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Review

Unravelling the folding of bacteriorhodopsin

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Abstract

The folding mechanism of integral membrane proteins has eluded detailed study, largely as a result of the inherent difficulties in folding these proteins in vitro. The seven-transmembrane helical protein bacteriorhodopsin has, however, allowed major advances to be made, not just on the folding of this particular protein, but also on the factors governing folding of transmembrane α-helical proteins in general. This review focusses on kinetic and equilibrium studies of bacteriorhodopsin folding in vitro. It covers what is currently known about secondary and tertiary structure formation as well as the events accompanying retinal binding, for protein in detergent and lipid systems, including native membrane samples.

Keywords: Bacteriorhodopsin; Protein folding; Membrane protein; Membrane lipid; Lateral pressure

1. Introduction

The folding of bacteriorhodopsin (bR) has been the most widely studied of any membrane protein. Much of this is thanks to the remarkable feats of two groups in the 1970s that placed folding studies of bR in a unique position: not only can aspects of its folding from a denatured state be studied in vitro in a variety of detergents and lipids, but also several important folding events can be followed in a native membrane environment. Following on from their successful isolation of bacteriorhodopsin [1,2] Oesterhelt, Stoeckenius and co-workers went on to show that binding of the retinal cofactor in a native purple membrane (PM) environment was reversible [3]. Since then, these workers have produced a sequence of detailed studies on this retinal binding event and the associated protein structural changes (for example [4–7] [8] and see a recent review [9]). Meanwhile Khorana and coworkers used bacteriorhodopsin to demonstrate for the first time that a membrane protein could be refolded from a denatured state in vitro [10,11]. Their approach proved to be invaluable in obtaining high yields of overexpressed protein from *Escherichia coli* [12]. Nearly every amino acid in the protein has been individually mutated and the effect on the generation and the function of the folded state investigated (for example see [13–18]). Several crystal structures have been reported for bR recently, the highest resolution being 1.55 Å [19] (see also [20]

Abbreviations: bR, bacteriorhodopsin; bO, bacterio-opsin; CD, circular dichroism; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio-1-propanesulfonate; DMPC, L-α-1,2-dimyristoylphosphatidylcholine; DHPC, L-α-1,2-dicaproylphosphatidylcholine; DPoPC, L-α-1,2-dipalmitoleoylphosphatidylcholine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PM, purple membrane; SB, Schiff base; SDS, sodium dodecylsulfate; TM, transmembrane

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and minireviews by E. Landau and H. Luecke in this issue).

2. How can bR be denatured and refolded in vitro?

Denaturants that destroy the structure of most water-soluble proteins have nowhere near the same effect on bR. For example, even completely delipidated apoprotein (bacterio-opsin, bO) is resistant to denaturation by 8 M guanidinium chloride [10]. Trifluoroethanoic acid or 88% methanoic acid have to be used to completely denature the delipidated protein to a random coil-like state. Such drastic denaturants have also been found to be effective at unfolding transmembrane helices of other membrane proteins. Refolding bR has of course to be achieved in detergents or lipids (in the presence of retinal). A problem arises in transferring the organic acid-denatured state to this refolding environment, as the organic acid will destabilise the detergent or lipid moieties. It is possible to transfer the acid-denatured bO state initially into SDS by neutralising the acid and dialysing against this detergent [10]. The SDS state remains denatured from the point of view that it cannot bind retinal, but it acquires α-helical structure equivalent to almost 4 transmembrane helices [11,21]. The protein can then be transferred into a variety of renaturing detergent micelles, mixed detergent/lipid micelles or lipid vesicles, by diluting or precipitating the SDS [10,11,22–24]. bR spontaneously refolds under these conditions in the presence of retinal. Refolding yields of about 95% can be readily obtained, for example in mixed DMPC/CHAPS micelles [24], DMPC/DHPC micelles [25], native lipid vesicles [23], DPoPC vesicles [26] and DMPC vesicles [23,26]. SDS can be omitted from the unfolding/refolding process by co-dissolving delipidated bO and lipids in organic solvent, drying off the solvent and re-dissolving the bO/lipid mixture. bR can then be regenerated by the addition of retinal and again yields of about 85% can be achieved (W. Meijberg, A.R. Curran, R.H Templer and P.J. Booth, unpublished data).

