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Review

Structural determinants of purple membrane assembly

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Abstract

The purple membrane is a two-dimensional crystalline lattice formed by bacteriorhodopsin and lipid molecules in the cytoplasmic membrane of *Halobacterium salinarum*. High-resolution structural studies, in conjunction with detailed knowledge of the lipid composition, make the purple membrane one of the best models for elucidating the forces that are responsible for the assembly and stability of integral membrane protein complexes. In this review, recent mutational efforts to identify the structural features of bacteriorhodopsin that determine its assembly in the purple membrane are discussed in the context of structural, calorimetric and reconstitution studies. Quantitative evidence is presented that interactions between transmembrane helices of neighboring bacteriorhodopsin molecules contribute to purple membrane assembly. However, other specific interactions, particularly between bacteriorhodopsin and lipid molecules, may provide the major driving force for assembly. Elucidating the molecular basis of protein–protein and protein–lipid interactions in the purple membrane may provide insights into the formation of integral membrane protein complexes in other systems.

Keywords: Membrane protein assembly; Two-dimensional crystallization; Protein–lipid interaction

1. Introduction

A major challenge in cell biology has been to understand the structural changes that a protein undergoes as it matures in the cell from a nascent polypeptide chain to its native functional state. This maturation process, termed protein biogenesis, can be analyzed from at least two perspectives: the structural features of a polypeptide that ultimately determine its folding and assembly, and the mechanisms by which cellular factors influence the efficiency of these processes. Although substantial progress has been made toward understanding the biogenesis of soluble proteins [1], less is known about the biogenesis of integral membrane proteins, which fold and assemble within the lipid bilayer.

Bacteriorhodopsin (BR), a retinal-containing integral membrane protein of the archaeon *Halobacterium salinarum*, is an excellent model for biogenesis studies. BR is induced by more than 50-fold under low-oxygen conditions [2,3] and accumulates at high levels in the cytoplasmic membrane. Under these conditions, BR assembles in two-dimensional crystalline patches known as the purple membrane (PM). High-resolution structural analysis of the PM (see below) and recent advances in *H. salinarum* molecular biology [4–7] have made it possible to investigate BR biogenesis at the molecular level [8–11]. This review focuses on studies of the structural features of BR that determine its assembly in the PM.

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2. PM composition

The PM is a protein–lipid complex of defined composition. The major protein of the PM is BR, which consists of the 248 amino acid polypeptide bacterioopsin (BO) [12] and a covalently bound molecule of all-*trans* retinal [2,13]. The only other proteins are BR precursors, which comprise ~30% of the total protein and arise from incomplete processing of a 13-amino acid presequence at the N terminus of the polypeptide [14–16]. The major lipid of the PM is PGP-Me (Fig. 1) [17], which is the main constituent of the *H. salinarum* cellular membrane. The minor lipids include two phospholipids (PG and PGS), a sulfated triglycosyl lipid (S-TGA-1) and squalene (Fig. 1). The apolar moeity of the phospholipids and sulfoglycolipids is archaeol (Fig. 1), consisting of two C_{20} isoprenoid chains in an ether linkage to glycerol. There are ~10 lipid molecules per molecule of BR in the PM, comprising 6–7 phospholipids, 2–3 sulfoglycolipids and 1 squalene [17,18].

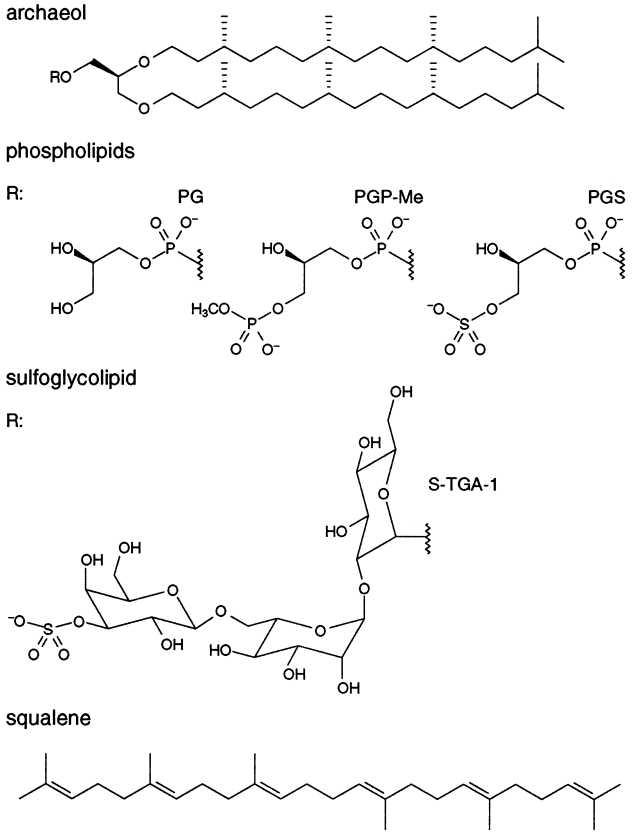

Fig. 1. The major lipids of the *Halobacterium salinarum* purple membrane. The polar lipids consist of the apolar moiety *sn*-2,3-diphytanylglycerol (archaeol) attached to polar headgroups (R). Phospholipids include PGP-Me (phosphatidylglycerol phosphate methyl ester), PGS (phosphatidylglycerol sulfate) and PG (phosphatidylglycerol). The sulfoglycolipid is 3-sulfate-Gal*p*β1–6Man*p*α1–2Glc*p*α-1-archaeol (S-TGA-1). Squalene is the major apolar lipid of the PM other than retinal.

