Conformational Changes in Bacteriorhodopsin Associated with Protein−Protein Interactions: A Functional $\alpha_I-\alpha_{II}$ Helix Switch? †

Jaume Torres, Francesc Sepulcre, and Esteve Padrós*

Unitat de Biofisica, Departament de Bioquímica i de Biologia Molecular, Facultat de Medicina, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain

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ABSTRACT: Fourier transform infrared spectra of bacteriorhodopsin samples were obtained in conditions in which the aggregation state of the protein (i.e., monomeric or trimeric) was modified by different treatments. Two approaches were followed: (1) renaturation of bacteriorhodopsin starting from bacterioopsin dissolved in SDS and (2) reconstitution of bacterioopsin in *Halobacterium* lipid liposomes at two different lipid/protein ratios. Concomitant with the gradual recovery of the native interactions between transmembrane helices, we observed clear and gradual changes in the infrared absorption spectra in the amide I band and also in the band at 1741 cm^{-1}. These processes were found to be compatible with the two-state oligomerization model. The whole set of experiments shows that the band at 1665 cm^{-1} in the deconvoluted spectra appears only when monomers interact forming trimers, even when the lattice is not present. This implies that the trimeric organization of bacteriorhodopsin is responsible for the unique features described in the amide I of purple membrane. The spectroscopic changes detected can be attributed to changes in secondary structure compatible with the interconversion of α_I and α_{II} helices. However, the exact nature and functional relevance of these changes is still unknown.

Bacteriorhodopsin (BR)[1] is found in the cytoplasmic membrane of *Halobacterium salinarium.* The individual protein molecules are organized into trimers, which in turn give rise to a rigid paracrystalline lattice known as purple membrane (PM). The lipids present in the purple membrane are located in the spaces between BR monomers and between trimers. BR binds a molecule of retinal that acts as a chromophore, triggering a sequence of conformational changes upon light absorption that lead to the pumping of protons from the cytoplasmic to the external side of the membrane.

A large amount of structural information on BR has become available throughout the past 20 years, which in combination with that obtained from site-directed mutagenesis has offered a detailed description about the phenomena that take place within the protein during the photocycle (Oesterhelt *et al.*, 1992; Rothschild, 1992; Ebrey, 1993; Krebs & Khorana, 1993; Lanyi, 1993). In spite of these efforts, little is known about the role of the lattice or the trimeric organization in the function of BR, although some form of communication between monomers is suspected. For example, such a close arrangement could facilitate the transmission of conformational changes in individual monomers to neighboring molecules during the photocycle, facilitating the regulation of proton transport. These conformational changes could involve either shifts in the transmembrane segments or changes in the dihedral angles of α helices. In this respect, the current proposal of the existence of significant amounts (around 50% of total helix) of α_{II} helix in BR (Krimm & Dwivedi, 1982; Gibson & Cassim, 1989; Earnest *et al.*, 1990; Cladera *et al.*, 1992) is particularly interesting since this helix differs from the α_I type only in the dihedral angles, the rest of the parameters remaining invariant. Krimm and Dwivedi (1982) worked out the frequencies corresponding to the parallel and perpendicular infrared modes for the α_{II} helix model (1659 and 1666 cm^{-1}). In bacteriorhodopsin, the deconvoluted spectrum of the amide I region shows two main bands centered at about 1665 and 1658 cm^{-1}. According to the above hypothesis, the band at 1658 cm^{-1} has been assigned to the unresolved parallel and perpendicular modes of α_I helix plus the parallel mode of α_{II} helix, and the band at 1665 cm^{-1} to the perpendicular mode of α_{II} helix (Krimm & Dwivedi, 1982; Earnest *et al.*, 1990; Cladera *et al.*, 1992). The use of polarized infrared spectroscopy has shown that α-helical segments absorbing at 1665 cm^{-1} are preferentially oriented perpendicularly to the membrane plane, in either dry (Rothschild & Clark, 1979; Nabedryk *et al.*, 1985) or hydrated films (Earnest *et al.*, 1990). Thus, the presence of α_{II} helix in BR could explain the unique characteristics of the IR amide I band, i.e., the large splitting of the parallel and perpendicular modes and the blue shift observed in the IR amide I spectra when compared to other proteins. A number of other spectroscopic data strongly support the existence of α_{II} helix in BR, *e.g.*, Raman (Vogel & Gartner, 1987), UVCD (Gibson & Cassim, 1989), far-UV oriented CD, and mid-IR linear dichroism (Draheim & Cassim, 1992).

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* Correspondence should be addressed to this author.

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[1] Abbreviations: BR, bacteriorhodopsin; BO, bacterioopsin; PM, purple membrane; PM/SDS, purple membrane solubilized in sodium dodecyl sulfate; PM/SDS/TRIT, purple membrane solubilized in sodium dodecyl sulfate and Triton X-100 added; PM/REG, purple membrane in sodium dodecyl sulfate/Triton X-100 after detergent removal with Bio-Beads; C1, helices CDEFG, obtained by chymotrypsin treatment; C2, helices AB; BO(10:1) and BO(1:1), reconstituted bacterioopsin in native polar lipids at a lipid/protein ratio of 10:1 (w/w) or 1:1 (w/w); IR, infrared; FTIR, Fourier transform infrared; UV, ultraviolet; CD, circular dichroism; DMPC, dimyristoylphosphatidylcholine.