The refolding yields are generally determined from the extent of recovery of a native-like, purple chromophore absorption band at about 560 nm [11,27]. The pK_a of the covalent Schiff base (SB) linkage between retinal and Lys-216 in the refolded protein is 11.8 [28], which is close to that of PM which has a SB pK_a of 13.3 [29]. The refolded bR is monomeric and shows native-like activity. The formation and decay of the M intermediate, which is a key step in the proton-pumping photocycle, exhibit similar characteristics to wild-type protein. Proton pumping has also been demonstrated for refolded bR in DMPC/CHAPS micelles [30]. The photocycle kinetics of refolded monomeric bR, as well as that isolated from PM, are dependent on the detergent/lipid environment. Thus, whilst over 95% of the refolded bR has native-like activity, the actual 'folded state' of the protein has slight conformational differences in different refolding environments. This probably reflects a change in the distribution of thermally accessible folded conformations with the detergent/lipid environment.

3. An in vitro folding mechanism

The refolding kinetics of SDS-denatured bO in DMPC/CHAPS, DMPC/DHPC micelles and DPoPC vesicles, in the presence of all-*trans* retinal, have been investigated by time-resolved fluorescence and absorption spectroscopy. The protein spontaneously refolds on diluting the SDS with the renaturing micelles or vesicles [31]. Stopped-flow mixing of the SDS and renaturing micelles occurs with a time constant of about 4 ms. The simplest reaction scheme that accounts for the kinetic data in lipid-based micelles is:

$$\mathrm{bO} \rightleftharpoons I_1 \rightleftharpoons I_2 \overset{R}{\rightleftharpoons} I_R \rightarrow \mathrm{bR}$$

bO is the SDS denatured state and I_1 and I_2 are intermediates that form prior to retinal binding. Retinal, R, binds in at least two steps, first non-covalently to give I_R and secondly via the covalent link to Lys-216 to give bR. I_R probably consists of at least two observable states (see below), one where the retinal absorption band is similar to that of free retinal at about 380 nm (I_{R380}) and one where the retinal band is red-shifted to 440 nm (I_{R440}). Both bO and I_2 also probably possess equivalent, additional states, although they have not been observed.

The intermediate I_1 could either be an apoprotein

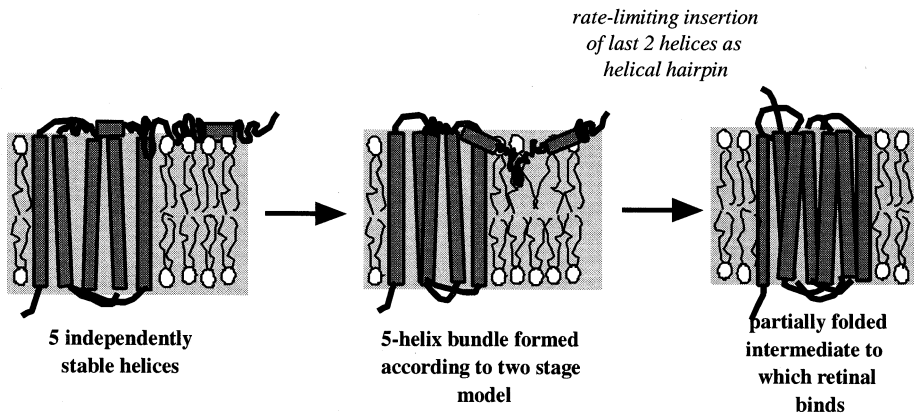

Fig. 1. A possible model for the folding of bacterio-opsin prior to the binding of retinal. Five TM helices, A–E, fold and partially pack, according to the two-stage model. Formation of this five-helical state then induces insertion and folding of the remaining two helices F and G. The resulting seven TM state has a loosely packed structure. The final helix packing and tertiary contacts form as the protein folds round retinal.

folding intermediate or reflect a change in the micelle/vesicle structure as a result of stopped flow mixing. A change in protein fluorescence occurs during I_1 formation with a time constant, τ1, of the order of hundreds of milliseconds in micelles. However there is also a change in the micelle structure which occurs with the same observed time constant, τ1, and is accompanied by an increase in the amount of light scattered by the micelles [32]. Thus τ1 may not be a bona fide folding event, but rather reflect the micelle structural change, which alters either the hydrophobic environment of the protein tryptophans and/or the apparent protein conformation. The change in protein fluorescence with time constant, τ1 is lost when arginine at position 175 is mutated to glutamine [31]. Arg-175 has been suggested to be involved in correct tilting and orientation of some transmembrane helices in PM, through an interaction of its positive charge with the lipid headgroups [33]. Thus I_1 formation could be a folding event which involves helix orientation. Alternatively, it is conceivable that the micellar structural change that occurs during I_1 formation, alters the interaction between Arg-175 and the detergent/lipid headgroups, and as a result no change in protein fluorescence is seen with this time constant in the Arg175Gln mutant.