The lipid composition of the PM differs from that of the surrounding cytoplasmic membrane. Chemical analysis indicates that carotenoids found in the cytoplasmic membrane are excluded from the PM, while the sulfated lipids PGS and S-TGA-1 are present only in the PM [19]. The absence of S-TGA-1 from cytoplasmic membrane fractions has been confirmed by mass spectrometric analysis [20]. These results raise the possibility that selective interactions occur between BR and certain lipid molecules, and that these interactions are essential for lattice assembly.

3. PM structure

The structure of BR was first determined by electron crystallography of isolated PM patches [21]. The protein contains seven transmembrane α-helices (A–G in Fig. 2A) surrounding the retinal chromophore and is arranged in trimeric units that pack in a hexagonal lattice (space group *P*3 with a unit cell dimension of ~62 Å) (Fig. 2B). Lipid molecules are located between trimers and in the space enclosed by each trimer (Fig. 2B). This structural model has been refined by higher resolution electron crystallography of the PM [18,22,23], by X-ray diffraction of BR crystallized from a lipidic cubic phase in the presence of endogenous lipids [24–27], and by X-ray diffraction of BR crystallized by heterogenous nucleation on benzamidine [28].

The highest resolution structural data have produced models that are in close agreement [23,26–28]. Except for the loop between transmembrane helices E and F (the EF loop), the polypeptide chain from residues 7 to 225 is resolved in all of the structures, which includes all of the transmembrane α-helices. The EF loop is disordered in all of the structures except one [28]; 1–6 amino acid residues at the N terminus and 17–23 residues at the C terminus are also disordered. The tertiary fold and trimeric organization of BR are similar in the highest resolution

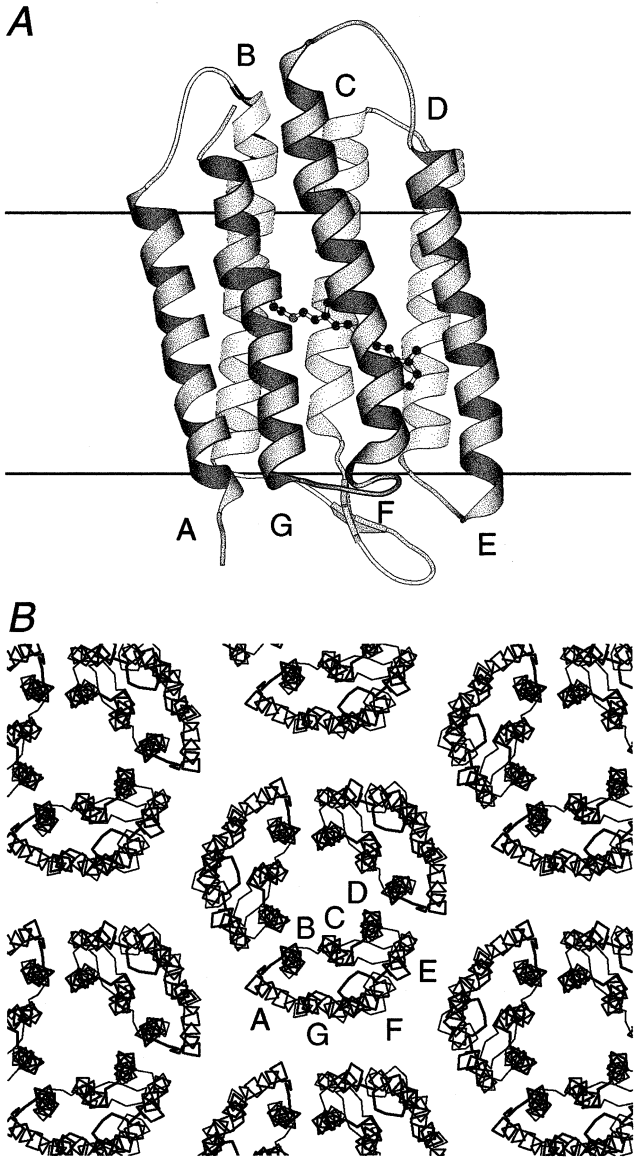

Fig. 2. Bacteriorhodopsin and the purple membrane. (A) Side view of the BR monomer as seen from outside the trimer. The seven transmembrane helices are labeled A–G and the retinal cofactor is drawn in ball-and-stick representation. Lines denote the boundaries of the apolar membrane core (as defined by the lipid ether oxygen atoms), with the cytoplasmic surface at the top. (B) Cytoplasmic view of the purple membrane. The BR polypeptide backbone is drawn with the transmembrane helices A–G labeled for one BR monomer. The 2at9 coordinates were used [23] and rendered with MOLSCRIPT [81].