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In the present work we monitored the changes in the infrared spectrum of BR when the aggregation state of bacteriorhodopsin was altered by different treatments. We took advantage of the fact that the process of formation of paracrystalline structure is spontaneous (Henderson & Unwin, 1975) and fully reversible (London & Khorana, 1982). The starting point was a sample of BR solubilized in SDS, in which case the chromophore is lost. Addition of Triton X-100 regenerates the chromophore, whereas the trimeric arrangement, and eventually the lattice, are also regained after elimination of the detergents. We used this procedure, as well as the reconstitution of bacteriorhodopsin at different lipid/protein ratios in *H. salinarium* polar lipid liposomes (Popot *et al.*, 1987), in order to analyze the possible relationship between the aggregation state of bacteriorhodopsin and the appearance of the band at 1665 cm^{-1} in the deconvoluted spectra.

MATERIALS AND METHODS

Sample Preparation. Purple membrane was isolated from *H. salinarium* strain S9 according to literature protocols (Oesterhelt & Stoeckenius, 1974).

Lipid Extraction. The native polar lipids of purple membrane were extracted following the method of Kates *et al.* (1982) as modified by Popot *et al.* (1987). The absence of double- and triple-conjugated double bonds [bands around 230 and 270−280 nm (New, 1990)] resulting from lipid oxidation was confirmed prior to the reconstitution experiments by acquiring a UV spectrum of the dried lipids dissolved in ethanol.

Reconstitution of BR in Native Polar Lipids of H. salinarium. Purple membrane was dissolved in chloroform/methanol (1:1 v/v) and 0.1 M $LiClO_4$ and passed through a LH-60 column equilibrated in the same solvent. The resulting delipidated protein was incorporated into liposomes at protein/lipid (w/w) ratios of 1:1 and 1:10 as in Popot *et al.* (1987). However, no taurocholate was used, as it is difficult to eliminate by dialysis and absorbs strongly in the IR amide I region. As a result, the elimination of the potassium dodecyl sulfate precipitate required up to 10 successive centrifugations (360*g* for 5 min) and exhaustive dialysis (4 days against 100 volumes of buffer). The content of the bags was centrifuged at 134000*g* for 1 h 30 min to obtain a suitable sample for IR spectroscopy. The percentage of correctly reconstituted BR molecules was estimated from the percentage of molecules able to regenerate the chromophore after the addition of *all-trans*-retinal, using $\epsilon_{560} = 63\,000\ M^{-1}\cdot cm^{-1}$.

Denaturation and Renaturation of BR with SDS and Triton X-100. The different samples were obtained following the methods of London and Khorana (1982). Typically, purple membrane was solubilized with SDS by adding 13 mL of 50 mM phosphate buffer and 0.25% (w/v) SDS, pH 6.5, to 2 mL of a purple membrane suspension (7.5 mg/mL). The sample was stirred in the dark at 45 °C for 30 min. The resulting yellow solution (PM/SDS) was stored in the dark at room temperature.

The reconstitution of BR was achieved by adding 20 mL of a buffer containing 0.5% (w/v) Triton X-100 and 50 mM sodium phosphate, pH 6.5, to 5 mL of the PM/SDS solution. After 1 min, the solution had its characteristic purple color.

Detergent removal was carried out in the following way: 30 mL of the above solution (PM/SDS/TRIT) was separated in 1-mL aliquots. Each aliquot was supplemented with 0.125 g of Bio-Beads SM2 prewashed in methanol and water (Holloway, 1973). After 3 h of agitation in the dark, the Bio-Beads were replaced and the agitation was continued overnight. The aliquots were put together and the suspension was centrifuged at 38000*g* for 25 min. The sediment was used to obtain a suspension concentrated enough to take IR spectra. The percentage of chromophore regeneration at this stage (about 80%) was estimated spectrophotometrically using $\epsilon_{max} = 55\,000\ M^{-1}\cdot cm^{-1}$ corresponding to BR incorporated in Triton X-100 micelles (London & Khorana, 1982).

Solubilization of PM in Triton X-100. A purple membrane suspension (7.6 mg of protein) was centrifuged at 50000*g* for 30 min. The sediment was resuspended in 0.4 mL of a 5% (v/v) Triton X-100 solution in 0.12 M acetate buffer (pH 5.0) and kept in agitation in the dark for 2 days. The suspension was centrifuged at 100000*g* for 30 min and the supernatant was concentrated with Centricon filters as described below.