All the kinetic folding experiments thus far point to the apoprotein intermediate I_2 being key to the folding process and a pre-requisite for retinal binding. Formation of I_2 is rate-limiting in apoprotein folding. The rate of this step can be controlled by manipulating particular characteristics of the refolding lipid environment, with the time constant ranging from 1 s to minutes (see below). Changes in intrinsic protein fluorescence also suggest the process is bi-exponential under certain lipid conditions, which probably reflects folding of slightly different conformations of the protein. The changes in protein secondary structure have been time-resolved during this stage of folding by far UV circular dichroism (CD) [21]. The SDS-denatured bO state has an α-helical content of about four TM helices, whilst the remaining equivalent of three TM helices are disordered. The secondary structure of I_2 is native-like and corresponds to seven TM helices. About half of the disordered SDS structure folds to form helices during the 20 s deadtime of these particular far UV CD experiments, whilst the remaining 30 or so amino acids form helices with a time constant equivalent to I_2 formation. The extent and nature of the tertiary interactions in I_2 are unknown. A possible model for the protein structural changes that occur during I_2 formation is shown in Fig. 1. This is largely based on a two-stage model that has been proposed for the folding of α-helical membrane proteins as well as indirect evidence form studies on helical fragments of bR, the time-resolved CD studies and the lipid dependence of the folding kinetics (see below).

Only one retinal-binding step has been identified in folding of bR in mixed micelle systems, when retinal binds non-covalently to I_2, probably within some sort of loosely formed binding pocket [27]. Although

this bi-molecular retinal binding reaction strictly follows second-order kinetics, for convenience it can be approximated to one or two exponential functions with time constants of about 1 s. Studies of this retinal binding process are complicated by the preceding formation of I_2. However, the binding reaction can be more readily investigated by allowing bO to prefold to a state equivalent to I_2 and then adding retinal [24]. There appear to be at least two non-covalent, retinal, protein I_R states. One retinal, protein intermediate (I_{R440}) is observed in transient absorption measurements because the retinal absorption band red shifts from 380 to 440 nm. This intermediate corresponds to a non-covalently bound state seen in studies of retinal binding to apomembrane (see below). However the formation of I_{R440} alone cannot account for the observed retinal, protein concentration dependence of the kinetics [64]. Another intermediate I_{R380} is suggested to form and decay in parallel with I_{R440} and with the same observed kinetics. This could result from a distribution of protein conformers in I_2 that have slightly different protonation equilibria of their side chains and can interconvert by thermal energy. Retinal binding to these I_2 conformers occurs via the same transition state (or distribution of interconverting transition states on a microscopic scale) to form I_R. I_R also contains a similar distribution of protein conformers as I_2, however the different protonation equilibria of the protein conformations affect the retinal absorption band and thus show up in this retinal bound state. I_{R440} and I_{R380} both decay with the same observed time constant of a few minutes to form refolded bR with a 560nm absorption band where the SB bond has formed and retinal is covalently bound. This bR state contains all-*trans* retinal within its binding pocket. The retinal then isomerises on a much slower time scale (time constant about 1 h) to a state equivalent to the dark-adapted state of bR that contains a mixture of all-*trans* and 13-*cis* retinal.

4. Transmembrane α-helix insertion, formation and the two-stage folding model

Experiments on bR are at the root of a 'two-stage' model that has been proposed for the folding of α-helical membrane proteins [34]. This model proposes that insertion and formation of transmembrane helices occurs as a first stage in folding, as these helices are independently stable entities within the membrane. Stage two then involves the correct packing of these helical segments to give the folded structure. Whilst this model does not necessarily reflect how membrane proteins actually fold in vivo, nor gives any prediction of the in vivo or in vitro folding mechanism, it does provide a thermodynamic base from which to consider the problem. Key features of the two-stage model have been tested with bR. Stage one predicts that each individual TM helix of bR ought to be independently stable in a lipid bilayer. Peptides corresponding to each of the independent helices have been synthesised and their propensity to form a stable transmembrane helix assessed [35]. As predicted, five of the peptides can form stable TM helices in bilayer vesicles of native lipids. However, a peripheral, membrane-bound conformation containing some α/β-structure was preferred by the peptide corresponding to helix F, and that for helix G formed a β-sheet structure within the membrane.