structures, except for differences in some of the loops [23,26–28]. Strikingly, BR in three-dimensional crystals grown from the lipidic cubic phase [26,27] is arranged in hexagonally packed trimers with the same unit cell dimensions as native PM, indicating that these crystals are an accurate model of the PM. In several of the structures, features of the endogenous lipid molecules, particularly the hydrocarbon chains, are resolved (see below). Taken together, these structures provide the most detailed view currently available of an integral membrane protein in its native lipid environment, and serve as a starting point for analyzing the structural features of BR that determine lattice assembly.

4. Pathway of lattice assembly

The PM lattice is probably assembled from BO or BR molecules that are fully integrated into the membrane, since the protein is inserted into the membrane co-translationally [8,9]. Because the integrated protein can diffuse only within the lipid bilayer, assembly likely occurs by addition of protein and lipid molecules to the perimeter of a PM patch, rather than to its interior. At early stages of induction, BR forms round PM patches with a diameter of $\sim$0.5 μm [29] containing $\sim$6000 unit cells and $\sim$18 000 BR monomers (three per unit cell). At later stages of BR induction, PM patches fuse, yielding irregularly shaped patches that may have more than one crystalline domain [29].

The substrate for lattice assembly is probably BR, not BO. BO produced in retinal-deficient strains forms a 'white membrane' that exhibits a low degree of hexagonal order [30,31], suggesting that the apoprotein retains some of the structural features needed for lattice assembly. However, the white membrane is poorly ordered compared to the PM. The white membrane exhibits a range of buoyant densities that are lower than that of the PM (1.18 g/ml) [32,33], suggesting that the protein-to-lipid ratio is both lower and more variable than in the PM. Moreover, membranes containing BO are not crystalline. Treatment of the PM with hydroxylamine, which converts BR to BO by disrupting the retinal linkage, yields a non-crystalline apomembrane [34]. Similarly, BO produced in cells grown in the presence of nicotine, which partially inhibits retinal biosynthesis, accumulates in a brown membrane that is non-crystalline [35–37]. The absence of a crystalline lattice in the white and brown membranes is due to BO, since

the PM can be restored in both cases by adding sufficient retinal to fully regenerate BR [30,38,39].

These results indicate that BR is necessary for lattice assembly and suggest that conformational changes known to occur upon retinal binding [40] are critical. In support of this, lattice assembly is perturbed by mutations that alter BR tertiary structure. D85N, which produces an equilibrium mixture of several conformational states [41], accumulates in membrane fractions of low buoyant density (M.P.K., unpublished results; G. Turner, personal communication) in addition to the lattice form [5]. Similarly, P186L, which has a visible absorption maximum that is blue-shifted by 90 nm, accumulates exclusively in the low-density membrane fraction and fails to form the lattice (M.P.K., unpublished results). In general, lattice assembly may be disrupted in BR mutants that exhibit major spectral shifts.

The oligomerization state of BR that adds to the growing lattice has not been identified. Although BR is generally assumed to form trimers prior to lattice assembly, there is little evidence that the trimer exists as a stable entity or as an intermediate in lattice assembly in vivo. Trimers have been observed in BR preparations solubilized from the PM in non-ionic detergent [42], in proteoliposomes containing native and non-native lipids [43,44], and in a crystallized form of BR [28], but not as isolated species in *H. salinarum* membranes. Thus, it is equally possible that lattice growth proceeds by addition of monomeric BR or a monomeric BR–lipid complex. Regardless of whether monomeric or trimeric BR is the substrate for assembly, it is apparent that the self-association of BR monomers, either in isolation or in the context of the lattice, is essential.

5. Energetics of lattice assembly

Lattice assembly is a thermodynamically favorable process as demonstrated by reconstitution studies in vitro. Monomeric BR can be prepared by solubilizing purified PM in a non-ionic detergent, such as Triton X-100, yielding mixed micelles that contain protein, detergent and native lipids. Removal of the detergent results in spontaneous formation of the crystalline lattice, indicating that lattice assembly is thermodynamically favorable in vitro [34,38,45]. However, it has been suggested that lattice assembly in vivo is not spontaneous. Reconstitution of the PM from hydroxylamine-bleached BR in intact cells requires metabolic energy [35], which is surprising in light of the in vitro results. A possible explanation for this observation is that energy may be required for the synthesis of lipids essential for PM assembly, which may be degraded when the lattice is disrupted.