Preparation of Samples for IR Spectra. In order to obtain a suitable signal-to-noise ratio for the FTIR spectra, the micelle suspensions, containing either Triton or SDS, were concentrated with Centricon-30 filters (cutoff 30 000 Da) at 5000*g* for 2 h. The detergents used in Scheme 1, SDS and Triton X-100, absorb in the IR amide I region. SDS has a wide band at 1640 cm^{-1}, whereas Triton X-100 shows a narrow band centered at 1610 cm^{-1} and another one at 1581 cm^{-1} (not shown), probably due to the ring. The contribution of the detergents to the IR amide I region was compensated by using as a reference the same concentration of detergent present in the sample. This was achieved by balancing the filter containing the sample with a filter containing a suspension control (*i.e.*, in the absence of protein). The fact that the control micelles were also trapped in the filter allowed us to obtain good spectral subtractions. For the reconstituted samples in native lipids, the lipid contribution to the IR spectrum[2] was subtracted digitally until the bands in the 3000−2800-cm^{-1} region, which correspond to the CH_2 and CH_3 vibrations, were compensated. To obtain spectra in D_2O, the samples were washed with D_2O buffer and allowed to exchange at room temperature for 2 days. The extent of exchange was calculated from the variation in the intensity of the amide II region as compared to the amide I.

Infrared Spectra Acquisition. The FTIR spectra were acquired with a Mattson Polaris FT spectrometer, at a resolution of 2 cm^{-1}. The samples were deposited onto CaF_2 windows fitted with 6-μm tin spacers for water samples and 25-μm Teflon spacers for samples in D_2O. The spectrum was obtained by digital subtraction of the reference until a flat line was observed in the range of 1800−2000 cm^{-1}. The spectrometer was purged continuously with dry air (dew point lower than −40 °C), and a shuttle was used to compensate the water vapor peaks. The IR spectra were

[2] As these lipids do not contain amide groups, they do not contribute to the IR amide region. However, three different preparations showed the presence of a band at 1656 cm^{-1} and another one, less intense, at 1549 cm^{-1} (not shown). These two bands probably correspond to the amide I and II, respectively, of some contaminant protein(s). The fact that the presence of this protein component cannot be detected by phosphor or iodine tinction, commonly used to assess the purity of these extractions, may explain that this fact has not been reported before. We roughly estimated the contribution of this component as about 6% of the dry lipid weight.

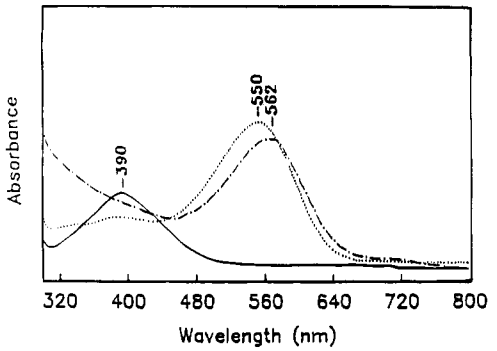
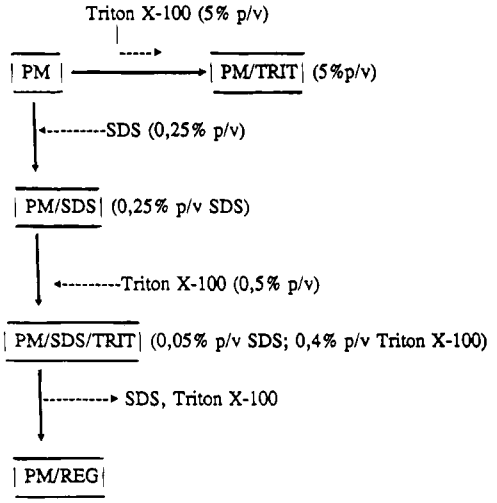


FIGURE 1: Visible absorption spectra of bacteriorhodopsin. (—) PM solubilized in 0.25% SDS (w/v) and 50 mM phosphate buffer, pH 6.5; bacteriorhodopsin concentration, 10 μM. (•••) The previous sample after addition of Triton X-100 (final concentration 0.4% (w/v) in 50 mM phosphate buffer, pH 6.5). (•-•-•) The previous sample after elimination of detergents with Bio-Beads, in 50 mM phosphate buffer, pH 6.5.

Scheme 1: Denaturation and Regeneration Processes of Bacteriorhodopsin



deconvoluted using the Kauppinnen *et al.* (1981) algorithm of the program SpectraCalc (Galactic Industries Inc.). The parameters used in the deconvolutions are described in the corresponding figure legends.

RESULTS

The reversibility of the renaturation process described in Scheme 1 can be observed by analyzing the spectra corresponding to the different species obtained, by either infrared or visible spectroscopy. Although the visible spectral changes are known (London & Khorana, 1982) we include the data presented in Figure 1 for the sake of clarity. The native chromophore band centered at 562 nm is lost after solubilization with SDS. Instead, a band at 390 nm appears, corresponding to retinal dissolved in SDS. After the addition of Triton X-100, the sample recovers its purple color in about 1 min, giving rise to a chromophore which absorbs at 550 nm. After elimination of the detergents, the maximum is shifted again to 562 nm, the same value as found in purple membrane. This regenerated sample (about 80% regeneration) can be sedimented by centrifugation, indicating that the sample is again organized in lipid bilayers.