Studies on TM fragments of bR began with the enzymatic cleavage of the BC loop to give two fragments consisting of the AB and C–G helices, respectively [10,22,34]. Both fragments form structures in DMPC/CHAPS micelles and native lipid or DMPC vesicles with the expected two TM or five TM helix content. The fragments are also able to re-associate and bind retinal to form functional protein. A recent extension of this approach has involved the overexpression of all two to five helix fragments of the protein [28]. Far UV CD studies show that the individual N terminal fragments, AB, A–C, A–D, A–E as well as that of C–G, have helix contents in DMPC/DHPC micelles that are equivalent to the corresponding regions of the native folded protein [36]. However the remaining C terminal fragments D–G, E–G, FG as well as the CD, C–E and DE fragments have lower helix contents at pH 6 than expected if they were in a folded state, and indeed the FG fragment has about 20% β structure. All pairs of complementary fragments (AB·C–G, A–C·D–G, A–D·E–G, A–E·FG) assemble in DMPC/CHAPS micelles in the presence of retinal to form functional protein with native-like α-helical content. Thus some of the C-terminal fragments misfold or

only partially fold on their own, whilst the N-terminal fragments each fold to attain their correct helical structure. The correct secondary structure of the C-terminal fragments is then induced by their interaction with their complementary N-terminal fragments and retinal.

The studies on bR fragments also go some way to answer a key question of the second stage of the two-stage model; can preformed, individual transmembrane helices pack correctly to form the functional state? The answer for helices A–E seems to be yes. It is harder to tell for F and G, because individually they do not form independently stable TM helices. Furthermore all C-terminal fragments with four or fewer TM helices do not fold to give the expected TM helix content.

On the basis of these studies, some slight refinements of the two stage model can be proposed [35]. The five N-terminal helices of bR seem to be able to fold according to the two-stage model. The remaining two C-terminal helices do not form stable TM helices on their own, and are probably stabilised in their folded TM helical state by interaction with the previously formed five helix bundle (see also Fig. 1). Therefore, it does not appear to be necessary for each individual TM helix of a membrane protein to be a stable entity in the absence of the rest of the protein. Only a certain proportion of the TM helices need exhibit such stability, which in the case of bR appears to be five out of its seven helices. This could leave room for the remaining helices to forego TM stability in favour of including amino acids that could be important for function or other stages of folding, such as helix association.

Correct helix packing and retinal binding are critical to the formation of a functional state of bR. The in vitro kinetic work suggests that retinal binds to a partially folded apoprotein state, I_2 that has some of the binding pocket already present, but it is unknown exactly how retinal gains access. It is also unknown what correct tertiary contacts between the helices are present in this I_2 state, as well as what controls the specific packing of the helices during folding. There are probably some specific helix–helix contact sites present in the partially folded state I_2. Retinal binding to I_2 would then allow the helices to pack round the bound retinal to form the folded state. The aqueous loops that connect the helices could also help to provide some specificity and close packing. However that fact that all the helix fragments discussed above can form a functional protein with breaks in the loops, shows that no individual loop is essential for correct folding of the protein. Mutations of each loop in turn to structureless linkers also results in a functional protein with only fairly subtle changes in the folding kinetics (J.-M. Kim, H.G. Khorana, S.J. Allen, H. Lu, P.J. Booth, unpublished data). Changes or cleavage in the BC loop, which seems to have some β-structure [37], can lead to a blue shifted chromophore [14,22]. Although this is temperature-dependent and under certain conditions it is possible to attain the purple chromophore for protein with a break in this loop, either generated from fragments expressed in *E. coli* [28] or by cleavage of native protein [23]. The recent high resolution crystal structures of bR [19,38] have also revealed hydrogen bonding between neighbouring helices, both between side chains and main chain to side chain bonds. The strength of such bonds within the bilayer presumably contributes to the stability of the folded state and could aid correct helix packing. On the other hand, they may hinder folding if non-native hydrogen bonds form en route.

