhan, W. 1997. Molecular basis for membrane phospholipid diversity: why are there so many lipids? *Annu. Rev. Biochem.* 66:199–232.

Ermler, U., G. Fritszch, S. K. Buchanan, and H. Michel. 1994a. Structure of the photosynthetic reaction center from *Rhodobacter sphaeroides* at 2.65-Å resolution: cofactors and protein-cofactor interactions. *Structure.* 2:925–936.

Ermler, U., H. Michel, and M. Schiffer. 1994b. Structure and function of the photosynthetic reaction center from *Rhodobacter sphaeroides. J. Bioenerg. Biomembr.* 26:5–15.

Essen, L.-O., R. Siegert, W. D. Lehmann, and D. Oesterhelt. 1998. Lipid patches in membrane protein oligomers: crystal structure of the bacteriorhodopsin-lipid complex. *Proc. Natl. Acad. Sci. U.S.A.* 95: 11673–11678.

Fleming, G. R., and R. van Grondelle. 1994. The primary steps of photosynthesis. *Phys. Today.* 47:48–55.

Francia, F., J. Wang, G. Venturoli, B. A. Melandri, W. P. Barz, and D. Oesterhelt. 1999. The reaction center-LH1 antenna complex of *Rhodobacter sphaeroides* contains one PufX molecule which is involved in dimerization of this complex. *Biochemistry.* 38:6834–6845.

Hoch, F. L. 1992. Cardiolipins and biomembrane function. *Biochim. Biophys. Acta.* 1113:71–133.

Hoff, A. J., and J. Deisenhofer. 1997. Photophysics of photosynthesis. Structure and spectroscopy of reaction centers of purple bacteria. *Phys. Lett.* 287:2–247.

Imhoff, J. F., and U. Bias-Imhoff. 1995. Lipids, quinones and fatty acids of anoxygenic phototrophic bacteria. *In* Anoxygenic Photosynthetic Bacteria. R. E. Blankenship, M. T. Madigan, and C. Bauer, editors. Kluwer Academic Publishers, Dordrecht, The Netherlands. 179–205.

Ioannu, P. V., and B. T. Golding. 1979. Cardiolipins: their chemistry and biochemistry. *Prog. Lipid Res.* 17:279–318.

Iwata, S., C. Ostermeier, B. Ludwig, and H. Michel. 1995. Structure at 2.8-Å resolution of cytochrome *c* oxidase from *Paracoccus denitrificans. Nature.* 376:660–669.

Komiya, H., T. O. Yeates, D. C. Rees, J. P. Allen, and G. Feher. 1988. Structure of the reaction center from *Rhodobacter sphaeroides* R-26 and 2.4.1: symmetry-relations and sequence comparisons between different species. *Proc. Natl. Acad. Sci. U.S.A.* 85:9012–9016.

Kraulis, P. J. 1991. Molscript : a program to produce both detailed and schematic plots of protein structures. *J. Appl. Crystallogr.* 24:946–950.

Lancaster, C. R. D., and H. Michel. 1997. The coupling of light-induced electron transfer and proton uptake as derived from crystal structures of reaction centers from *Rhodopseudomonas viridis* modified at the binding site of the secondary quinone, Q_B. *Structure.* 5:1339–1359.

Lancaster, C. R. D., and H. Michel. 1999. Refined crystal structures of reaction centers from *Rhodopseudomonas viridis* in complexes with the herbicide atrazine and two chiral atrazine derivatives also lead to a new model of the bound carotenoid. *J. Mol. Biol.* 286:883–898.

Loach, P. A. 2000. Supramolecular complexes in photosynthetic bacteria. *Proc. Natl. Acad. Sci. U.S.A.* 97:5016–5018.

Luecke, H., B. Schobert, H.-T. Richter, J.-P. Cartailler, and J. K. Lanyi. 1999. Structure of bacteriorhodopsin at 1.55 Å resolution. *J. Mol. Biol.* 291:899–911.

McAuley, K. E., P. K. Fyfe, R. J. Cogdell, N. W. Isaacs, and M. R. Jones. 2000. X-ray crystal structure of the YM210W mutant reaction center from *Rhodobacter sphaeroides. FEBS Lett.* 467:285–290.

McAuley, K. E., P. K. Fyfe, J. P. Ridge, N. W. Isaacs, R. J. Cogdell, and M. R. Jones. 1999. Structural details of an interaction between cardiolipin and an integral membrane protein. *Proc. Natl. Acad. Sci. U.S.A.* 96:14706–14711.