Figure 2 presents the IR absorption spectra of the species described above. The amide I maximum found in native

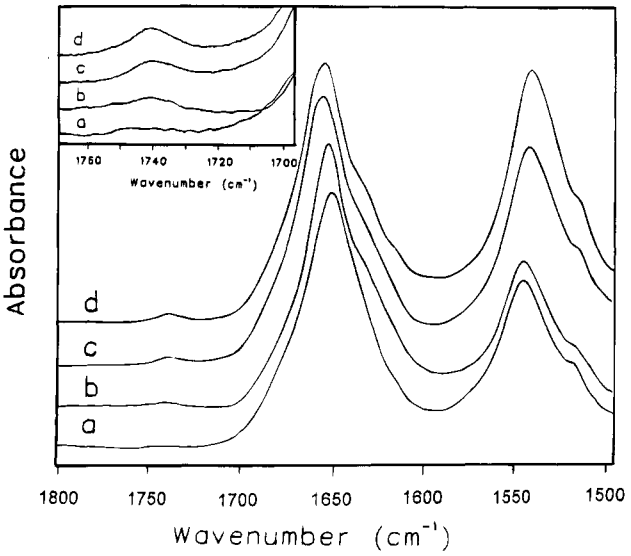


FIGURE 2: Infrared spectra of purple membrane samples. (a) PM dissolved in 0.25% SDS (w/v) and 50 mM phosphate buffer, pH 6.5; (b) PM dissolved in SDS (sample a) with Triton X-100 added to a final concentration of 0.4% (w/v) in 50 mM phosphate buffer, pH 6.5; (c) PM in SDS/Triton X-100 (sample b), after detergent removal with Bio-Beads, in 50 mM phosphate buffer, pH 6.5; (d) native PM suspended in 50 mM phosphate buffer, pH 6.5. The amide I intensity was normalized for all the samples. Inset: The 1700−1760-cm^{-1} zone expanded to show the carboxylate region.

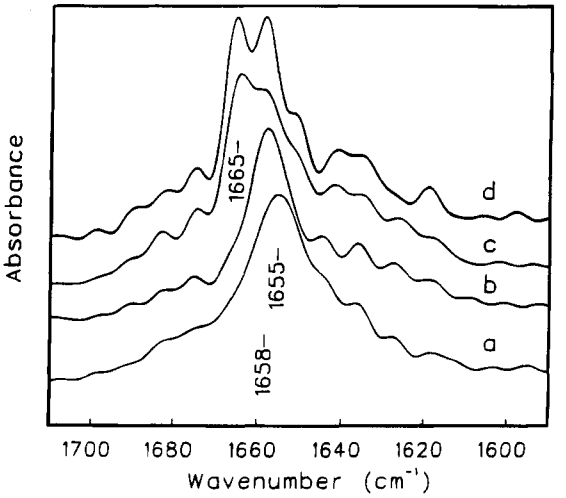


FIGURE 3: Infrared deconvoluted spectra corresponding to the amide I region of the samples in Figure 2. (a) PM solubilized in SDS; (b) PM solubilized in SDS after addition of Triton X-100; (c) regenerated sample obtained by elimination of detergents; (d) native purple membrane. Deconvolutions were done using a bandwidth of 14 cm^{-1} and a k factor of 2.2.

purple membrane (1661 cm^{-1}) is red-shifted to 1655 cm^{-1} in PM/SDS and to 1658 cm^{-1} in PM/SDS/TRIT. The original spectrum is almost recovered after the detergents have been removed and substituted by native lipids (PM/REG). Moreover, as shown in the inset of this figure, the band at 1741 cm^{-1}, corresponding to protonated carboxylic groups, is lost in PM/SDS, being recovered partially in PM/SDS/TRIT and totally recovered in the regenerated sample (PM/REG).

The amide I deconvoluted spectra corresponding to these samples (Figure 3) show that, after solubilization with SDS, the main bands at 1665 and 1658 cm^{-1} in native BR have been replaced by one band centered at 1655 cm^{-1}. After

the addition of Triton X-100, this band has been substituted by a narrower one, centered at 1658 cm^{-1}. At this stage, the deconvoluted spectrum is very similar to that observed for the purple membrane solubilized directly in Triton X-100 (not shown), indicating that the effect of the SDS on the protein secondary structure is not significant at this molar ratio (SDS/Triton X-100 1:1.5).

When the detergents are removed and substituted by the native lipids, the deconvoluted spectrum is similar to that of the native BR (Figure 3). These spectra, however, are not identical, showing differences in intensity in the band centered at 1658 cm^{-1} as well as in the 1640−1620-cm^{-1} region, usually assigned to β structures. The fact that the regeneration has not been complete (about 80%) and that, throughout the process, some irreversible modifications can have taken place could explain these small differences. In this respect, the elimination of the detergent is not totally achieved, as about 10 molecules of Triton X-100/BR molecule can still be present (London & Khorana, 1982).