Bacteriorhodopsin is remarkably tolerant of changes in its amino acid sequence [16]. Many single point mutations, as well as the changes in the loops discussed above, have been studied using the *E. coli* expression system with a synthetic bO bop gene, and then folding the protein from its SDS-denatured state. These studies have shown that in vitro many of the mutant proteins are capable of folding to a functional state with a native-like purple absorption band (see Fig. 2). Some of the mutants have also been studied following homologous expression in *Halobacterium salinarium*, where they are also able to fold to a bR-like state, although different phenotypes and chromophore bands have been observed. The overall folding rate of the mutants expressed in *E. coli* has been determined in mixed DMPC/CHAPS micelles and generally they fold only slightly slower than wild-type. The detailed folding kinetics in DMPC/CHAPS micelles have only been studied for a few mutant proteins. Thus, for example, single point mutations of the charged amino acids arginine and aspartic acid to glutamine and asparagine, respectively, near the helix ends and in the helix-con-

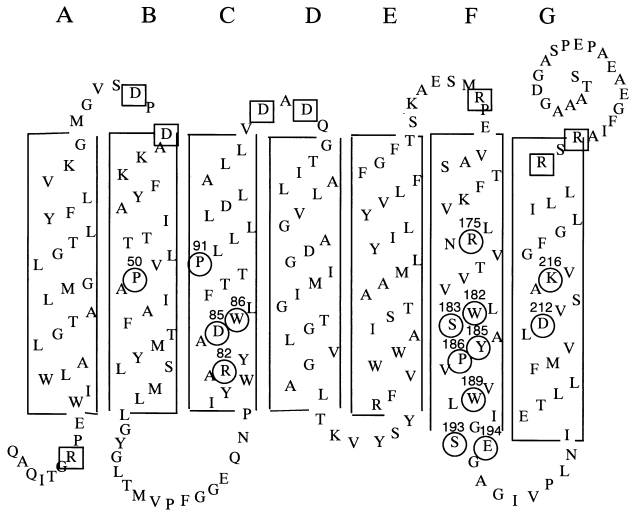

Fig. 2. A representation of the secondary structure of bR, highlighting some of the sites of mutations referred to in the text. □, Site of mutation that has so far been found to have little effect on folding; ○, Site of mutation that has a greater effect on folding.

necting loops have little affect on folding. In each case, the kinetics of apoprotein folding to I_2, retinal binding to form I_R and overall bR formation are similar to wild-type protein (P.J. Booth, S.L. Flitsch, H.G. Khorana, unpublished data) [39]. This is perhaps not surprising in view of the fact that no individual loop seems necessary for correct folding (see above). Protein with single point mutations of Ser183Ala, Tyr185Phe, Ser193Ala and Glu194Gln on helix F also all fold to functional states with native-like bound chromophores, but with slower overall folding rates. Some single point mutations have greater effects on bR folding, as shown by other single point mutations on helix F of Arg-175, Trp-182, Pro-186, Trp-189. For example, Arg175Gln folds to a native-like state about ten times slower than wild-type [40] and has altered early apoprotein kinetics that result in the loss of the kinetic phase $\tau 1$, which is associated with formation of the intermediate I_1 (P.J. Booth, S.L. Flitsch, H.G. Khorana, unpublished data). Mutations of amino acids that line the retinal binding pocket can lead to blue shifts in the bound chromophore absorption band (e.g. Trp86Phe, Trp182Phe, Trp189Phe, Pro186Gly/Val/Leu [18] [41]). In contrast, some mutations of Asp acids 212 and 85, which are involved in proton pumping give rise to red shifts in the bound chromophore absorption band (Asp212Glu, Asp85Glu,Asp85Asn) [39]. Although Asp212Asn has an unshifted, wild-type-like chromophore. The Asp mutations, together with Pro186Gly show more or less wild-type apoprotein folding kinetics to I_2, but altered retinal binding (H. Lu, T. Marti, P.J. Booth, S.L. Flitsch, H.G. Khorana, unpublished data). The molecular origin of these various shifts in the bound chromophore band of the mutants as compared to wild-type is unknown. They could reflect slight differences in the folded structure to wild-type, which results in a modified retinal binding pocket, or an altered electrostatic environment of the bound retinal. In certain cases (e.g. Pro186Gly) they may reflect two bound species, possibly in equilibrium, one of which is the normal purple chromophore. The blue shifted chromophore of Pro186Gly can be converted to the normal purple species at high salt. Thus different folding conditions may well alter the observed chromophore band(s), but this has not been investigated in detail in many cases. Altered chromophore absorption bands have also been observed in certain cases when the mutants are expressed in *H. salinarium*. For example, Asp212Asn has a wild-type chromophore when expressed from *E. coli*, but a red shifted chromophore when expressed in *H. salinarium* [42]. Trp182Phe from *E. coli* has a blue-shifted band, but has been reported as unshifted when homologously expressed [43]. These homologous expressions use a bacterial strain where the wild-type bop gene is present in an inactive form, together with the mutant bop gene, although recombination frequencies between the two genes seem to be sufficiently low that the mutant phenotype dominates. The position of the chromophore band in purple membrane is also dependent on ionic strength and pH [44].