PM thermal stability has been investigated by differential scanning calorimetry. Two calorimetric transitions are observed at physiological pH: a reversible transition at 70–80°C, and an irreversible denaturational transition at $\sim$100°C [46–49]. The reversible transition was interpreted as a structural reordering of the lattice [46]. This has been confirmed by X-ray diffraction studies of hydrated PM films, which indicate a reversible melting of the lattice at $\sim$75°C [50,51]. A low degree of order is retained above the melting temperature, perhaps corresponding to BR trimers [50,51]. The denaturational transition at $\sim$100°C exhibited an enthalpy change of 100 kcal/mol and was cooperative, consistent with the idea that the protein denatures as a trimer [46,49,52]. Remarkably, monomeric BR in detergent is denatured with a nearly identical enthalpy change (95 kcal/mol), although at a lower temperature of 80°C [53]. This suggests that BR is more stable in the trimer, and that the greater stability is due to entropic factors [54].

The stabilizing effect of the trimer may be estimated by calculating the ΔS values of denaturation of the monomeric and trimeric species at the transition temperature, where $\Delta H = T\Delta S$. The ΔS value can be used to calculate the ΔG of denaturation of the two species at the same temperature [54]. This calculation indicates that trimeric BR is $\sim$5 kcal/mol more stable than monomeric BR. Thus, BR in the lattice is stabilized by at least 5 kcal/mol due to trimer formation, and possibly more due to favorable energetics of the association between trimers in the lattice.

6. Role of BR–BR interactions in lattice assembly

PM structural models can be used to identify intermolecular protein–protein and protein–lipid contacts that are potentially important for lattice assem-

bly. We first consider BR–BR interactions, which may be critical for the formation of the trimer and thus the assembly of the lattice. Close contacts between monomers are confined to an interface with a buried surface of ~520–650 Å^2 per BR [26,28]. Most of this interface is located within the hydrocarbon core of the membrane and includes contacting amino acid residues in transmembrane helix B of one monomer and helices D and E of an adjacent monomer. Contacting amino acid residues are defined as those that contain heavy atoms separated by <4 Å, which is sufficient to include residues engaged in van der Waals contacts, hydrogen bonding and salt bridges. In the transmembrane helical regions, the contacting residues are almost entirely apolar and appear to engage in van der Waals packing (Fig. 3A). Additional contacts occur among polar amino acid residues in the BR loop regions (see below).

6.1. Helix–helix interactions

In initial mutational studies of lattice assembly [10,11], the helix–helix interface between BR monomers was suspected as a likely site of key interactions. This view was based on the two-stage model of membrane protein assembly, in which interactions between independently stable transmembrane α-helices provide a driving force for membrane protein folding and oligomerization [55,56]. Systematic studies of the glycophorin A dimer have established that packing interactions between apolar amino acids in adjacent transmembrane α-helices contribute significantly to membrane protein oligomerization [57–60].

To test the importance of helix–helix interactions in lattice assembly, site-specific mutagenesis of BR was carried out in *H. salinarum* [10,11]. Single amino acid substitutions were made that perturb lattice assembly while causing minimal effects on BR tertiary structure. Lattice accumulation was determined by examining the distribution of BR in membrane fractions obtained by lysing *H. salinarum* cells in water [10]. At low ionic strength, most of the cell membrane of this extreme halophile is disrupted into small fragments while the PM remains intact [61]. The small fragments have a lower buoyant density than the PM and can be separated by equilibrium sucrose density centrifugation. The amount of BR

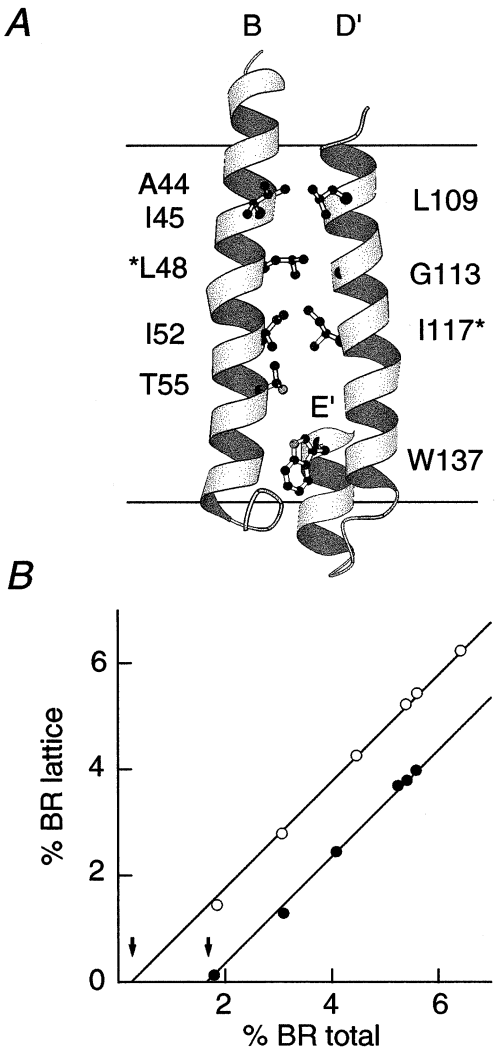

Fig. 3. Substitutions at the helix–helix interface destabilize the lattice. (A) The interface between transmembrane helices of neighboring BR monomers (ribbons) and residues chosen for substitution (ball-and-stick). The view is parallel to the membrane plane as seen from the trimer interior. Horizontal lines denote the boundaries of the membrane core as defined by the lipid ether oxygen atoms of the 2brd structure when superimposed on the 1at9 structure. Asterisks mark the locations where small substitutions cause a substantial decrease in lattice stability. (B) The concentration of BR in the lattice was graphed against total BR for wild-type (open circles) and I117A (filled circles). The data were fit with a self-assembly model to determine critical concentration values (denoted by arrows) for wild-type and I117A lattices. An increase in the critical concentration indicates a less-stable lattice. BR concentrations are expressed as a weight percentage of total cellular protein.