The band appearing at 1665 cm^{-1}, as we will show, can be related to the changes in the aggregation state of the sample that take place during the process (Scheme 1). As shown by London and Khorana (1982), BR in PM/SDS and in PM/SDS/TRIT is monomeric, whereas in the regenerated sample (PM/REG) BR is forming an oligomeric lattice. This indicates that whereas in PM/SDS and PM/SDS/TRIT the monomers do not interact, after the substitution of the detergents by the native lipids the trimeric organization is regained. Looking at Figure 3 we can see that as long as the interaction between molecules is lost (PM/SDS and PM/SDS/TRIT), the band at 1665 cm^{-1} is absent. In the regenerated sample (PM/REG) or in native PM, where the exciton coupling effect is detected, the band at 1665 cm^{-1} is present in the deconvoluted spectrum.

The possibility that these effects could be caused merely by the presence or absence of the native lipids and not by changes in the aggregation state made it necessary to check this conclusion using an independent system. We reconstituted BO in native polar *Halobacterium* lipid liposomes at two very different lipid/protein ratios, 1:1 (w/w) and 10:1 (w/w). In one case, the lipid/protein ratio is close to that of the native PM (0.3:1); in the other, the BR molecules can hardly interact (Popet *et al.*, 1987). Figure 4 shows the deconvoluted spectra corresponding to the samples of BR reconstituted at lipid/protein ratios of 10:1 and 1:1 (w/w). The sample BO(10:1) in aqueous buffer has a wide band at 1656 cm^{-1} and a shoulder at 1665 cm^{-1}, whereas the sample BO(1:1) has narrower bands in identical frequencies to those corresponding to the native purple membrane (1665, 1658, and 1651 cm^{-1}). The intensity of the band at 1665 cm^{-1} in the sample BO(1:1) is around half of that observed for bleached or native purple membranes (Cladera *et al.*, 1992; Cladera, 1993). After deuteration, the band at 1665 cm^{-1} [a shoulder in the case of BO(10:1)] is still present in both samples, indicating that it is originated from a structure embedded in the membrane bilayer. The extent of exchange for the two samples (around 40%) was similar to that reported for native purple membrane (Cladera *et al.*, 1992). This is consistent with the fact that only extramembraneous regions have been exchanged. Thus, structures normally found in the extramembraneous space (*e.g.*, turns, β structure, disordered sequences) do not contribute significantly to this band. On the other hand, this fact supports α_{II} helix as a major

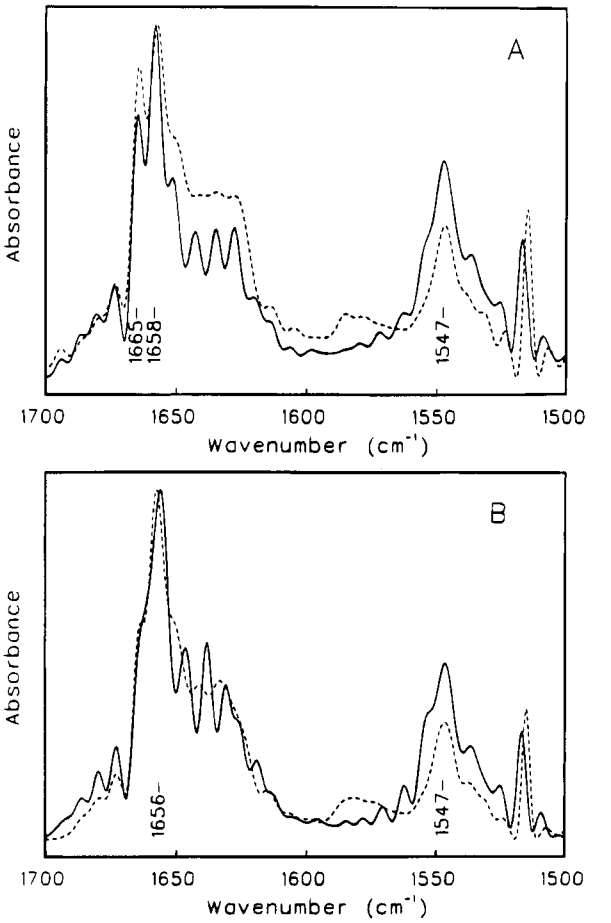


FIGURE 4: Infrared deconvoluted spectra in the amide I and II of bacteriorhodopsin samples reconstituted in *Halobacterium* polar lipids in H_2O buffer (—) and D_2O buffer (- - -). (A) Lipid to BR ratio of 1:1 (w/w); (B) lipid to BR ratio of 10:1 (w/w). Deconvolution was done using a bandwidth of 14 cm^{-1} and a k factor of 2.5.

contributor, in agreement with others (Krimm & Dwivedi, 1982; Earnest *et al.*, 1990; Cladera *et al.*, 1992).

DISCUSSION

Effect of the Solubilization of BR with SDS. The IR spectral changes observed after the solubilization of Br with SDS can be associated with alterations in carboxylic amino acids and the helical structures, which will be considered in some detail in the following.