Lys-216 has also been mutated to investigate the effect of the covalent link to retinal on the folding and function of the protein. Studies on a Lys216Ala mutant expressed in *H. salinarium* and then reconstituted with retinylidene alkylamines show that bR can function with these retinylidene SB compounds, but without the covalent link to Lys-216 [7]. Kinetic studies on Lys216Cys in DMPC/CHAPS micelles show that this protein has altered folding kinetics to I_2, but wild-type retinal binding to give a non-covalently bound intermediate state, I_{R440} with an

absorption maximum at 440 nm (H. Lu, P.J. Booth, S.L. Flitsch, H.G. Khorana, unpublished data).

Bacteriorhodopsin, like many membrane proteins, has proline residues near the middle of transmembrane helices. This is unexpected in view of the large body of data that exists on water soluble proteins which indicates that Pro is a helix breaker that is rarely found at the centre of helices [45,46]. Prolines are unique amongst amino acids in that a *cis* as well as *trans* arrangements of the peptide bond can readily occur, especially in unfolded states. Studies of water soluble proteins have shown that isomerisation of non-native (generally *cis*) prolines to the native isomer is frequently rate-limiting in folding and occurs with a time constant of the order of seconds. Although there have been no studies on *cis–trans* proline isomerisation during membrane protein folding, *cis-trans* isomerisations of the transmembrane or the loop prolines of bR could be the origin of some of the kinetics that have been observed. The isomerisation state of the prolines in the SDS-denatured bO state is unknown, but they are all *trans* in folded bR [19]. The three transmembrane prolines of bR have been mutated to both Gly and Ala and their overall folding from an SDS-denatured state to a folded state assayed in DMPC/CHAPS micelles [41]. The mutations of Pro-50 and Pro-91, which are on helices B and C, respectively, resulted in normal purple chromophore formation and protein activity. The overall folding rates were slower than those of wild-type, except for Pro50Gly that folded faster. Pro-186 lies on helix F and seems to form a very close contact with the retinal. Mutation of Pro-186 to Gly resulted in a blue-shifted chromophore band. Thus these transmembrane prolines, and particularly Pro-50 and Pro-186 do contribute to the folding of bR, and more detailed studies of this are underway.

5. Folding and reconstitution in a native membrane environment

Folding studies in a more native-like environment have been tackled in three main ways. Two approaches have covered detailed biophysical studies on retinal binding to apomembrane. This apomembrane state is formed either by bleaching PM or from a retinal deficient bacterial strain. The apomembrane contains bO that is probably arranged in trimers, but lacks the characteristic crystalline array of PM. Formation of PM is then achieved either by addition of all-*trans* retinal to the apomembrane, or by photoisomerisation of 9-*cis* retinal already non-covalently bound to the apomembrane. This 9-*cis* isomer cannot bind to give functional protein, but the formation of PM is triggered by the photoisomerisation to all-*trans* retinal that does bind to give a functional state. The third native membrane approach has been to study the formation of the PM crystalline lattice in vivo by investigating the effect of mutations expressed in *H. salinarium*, where the wild-type bop gene has been deleted and completely replaced by the mutant gene ([47,48] and see minireview by MP Krebs in this issue).

The apomembrane studies show that all-*trans* retinal binds in two stages to form PM [4,5,49]. Firstly retinal is fixed within its binding site and ring-chain planarisation of the molecule occurs, resulting in a red shift of the retinal absorption band from about 380 to about 400 nm. A further red shift of the absorption band is then observed giving an intermediate with an absorption band with maxima at 430 and 460 nm. Retinal is non-covalently bound within its binding pocket in both the 400 and 430/460 nm states. Finally, the covalent SB forms and the characteristic 560 nm purple band is observed. The protein secondary structure in the apomembrane and PM states appears to be the same. The tertiary structures of the apomembrane state has not been investigated in detail, but significant tilting of the helices from the membrane normal has been noted in this state [50]. The photoisomerisation of 9-*cis* retinal as a trigger of PM formation has allowed the secondary structure and side chain protonation changes that occur during these retinal binding reactions to be probed by FTIR difference spectroscopy [6,51]. Protonation changes were observed with pK_a shifts for two apparent proton-binding groups from pK_a's of 4.6 and 7.1 in the apomembrane state to 2.8 and 8.9, respectively, in purple membrane. These proton binding groups could not be assigned to particular amino acids and were suggested to result from a hydrogen-bonded network in the protein (possibly involving E204, D85, R82, and Y57 and bound water) that is capable of binding two protons. Evidence for the participation of water in SB formation