in membrane fractions is determined spectrophotometrically [10].

This assay was used to establish qualitatively that the lattice could be disrupted by single amino acid substitutions at the BR–BR interface [10]. The introduction of both larger side chains (G113L and I117F) and smaller side chains (I117A) was disruptive. These results suggested that packing interactions at the interface contribute to lattice stability.

More recently, a quantitative assay of stability was developed in which lattice formation is modeled as a self-assembly process [11]. The distribution of BR in membrane fractions was determined as a function of total cellular BR, which was varied by preparing samples containing increasing amounts of BR over the course of its induction. Plotting the amount of BR in the lattice against total BR yields a critical concentration value that can be used to compare the effects of mutations (Fig. 3B). With this assay, substitutions at the helix–helix interface between BR monomers were examined, including a series in which large residues were substituted with smaller ones [11]. Ala or Gly substitution of residues that contribute to favorable packing interactions would be expected to destabilize the lattice. Surprisingly, substitutions at only two positions, Leu-48 and Ile-117, significantly increased the critical concentration of lattice assembly. From the critical concentration values of the wild-type and mutant proteins, L48A and I117A were estimated to destabilize the lattice by $\sim$1.0 kcal/mol. It was concluded that helix–helix interactions play a role in lattice assembly. However, the contribution of these interactions is relatively small, suggesting that they are not a major determinant of lattice assembly.

One reservation in concluding that helix–helix interactions are not a major determinant of lattice assembly is that only a subset of helical residues at the BR–BR interface has been examined. Additional contacting amino acid residues not examined in the study have been identified in recently refined BR structures [23,26–28]. Also, residues that appeared to interact with non-helical residues were excluded because the analysis focused on residues that participated only in helix–helix interactions. One residue, Trp-137, was excluded because its visible absorption maximum was shifted by more than 10 nm relative to wild-type BR when substituted with Ala ([11]; T.A.I. and M.P.K., unpublished results). W137A did not form lattice, but the spectral shift suggested that substantial conformational changes had occurred in the mutant protein, making it difficult to interpret the effect of the substitution. Given the importance of Trp residues at protein–protein interfaces [62], Trp-137 may be critical for lattice assembly.

This reservation aside, it is striking that most of the substitutions at the helix–helix interface have little effect on lattice stability. For this reason, other regions of the protein must be considered as potential sources of stabilizing interactions.

6.2. Loop–helix and loop–loop interactions

Lattice assembly may require interactions that involve amino acid residues in non-helical regions, which include the N and C termini and the loops between transmembrane α-helices. The N and C termini are unstructured in the crystallographic models, so it is unlikely that they participate in stabilizing the lattice. The loops contain fewer than 10 amino acids, except for the BC loop, which contains 14–21 amino acids. Because the loops are short, potential loop–loop and loop–helix interactions occur mostly within the boundaries of the lipid bilayer, which include an interfacial region of $\sim$15 Å on each side of a $\sim$30 Å hydrocarbon core [63].

The precise properties of the interfacial region are unusual and are expected to depend on the composition and mobility of the lipid headgroups. A gradient of electrical polarity exists in this region, decreasing from a dielectric constant of $\varepsilon \approx 80$ in the bulk aqueous medium to $\varepsilon \approx 2$ in the hydrocarbon core of the membrane [63]. Since little is known about the relative strengths of van der Waals, hydrogen-bonding and electrostatic interactions in the interfacial region, it cannot be predicted which loop residues will prove critical for lattice assembly.

Potential loop–loop and loop–helix interactions are evident in the structural models. Most of the interactions occur in loops near the helix–helix interface. On the cytoplasmic face, all of the structural models show a close association of the AB and CD loops from neighboring BR monomers and a specific interaction in the form of a salt bridge between Asp-104 and Arg-40 (2.2–3.3 Å) [23,26–28]. On the extracellular face, the side chains of loop residues Tyr-64

and Leu-127 make contacts with each other and with Leu-58, Ser-59, Val-124, Tyr-133, and Trp-137, which are helical in most of the structures. These residues appear to interact largely via van der Waals packing.