Effect of SDS on Carboxylic Amino Acids. One of the effects produced by SDS solubilization is the alteration of the protonated carboxylic residues, as seen by the disappearance of the band at 1741 cm^{-1} (Figure 2, inset). It is known that in BR two internal carboxylic side chains are protonated (Asp 96 and Asp 115), absorbing at 1742 and 1734 cm^{-1}, respectively (Braiman *et al.*, 1988; Sasaki *et al.*, 1994). Other carboxylic side chains are involved in conformational changes during the photocycle, giving rise to absorptions in the zone 1700−1760 cm^{-1}. Finally, several carboxylic side chains are involved in cation binding and also absorb in the region 1741−1730 cm^{-1} (Duñach *et al.*, 1989). The remaining Asp and Glu residues, mostly located in the loops or in the N- and C-terminal regions, are likely to be ionized and thus must absorb in the 1567−1584-cm^{-1} region (Chirgadze *et al.*, 1975; Venyaminov & Kalnin, 1990). The fact that the absorption in the region 1740−1730 cm^{-1}

is lost upon SDS solubilization indicates that the functionally important residues become ionized. Thus, the solubilization leads to the disruption of the helix−helix interactions that in normal conditions provide the adequate environment for the increased pK_a of Asp 96 and Asp 115.[3] This conclusion agrees with other known effects of SDS (e.g., loss of retinal), or with the observation that the chymotryptic fragments C1 and C2 are not tightly associated in SDS solution (Huang *et al.*, 1981).

Effect of SDS on α-Helices. The deconvoluted amide I spectrum corresponding to BR solubilized in SDS shows a broad band centered at 1655 cm^{-1}. One of the reasons for the bandwidth increase is the increase in the environment heterogeneity. Thus, the large width found in the band at 1655 cm^{-1} could be due either to a lack of interaction between the different transmembrane fragments or to an increase in disordered structure. In this respect, a decrease in the content of α helix and a concomitant increase in disordered structure was suggested for the samples BO, C1, and C2 incorporated in SDS micelles (London & Khorana, 1982; Liao *et al.*, 1983). This hypothesis was based on the observed loss in ellipticity in the CD spectra. However, NMR studies made on isolated helices A (Pervushin & Arseniev, 1992) and B (Lomize *et al.*, 1992) and the proteolytic fragment C2 in SDS (Pervushin *et al.*, 1994) show that the helix-forming segments are very similar to those found by Henderson *et al.*, (1990) for native BR. Thus, although a small decrease in helical structure could still be attributed in those samples to the disruption of the possible short helical segment in the loop B−C (Henderson *et al.*, 1990), the most reasonable explanation for the observed broadening in the 1655-cm^{-1} band comes from the fact that transmembrane segments no longer interact in SDS micelles. That is, noninteracting fragments can fluctuate among several slightly different helical conformations due to the lack of constraints, which are imposed by helix−helix interactions. In this respect, Pervushin *et al.* (1994) reported that at least two different helical conformations are present in the C2 fragment in SDS micelles, where the helical segments do not interact.

On the other hand, the frequency observed (1655 cm^{-1}) lies in the region typical of α helices (Fraser & McRae, 1973; Byler & Susi, 1986; Surewicz & Mantsch, 1988; Arrondo *et al.*, 1993). This indicates that, upon SDS solubilization, the helical structure is retained in BR, but the helix type is different as compared to the native form. The whole of these facts indicates that although a slight increase in disordered structures cannot be discarded, the SDS micelles preserve the α-helical segments of native BR. As in the case of the Asp side chains, the spectral changes described above suggest that the interactions between the helices have been lost in SDS.

Recovery of Helix−Helix Interactions in Triton X-100 and the Two-State Oligomerization Model. In mixed Triton X-100/SDS micelles, the purple color is recovered and the principal IR amide I band appears now at 1658 cm^{-1} (like one of the two main α-helical bands in native BR). This is accompanied by a partial recovering of the carboxylate absorptions in the 1740−1730-cm^{-1} region. These changes indicate that the helix−helix interactions present in the native state are better preserved in Triton X-100 than in SDS. It is likely that the nonionic character of Triton X-100 as compared to the highly ionic SDS weakens the detergent−helix interactions, thus allowing the formation of specific helix−helix interactions like those found in the native state.

These results can be related to the two-state oligomerization model for α-helical membrane proteins (Popot & Engelman, 1990). In this model, isolated α helices would first be formed in the bilayer. This first step could be analogous to the noninteracting α helices we observe when BR is dissolved in SDS. The second step in the model corresponds to the formation of helix−helix interactions, giving rise to the native state. Our results are consistent with the assumptions of this model, in the sense that helix−helix interactions of preformed helices are required to conform the adequate environment for the retinal active site. This step should correspond to BR in SDS/Triton X-100, in which medium a re-formation of helix−helix interactions occurs.

In the case of BR, another level of interaction would constitute a third step involving intermolecular interactions giving rise to trimers. Interestingly, as we have seen, this step is accompanied by significant changes in the IR (*e.g.*, the appearance of the intense band at 1665 cm^{-1}).