has also been found in studies of bR at different humidities [52,53]. The pK_a of the PM SB also varies with conditions. The SB of native PM has a high pK_a of about 13. In contrast, lower pK_a values of 9 or 8.5 have been found for PM that has been generated by addition of retinal to apomembrane, prepared from bleaching PM or from retinal-deficient cells, respectively [8]. In the latter case, a higher pK_a of >10 was found for the in vivo formation of PM, when retinal was added to the retinal-deficient cells during cell growth. This was suggested to reflect a different packing of the helices in the apomembrane state as compared to PM, and may be connected with the lack of the crystalline array of bR in the apomembrane state. Thus when retinal is present during synthesis of the PM the helices can fold more tightly round the retinal than when retinal is added to apomembrane after cell growth and apomembrane isolation. When monomeric bR is formed by addition of retinal to SDS-denatured apoprotein in DMPC/CHAPS micelles, the resulting pK_a of the SB is about 11.5 [28]. This suggests that the intermediate state I_2 that retinal binds to in the micelles may be less compact with fewer tertiary interactions than the apomembrane state. A less compact state could arise as a result of the higher detergent/lipid:protein ratio in DMPC/CHAPS micelles. Thus, in the micelle system, the protein folds more tightly around retinal, resulting in a high pK_a, as observed when retinal is present during PM synthesis in vivo.

What are the parallels between the refolding of monomeric bR and reconstitution in the membrane? The folding events accompanying retinal binding appear to be very similar in the two instances. Retinal binds first non-covalently in its binding pocket, resulting in an observed absorption shift of retinal to about 440 nm. The final step is then covalent binding of retinal and formation of the 560 nm chromophore. The pH dependence of the refolding kinetics of monomers in DMPC/DHPC micelles also agree with the FTIR studies on retinal binding to apomembrane, and are consistent with a change in the pK_a of two apparent proton-binding groups. The pK_a of the SB seems to depend on the refolding/reconstitution conditions. Refolding in DMPC/CHAPS micelles gives pK_a of over 11, as in native PM, whereas addition of retinal to apomembrane results in a lower pK_a of about 8–9, unless retinal is present during cell growth and PM synthesis. The state to which retinal binds is similar in both apomembrane and micellar samples to the extent that all seven TM helices are present. However, these two apoprotein states could differ in terms of their helix packing. The monomeric micelle state may be rather more unfolded and then packs tightly round the retinal, whilst the apomembrane state has a loosely packed, but stable structure that does not rearrange as much as retinal binds.

Very little is known about the mechanism of bR insertion and folding in vivo. A recent kinetic assay has been reported for this process where halobacterial strains were created with a unique cysteine in the N-terminal extracellular domain [54]. The translocation of this domain across the membrane was then probed by derivatising the cysteine with AMS that is membrane impermeant. Almost 80 amino acids were found to have been synthesised before the cysteine was translocated, showing that the N terminus inserts co-translationally. This could suggest that co-translational insertion and folding of the N-terminal, A helix aids folding of the remaining helices. This is also consistent with the results from the in vitro folding and fragment work where the A and B helices seem to facilitate folding of the remainder of the protein. The authors point out that the results are consistent with the membrane insertion being either spontaneous or mediated by a signal recognition particle. Halobacteria appear to contain a signal recognition-like particle that may be involved in translation and insertion of membrane-associated proteins [55].

6. Effect of the lipid environment

The lipid environment is known to affect bR folding and functionality. Fairly subtle effects of a functional bR, but with altered dynamics, have been observed following solubilisation by different detergents or by dehydration of the PM [56–58]. The latter will alter the lipid phase properties as well as internally bound water within bR. Despite the role of the lipids in such fine tuning of function, and presumably also the final structure of the folded state, bR can refold and function in a number of non-native-like lipids and detergents, and thus is rather insensitive to the

exact nature of the refolding environment as long as certain criteria are met. It should, however, be noted that although the delipidated protein used in the refolding work contains $<5\%$ native lipids, or <1 lipid per bR monomer, there may be some bR monomers that are stabilised by the binding of a native lipid. Recent crystal structures of bR have reported native lipids bound to the protein [19,38,59]. The organic phase extraction method used to delipidate the protein [12] in the refolding work probably removes more of the native lipids than the detergent solubisation in the crystallographic studies.