A systematic mutational analysis of the role of loop residues in lattice assembly has not been conducted. However, two of the residues listed above have been mutated in *H. salinarum*. Asp-104 has been substituted with Cys, Arg and Asn [64,65]. The mutant proteins apparently could be purified in the lattice form and had no reported effect on the amount of lattice produced in *H. salinarum*, suggesting that the proposed Lys-40–Asp-104 salt-bridge is not critical for lattice assembly. Tyr-64 has been replaced with Ala, resulting in a severe defect in lattice assembly (T.A.I. and M.P.K., unpublished results). This suggests that a loop residue is important for lattice assembly, though further experiments are needed to establish whether the defect in Y64A is due to a loss of favorable BR–BR contacts or disruption of lipid binding (see below).

6.3. Intertrimer interactions

Interactions between BR molecules in adjacent trimers may also contribute to lattice assembly. Since trimers are not close enough to allow amino acid contacts within 4 Å, interactions are limited to long-range electrostatic interactions. It might be argued that such interactions would contribute little to lattice assembly if the interacting residues were exposed to the high dielectric of the aqueous environment. In the PM, electrostatic interactions might also be shielded by potassium or sodium ions, which are present at concentrations of 3–4 M under physiological conditions in *H. salinarum*. However, the structural models show that many of the charged groups of BR are located in the membrane interfacial region. As discussed above, the strength of electrostatic interactions in this region is unknown, and may be substantial due to the reduced electrical polarity. Indeed, regions of negative electrostatic potential in each BR trimer are paired with regions of positive potential in adjacent trimers [66], suggesting that the relative orientation and distance between trimers in the lattice is influenced by long-range electrostatic interactions between BR molecules.

7. Role of BR–lipid interactions in lattice assembly

The importance of BR–lipid interactions in lattice assembly is apparent from studies of PM composition and structure, which suggest a tight association between unique *H. salinarum* lipids and BR. Lipid locations have been determined by electron diffraction of the PM [18,23] and by X-ray diffraction studies of crystals grown from the lipidic cubic phase, which form a hexagonal lattice identical to that of the PM [26,27]. Although lipid headgroups have not been resolved in these studies, one or both phytanyl chains of several lipid molecules have sufficiently low crystallographic temperature factors (< 60) to assign a continuous chain. In the highest resolution structure, four pairs of full-length C_{20} phytanyl chains could be linked with glycerol, indicating that they are part of the same archaeol molecule [26]. In other structures, the lipids have been modeled to varying degrees by including the glycerol moiety or the PGP-Me headgroup [18,23,27].

Many of the lipid hydrocarbon tails are highly ordered and fit into cavities lined with apolar residues on the membrane-embedded surface of BR [26]. A similar apolar cavity accommodates an S-shaped molecule that is completely buried within the membrane and has been tentatively identified as squalene [26]. These findings suggest that lipids bind tightly to BR, stabilized in part by van der Waals packing of the hydrocarbon chains against the apolar surface of the protein. Specific, high-affinity binding of lipids to BR may be critical for lattice assembly.

7.1. Specific interactions: S-TGA-1

Although the precise distribution of PM phospholipids has not been determined, S-TGA-1 molecules have been localized by several elegant studies. S-TGA-1 was shown to be distributed exclusively on the PM extracellular face by ferritin-avidin decoration of PM patches biotinylated with a reagent specific for sugar residues [67]. Neutron diffraction studies of PM containing S-TGA-1 metabolically labeled with deuterated glucose showed density at two locations per BR monomer, one in the interior of the BR trimer and the other in the intertrimer space [20]. Phytanyl chains at these locations are among the most highly ordered in the BR lattice [18], suggesting