Interpretation of the IR 1665-cm^{-1} Band. According to the results obtained in the process of renaturation (Scheme 1), the presence of the band at 1665 cm^{-1} in the deconvoluted spectrum could be associated either with the nature of the environment surrounding the protein (*i.e.*, the presence of native lipids) or with the aggregation state (*i.e.*, not present when BR is solubilized as monomers in detergent). Clearly, the data obtained after the reconstitution of BR in native lipid liposomes indicates that the appearance of the band at 1665 cm^{-1} is directly related to the aggregation state. Whereas in BO(10:1) the band at 1665 cm^{-1} is very weak, this band is clearly detected in the sample BO(1:1). By using the known different characteristics in the visible CD spectrum between monomeric BR and the native purple membrane, Popot *et al.* (1987) found that BO(1:1) contained a mixture of monomeric and trimeric BR. In addition, these authors suggested that the molecules well reconstituted would give rise to aggregates (trimers), whereas the molecules incorrectly incorporated would give rise to monomers. The percentage of BR molecules able to form the chromophore was around 50% in our samples for both BO(1:1) and BO(10:1), and similar results were found by Popot *et al.* (1987). This indicates that the difference in the intensity of the 1665-cm^{-1} band between the two samples cannot be associated with differences in the percentage of molecules correctly reconstituted. Moreover, the fact that the intensity of the band at 1665 cm^{-1} in BO(1:1) is about half of that present either in native BR or in the bleached membrane [where the intensity of the 1665-cm^{-1} band is similar to that of the native BR (Cladera, 1993)] suggests that (a) around 50% of the molecules in BO(1:1) are forming trimers and (b) the band at 1665 cm^{-1} has the same intensity for the correctly reconstituted molecules in BO(1:1) as in the native sample, as expected.

The reported differences observed in the intensity of this band cannot be due to orientational effects because the samples used in this work were analyzed in solution and not oriented in films. Thus, it is likely that the band at 1665 cm^{-1} originated from a distinct type of secondary structure.

[3] BR solubilized in chloroform/methanol (1:1 v/v) and 0.1 M $LiClO_4$ loses the absorption band at 1741 cm^{-1} (Torres & Padrós, 1995), indicating that these interactions are also lost in this organic medium.

Although the existence of α_{II} helix in bacteriorhodopsin has not been proved yet by X-ray diffraction methods, there exist considerable data supporting this hypothesis (see introduction). Meanwhile, new data point in the same direction; for example, the NMR peaks of the α_{II} helix have been distinguished from those of the α_I helix by deconvolution of the respective peaks of the hydrated [3-^{13}C]Ala-BR (Tuzi *et al.*, 1994), as referred to the conformation-dependent ^{13}C chemical shift of polyalanine in hexafluoroisopropyl alcohol solution (Krimm & Dwivedi, 1982). In another work, Zhang *et al.* (1995) have shown that, in certain conditions, the insertion of a hydrophobic transmembrane peptide in phosphatidylcholine bilayers causes the appearance of the 1665-cm^{-1} band in the gel phase. These authors suggested that some distortion of the α-helical domains compatible with α_{II} helix could account for this effect. In addition, the differences detected in the rate of isotopic exchange for the chymotryptic fragments C1 and C2 of BR dissolved in organic solvent (Torres & Padrós, 1995) have been interpreted as the presence of weaker C=O···H−N bonds in the transmembrane segments of C1 with respect to C2. Similar results suggesting this type of heterogeneity between transmembrane segments in BR have been obtained using NMR (Orekhov *et al.*, 1992). These studies, although performed in a nonnative environment, point out that the conformational tendencies of the different transmembrane segments are not the same and that similar behavior giving rise to α_I and α_{II} helices could be expected in the lipid bilayer.

Appearance of the Band at 1665 cm^{-1} as a Consequence of Trimer Formation. As referred to above, we assume that in BO(10:1), the protein molecules are not interacting forming trimers. This is indicated by different lines of evidence. First of all, it is known that the state of aggregation of BR when reconstituted in liposomes with exogenous lipids (*e.g.*, dimyristoyl- or dipalmitoylphosphatidylcholine) depends on the lipid:protein ratio used in the reconstitution as well as on the temperature (Cherry *et al.*, 1978). In these conditions, lipid:protein ratios higher than 4:1 (w/w) lead to monomeric BR even below the T_c of the lipid (Cherry *et al.*, 1978). Thus, it is reasonable to think that in the sample BO(10:1) the molecules will be sufficiently dispersed by the lipids, and the formation of trimers will not be significant. Indeed, the presence of a shoulder at 1665 cm^{-1} in BO(10:1) could originate from other structures or from a small percentage of aggregated molecules. Other evidence concerning the aggregation state of these samples is related to the ability to form a lattice under certain conditions. When either BO(1:1) or BO(10:1) is incorporated into liposomes, it does not form a lattice (Popot *et al.*, 1987); similar results have been reported for BO(2:1) (Kahn *et al.*, 1992). According to Popot *et al.* (1987), the lattice is formed when the pellet of BO(1:1) is partially dried, but it is not formed in the case of BO(10:1). As these authors detected the existence of trimers in BO(1:1) in solution, it is likely that the existence of trimers in the liposome suspension promotes the formation of the lattice upon drying. The fact that the lattice was not detected in the case of BO(10:1) argues that the aggregation state of the molecules in BO(10:1) is predominantly monomeric.