A factor that seems likely to be important for folding membrane proteins is a good match between the hydrophobic thickness of the protein and lipid bilayer, although it appears this does not have to be tightly controlled for bR. bR appears to be able to fold and function in lipid bilayers with C12–18 hydrocarbon chains and hydrophobic thicknesses that are probably within a few angstroms of that of bR. Reconstitution of PM into these C12–18 phosphatidylcholine (PC) lipid bilayers show that the surrounding alkyl chains are elongated or compressed when their hydrophobic thickness is less than or greater than that of bR, respectively [60]. Certain native lipids remained bound to the protein in this study and their effect on the protein was not investigated. SDS-denatured bO is also able to refold to about 100% yield in PC bilayer vesicles with C14–C18 chains [26].

A lipid property that also seems to be important for the folding, stability and function of bR is the curvature elastic energy within the bilayer. This has been shown in a variety of lipid mixtures containing phosphatidylcholine (PC) and phosphatidylethanolamine (PE) lipids with the same chain length, or PC lipids with chains of similar length, but different degrees of saturation. In these two lipid mixtures, the introduction of the PE lipid in the former, or the unsaturated lipid chain in the latter, imparts an increased desire for monolayer curvature towards the water (see Fig. 3). Since the monolayers are constrained flat in the bilayer, this increases the stored curvature elastic energy within the bilayer [61–63]. One effect of this is to increase the number of collisions between the lipid chains within the bilayer, which increases the pressure these chains exert on the refolding protein. Several effects of this increased chains pressure have been observed for bR. Firstly, it appears to slow the rate-limiting folding step (i.e. formation of I_2) [25] (P.J. Booth, A.R. Curran, R.H. Templer, unpublished data). In terms of the model proposed in Fig. 1, this can be explained by the increase in the chain pressure hindering the insertion and formation of the F and G helices. A second effect of this increased lipid chain pressure is that once a certain pressure is reached, the yield of refolded protein decreases [26]. This is probably a combination of the fact that the rate-limiting step is slowed (possibly allowing other events to compete with folding) and that once the bilayer gets too rigid, it becomes harder for the SDS denatured bO to insert into it. The increased pressure also affects the folded state stability. Increasing the pressure the lipid chains exert on the helical bundle increase the temperature at which the refolded monomeric bR denatures (W. Meijberg, R.H. Templer, P.J. Booth, unpublished data).

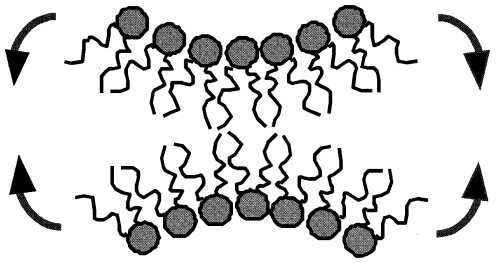

Fig. 3. The inherent desire for monolayer curvature in bilayers of biological lipids. Most biologically relevant lipids form monolayers that wish to bend towards water. The desire for curvature in monolayers of PC lipids with saturated chains is increased by the introduction of either PE lipids or PC lipids with unsaturated chains. However, since the monolayers are back to back in the bilayer, they cannot bend and are forced to lie flat. As a result the curvature elastic energy of the bilayer increases.

7. Conclusions and future directions

Investigations on bR seem likely to continue to lead the way in detailed molecular level study of the folding of α-helical membrane proteins for some years to come. The large body of structural, functional and dynamic data as well as the ability to perform biophysical studies on aspects of the folding of bR in vivo and in vitro, place bR in a unique

position amongst integral membrane proteins. This holds much promise for molecular level information on the folding of this protein. The robustness of bR also makes it an ideal testing ground for developing techniques to study membrane protein folding that can then be adapted for other membrane proteins. The information that currently exists on bR folding already far exceeds that of any other membrane protein, except possibly outer membrane β-barrel proteins. However, despite this, many central questions remain to be addressed. Areas that merit particular attention are to further the development of approaches to probe the dynamics and structural changes of both the protein and surrounding membrane environment during folding in vitro as well as during certain biogenesis events in vivo.

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