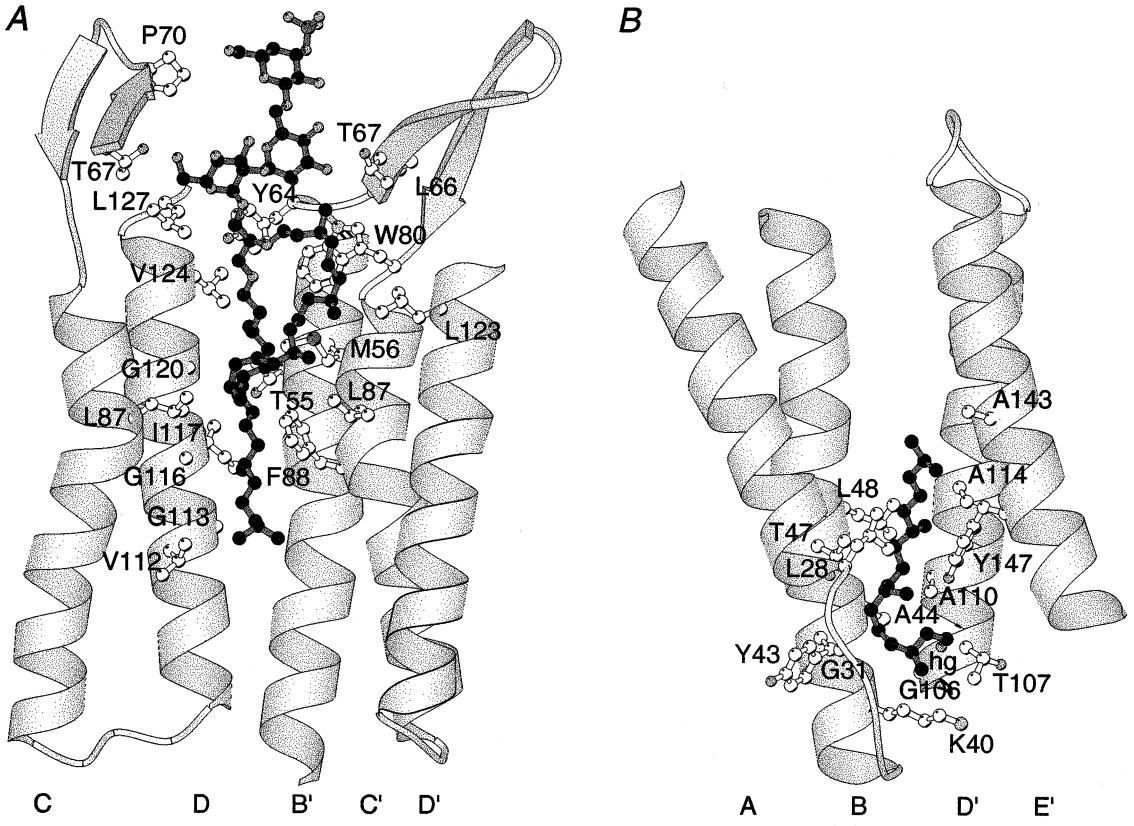

Fig. 4. Ordered lipid molecules associated with the BR trimer. (A) S-TGA-1 lipid in the trimer interior (ARC 3 in 1brr) as viewed from the trimer interior. (B) The lipid phytanyl tail at the BR–BR interface (ARC 8 in 1brr) as viewed from the trimer exterior. The second lipid tail and the headgroup are not resolved in the model, so the position of the headgroup is denoted by 'hg'. In both panels, the extracellular membrane surface is at the top and primed labels denote helices from neighboring BR monomers. Side chains containing atoms within 4.0 Å of the lipid are shown. Both panels are based on the 1brr coordinates [28] and were rendered with MOLSCRIPT [81].

that S-TGA-1 is tightly bound to BR. X-ray diffraction studies of BR trimers crystallized in an arrangement different from that of the PM have revealed the complete structure of S-TGA-1 molecules in the interior of the BR trimer [28]. The X-ray studies are particularly valuable in providing information about the contacts between the S-TGA-1 headgroup and the surrounding protein. However, some caution is warranted in interpreting the contacts, since the crystallographic temperature factors of the lipid headgroups range from 50–100, which may be too high for unambiguous assignment of atomic positions.

Specific interactions between BR and S-TGA-1 have been proposed based on the structural models. From the neutron diffraction data, aromatic residues near S-TGA-1 headgroups were proposed to stack against sugar residues in the headgroup [20]. This proposal is not supported by the higher-resolution structure of S-TGA-1 in the interior of the BR trimer [28], which indicates that aromatic residues near the headgroup (Tyr-64 and Trp-80; Fig. 4A) are not oriented appropriately for stacking [68]. Nevertheless, stacking may occur between BR and S-TGA-1 molecules in the intertrimer space. Other types of interactions are possible between BR and S-TGA-1 in the interior of the trimer. In the highest resolution structural models [23,26–28], the Tyr-64 hydroxyl group appears to form a hydrogen bond with the glycerol O2 oxygen of this lipid. Hydrogen bonds may also occur between the Thr-67 hydroxyl and hydroxyl groups of the S-TGA-1 glucose and mannose units, and between the backbone carbonyl oxygens of Tyr-64 and Leu-66 and the 2-OH group of mannose [28]. A long-range electrostatic interaction

may exist between Lys-129 and S-TGA-1 (this was proposed to be a salt-bridge [28], although the >5 Å distance between the Lys ε-amino and the lipid sulfate group would not allow the hydrogen bonding characteristic of salt-bridges). In addition to interactions between BR and the S-TGA-1 headgroup, one of the phytanyl chains of S-TGA-1 in the trimer interior is packed in a cleft formed by Gly residues in helix D (Gly-113, Gly-116 and Gly-120) and lined by bulky apolar residues [23,26–28] (Fig. 4A).

These results point to a 'structural complementarity' between the cavity formed by the BR trimer and lipids located in this cavity [28]. The evidence for specific interactions between BR and S-TGA-1 molecules as well as the high degree of order of these lipids implies that tight association of BR and S-TGA-1 is critical for lattice assembly. Since S-TGA-1 molecules are located both in the interior of the BR trimer and in the intertrimer space, they may promote the association of BR monomers to form trimers as well as the association of trimers to form the lattice.