Although in native PM or in PM/REG the lattice is present in addition to the trimers (Hwang & Stoeckenius, 1977; Cherry *et al.*, 1978), the presence of the band at 1665 cm^{-1} cannot be related to the presence of the lattice itself. In fact, in samples where the lattice is not present, as in the bleached membrane (Stoeckenius *et al.*, 1979), the intensity of this band is comparable to that found in the native PM (Cladera, 1993).

Table 1: Relationship between the State of Aggregation of Bacteriorhodopsin and the Appearance of the 1665-cm^{-1} Band in the IR Deconvoluted Spectrum

sample	state of aggregation	1665-cm^{-1} band
native PM	oligomeric	yes
PM/SDS	monomeric[a]	no
PM/SDS/TRIT	monomeric[a]	no
PM/TRIT	monomeric[b]	no
PM/REG	oligomeric[a]	yes
native BO	trimeric?	yes
BO(1:1)	both[c]	~50%
BO(10:1)	probably monomeric[c]	shoulder
BR (C/M)[d]	monomeric[d]	no

[a] London & Khorana (1982). [b] Dencher & Heyn (1978). [c] Popot *et al.* (1987). [d] BR dissolved in chloroform/methanol (1:1 v/v) and 0.1 M ClO_4Li (Torres & Padrós, 1993).

As summarized in Table 1, the presence of the band at 1665 cm^{-1} in the deconvoluted spectra is closely related to the existence of interactions between BR monomers. In particular, this band does not appear in samples where the interactions do not exist (PM/SDS, PM/SDS/TRIT, PM/TRIT) and is found again when the oligomeric form (*i.e.*, trimeric) is obtained, either in the untreated sample (native PM) or after detergent elimination (PM/REG). In addition, it is nearly absent in BO(10:1), where the monomers are dispersed, and is intense in BO(1:1), where monomers interact, forming trimers. The relation between the band at 1665 cm^{-1} and the aggregation state of BR is supported also by previous works, using other conditions. Lee *et al.* (1985) reconstituted BR in DMPC liposomes [lipid/protein 4:1 (w/w)], in which case the BR molecules should be in the monomeric state (Cherry *et al.*, 1978). According to our hypothesis, in these conditions the band at 1665 cm^{-1} in the deconvoluted amide I region should not be present. Not surprisingly, these authors reported that the second derivative maximum corresponding to the main component of the amide I had been shifted from 1664 cm^{-1} in the purple membrane to 1659 cm^{-1} either below or above T_c.

Consequently, we conclude that an important condition for the appearance of the band at 1665 cm^{-1}, and hence α_{II} helix, is the existence of interactions between monomers forming trimers. However, the formation of the lattice is not required. One logical implication of this conclusion is that, although in the bleached membrane the lattice is absent (Stoeckenius *et al.*, 1979), the presence of the band at 1665 cm^{-1} (Cladera, 1993), and hence α_{II} helix, is indicative of the trimeric organization of this sample.

About the Functional Role of the Putative α_{II} Helix. The possible functional role for the α_{II} helix in BR was first suggested by Krimm and Dwivedi (1982). These authors proposed that the weak hydrogen bonds present in α_{II} helix, as compared to those found in α_I helix, could provide a pathway for the transference of protons across the protein. Obviously, this proposal has long been discarded in view of later studies. The hypothesis described in our work results in a new perspective from which we can discuss the functional relevance of this structure. First of all, in samples where α_{II} helix is not present (*e.g.*, BR solubilized in Triton

X-100), BR presents the same light-adapting behavior and is functionally active, because its photocycle is similar to the native one (Dencher & Heyn, 1978). Also, in BR reconstituted in DMPC vesicles, where the aggregation state can be modified by varying the temperature (Cherry *et al.*, 1978), the monomeric BR is able to pump protons with an efficiency similar to that of native purple membrane (Dencher & Heyn, 1979). Thus, the function of α_{II} seems to have no direct relation to the photocycling or pumping abilities of BR. However, the low energy barrier between α_I and α_{II} helices (Krimm & Dwivedi, 1982) makes it possible that this putative structure is involved in conformational mobility between monomers or between different transmembrane segments. In this respect, results obtained by recent time-resolved photoinduced dichroism (Wan *et al.*, 1993) are compatible with rotational movements of monomers within the trimers. Evidence of changes in the spatial disposition of transmembrane segments during the photocycle has also been reported based on experimental results (Rothschild *et al.*, 1993; Subramaniam *et al.*, 1993; Han *et al.*, 1994) or by comparing computational models of *cis*- and *trans*-BR (Chou, 1993).

From the experimental evidence presented in this work, we can conclude that the interaction between monomers concomitant with the formation of a trimer leads to the conversion of substantial amounts of α_I into α_{II} helix. The conformational changes occurring during the photocycle could be facilitated by a transition between these two helical conformations. Finally, it is interesting to note that the tight packing of BR in the purple membrane, which is needed in order to harvest light with higher efficiency, could have created the conditions which facilitated the integration of the chromophore units in a coordinated system.

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