Merritt, E. A., and D. J. Bacon. 1997. Raster3D: Photorealistic molecular graphics. *Methods Enzymol.* 277:505–524.

Michel, H., K. A. Weyer, H. Gruenberg, I. Dunger, D. Oesterhelt, and F. Lottspeich. 1986. The light and medium subunits of the photosynthetic reaction center from *Rhodopseudomonas viridis*: isolation of the genes, nucleotide and amino-acid-sequence. *EMBO J.* 5:1149–1158.

Mizushima, T., M. Yao, N. Inoue, H. Aoyama, E. Yamashita, H. Yamaguchi, T. Tsukihara, R. Nakashima, K. Shinzawa-Itoh, R. Yaono, and S. Yoshikawa. 1999. Structure of phospholipids in a membrane protein complex, bovine heart cytochrome *c* oxidase. *Acta Crystallogr. A55 Supplement.* Abstr. P06.04.069.

Morrison, L., J. Runquist, and P. A. Loach. 1977. Ubiquinone and photochemical activity in *Rhodospirillum rubrum. Photochem. Photobiol.* 25:73–84.

Nicholls, A., K. A. Sharp, and B. Honig. 1991. Protein folding and association: insights from the interfacial and thermodynamic properties of hydrocarbons. *Proteins.* 11:281–296.

Ostermeier, C., A. Harrenga, U. Ermler, and H. Michel. 1997. Structure at 2.7 Å resolution of the *Paracoccus denitrificans* two-subunit cytochrome *c* oxidase complexed with an antibody F-V fragment. *Proc. Natl. Acad. Sci. U.S.A.* 94:10547–10553.

Parson, W. W. 1991. Reaction centers. *In* Chlorophylls. H. Scheer, editor, CRC Press, Boca Raton, FL. 1153–1180

Parson, W. W. 1996. Photosynthetic bacterial reaction centers. *In* Protein Electron Transfer. D. S. Bendall, editor, BIOS Scientific Publishers, Oxford. 125–160.

Robinson, N. C. 1982. Specificity and binding-affinity of phospholipids to the high- affinity cardiolipin sites of beef-heart cytochrome *c* oxidase. *Biochemistry.* 21:184–188.

Russell, N. J., and J. L. Harwood. 1979. Changes in the acyl lipid composition of photosynthetic bacteria grown under photosynthetic and non-photosynthetic conditions. *Biochem. J.* 181:339–345.

Stowell, M. H. B., T. M. McPhillips, D. C. Rees, S. M. Soltis, E. Abresch, and G. Feher. 1997. Light-induced structural changes in photosynthetic reaction center: implications for mechanism of electron-proton transfer. *Science.* 276:812–816.

Thompson, J. D., D. G. Higgins, and T. J. Gibson. 1994. Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 22:4673–4680.

Tsukihara, T., H. Aoyama, E. Yamashita, T. Tomizaki, H. Yamaguchi, K. Shinzawa-Itoh, R. Nakashima, R. Yaono, and S. Yoshikawa. 1996. The whole structure of the 13-subunit oxidized cytochrome *c* oxidase at 2.8 Å. *Science.* 272:1136–1144.

Vermeglio, A., P. Joliot, and A. Joliot. 1993. The rate of cytochrome c_2 photooxidation reflects the subcellular-distribution of reaction centers in

Rhodobacter sphaeroides GA cells. *Biochim. Biophys. Acta.* 1183: 352–360.

Welte, W., and W. Kreutz. 1982. Formation, structure and composition of a planar hexagonal lattice composed of specific protein-lipid complexes in the thylakoid membranes of *Rhodopseudomonas viridis. Biochim. Biophys. Acta.* 692:479–488.

Williams, J. C., L. A. Steiner, and G. Feher. 1986. Primary structure of the reaction center from *Rhodopseudomonas sphaeroides*. *Proteins Struct. Funct. Genet.* 1:312–325.

Woodbury, N. W., and J. P. Allen. 1995. The pathway, kinetics and thermodynamics of electron transfer in wild type and mutant bacterial reaction centers of purple nonsulfur bacteria. *In* Anoxygenic Photosynthetic Bacteria. R. E. Blankenship, M. T. Madigan, and C. Bauer, editors. Kluwer Academic Publishers, Dordrecht, The Netherlands. 527–557.

Youvan, D. C., E. J. Bylina, M. Alberti, H. Begusch, and J. E. Hearst. 1984. Nucleotide and deduced polypeptide sequences of the photosynthetic reaction-center, B870 antenna, and flanking polypeptides from *R. capsulata. Cell.* 37:949–957.