7.2. Specific interactions: phospholipid

With knowledge of the location of S-TGA-1, the remaining polar lipids can be tentatively assigned to the phospholipids PGP-Me, PGS or PG. One of these phospholipids contributes a phytanyl chain to a crevice formed at the interface between BR monomers on the cytoplasmic face of the protein. This chain is highly ordered and appears in all of the high-resolution structures [23,26,27], including that obtained from crystallographic studies of BR trimers that were not organized as in the native lattice [28]. A phospholipid with a phytanyl chain in the same position was also noted in an electron diffraction study of BR in a novel honeycomb lattice [69]. These results suggest that the phospholipid at the cytoplasmic side of the BR–BR interface is critical for assembly of the BR trimer. The binding of this lipid may be stabilized by apolar interactions between the phytanyl chain and apolar groups at the BR–BR interface. An interaction between the phospholipid headgroup and Lys-40 has also been proposed [69].

PM phospholipids may also mediate interactions between trimers. The phytanyl chains of four of the phospholipids resolved in the intertrimer space contact BR molecules in adjacent trimers [27]. These bridging contacts may contribute significantly to lattice assembly.

7.3. Tests of BR–lipid interactions

Biochemical studies have indicated a strong interaction between BR and lipid molecules, and have suggested that certain lipids are required for lattice formation. Solubilization of the PM with mild nonionic detergents often does not completely remove endogenous lipids from the protein. At least some of the lipid remaining after solubilization of the PM and subsequent detergent exchange is S-TGA-1, as indicated by FTIR [70] and NMR spectroscopy of BR solubilized from the PM in dodecyl maltoside [71]. The highly negatively charged lipids PGP-Me or PGS were found to be essential for formation of a BR lattice in dimyristoyl phosphatidylcholine proteoliposomes [44,72,73]. This requirement may reflect a need for negative charge at the membrane surface to balance the repulsive interactions among positively charged BR residues in this region [72]. Alternatively, the presence of a phospholipid at the BR–BR interface may be essential for trimer assembly as discussed above.

Surprisingly, S-TGA-1 was not required for lattice assembly. However, the diameter of the BR trimeric unit in the lattice formed in these experiments was $\sim$30% larger than the native trimer [44], perhaps indicating that a different form of the trimer was assembled. Optimal trimerization may require S-TGA-1 in a single leaflet of the lipid bilayer as in the native PM, and this may be difficult to achieve in proteoliposomes.

Mutational evidence that BR–lipid interactions are critical for lattice assembly is limited. This is partly due to the difficulty in establishing whether the effect of a substitution is due to a loss of BR–lipid or BR–BR interactions. Nevertheless, substitution of several residues proposed to play a role in lipid binding has been shown to affect assembly. Bulky substitutions of Gly-113 and Gly-116 within the groove on the interior surface of the BR trimer disrupt lattice assembly ([10,11]; M.P.K., unpublished results). Although the effect of changes at Gly-113 may be due in part to steric clash between BR monomers (see above), the Gly-116 substitutions are not likely

to affect BR–BR contacts. These substitutions may reduce the affinity of BR for S-TGA-1 in the trimer interior. As discussed above, Y64A severely disrupts lattice assembly (T.A.I. and M.P.K., unpublished results), which may be due to the loss of hydrogen bonding with S-TGA-1 in addition to a loss of favorable BR–BR packing interactions. The BR trimer is disrupted by substitution of Trp-80 with smaller amino acids (H. Besir and D. Oesterhelt, unpublished results). This suggests that the interaction between S-TGA-1 in the trimer interior and Trp-80 is crucial for lattice assembly. In contrast, Cys substitution of Lys-129 [9], which was proposed to form a salt-bridge with the sulfate of S-TGA-1 in the BR trimer interior [28], does not disrupt the lattice.

Functional studies also suggest that PM lipids interact intimately with BR in the lattice. Brief treatment of PM with a small amount of the detergent Triton X-100 extracts a subset of PM lipids and causes drastic effects on the BR photocycle [74,75]. These effects occur without disrupting the trimeric arrangement of BR [76]. Subsequent incubation of detergent-treated PM with the complement of PM lipids, with a combination of near-native amounts of PGP-Me and squalene, or with high amounts of PGP-Me, restore proper photocycling kinetics [77]. These results suggest that specific interactions between BR and the lipids PGP-Me and squalene are critical for normal BR photocycling behavior.

8. Summary

The assembly of BR in the crystalline lattice of the *H. salinarum* PM provides a superb opportunity for understanding the structural determinants of membrane protein oligomerization. Helix–helix interactions between BR monomers that contribute to lattice stability have been identified, but they are unlikely to account fully for lattice stability. Loop–loop and loop–helix interactions between BR monomers may also be important for formation of the trimeric unit. However, since there are limited contacts between trimers, and since unique lipids appear to be recruited to the lattice, it is clear that BR–lipid interactions play a major role in lattice stability. Specific protein–lipid interactions are crucial for the assembly of other membrane protein complexes [78–80], and may be more widespread than is generally recognized. Future studies of the nature and strength of protein–lipid interactions important for PM assembly may greatly improve our general understanding of integral membrane protein biogenesis.

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