Fourier-Transform Infrared Studies on Cation Binding to Native and Modified Purple Membranes†

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ABSTRACT: Fourier-transform infrared spectroscopy has been used to examine the structural differences in the protein moiety between the native purple and the deionized blue membranes, both at pH 5.0. The spectra demonstrate that deionization of purple membrane decreases the content of the distorted α_{II}-helices in favor of the more common α_I-helices. Changes in the signals from β-turns are also observed. The changes corresponding to the carboxyl groups suggest that deionization leads to a decrease in the strength of the hydrogen bonds involving carboxyl groups. Most of these effects are reversed progressively upon binding of one to five Mn^{2+} per bacteriorhodopsin to the deionized membrane. Binding of Hg^{2+} to the deionized membranes does not restore the purple color but induces global changes similar to, but less intense than, those brought about by Mn^{2+} binding. However, the effects attributed to the carboxyl groups are opposite to those found for Mn^{2+}. Schiff base reduction or bleaching induces a decrease of the content of the α_{II}-helix in favor of the α_I-helix and a decrease in the strength of hydrogen bonds to carboxyl groups. Deionization of these modified membranes leads to a further loss in the α_{II} content. These results indicate a conformational rearrangement of the protein structure between the native purple membrane and the deionized membrane, which could arise from surface potential changes elicited by bound cations. The changes observed in the carboxyl groups suggest that some of them are located structurally close to the retinal environment and may be involved in cation binding.

The purple membrane of *Halobacterium halobium* contains a unique protein, bacteriorhodopsin (BR),[1] which upon illumination translocates protons from the inside to the outside of the cell. This creates an electrochemical gradient that is used to maintain the essential functions of the cell [for recent review, see Dencher (1983) and Stoeckenius (1985)]. Despite extensive investigations, several fundamental questions remain about the molecular mechanism of proton transport.

Visible spectral properties of BR depend on the pH and on bound cations to the purple membrane. Native purple membrane (λ_{max} 558 nm) contains one bound Ca^{2+} and four Mg^{2+} per BR molecule (Chang et al., 1985; Ariki & Lanyi, 1986). The removal of these cations from the purple membrane or the lowering of the pH induces a reversible transition of BR to a blue form (λ_{max} 605 nm) which does not pump protons (Mowery et al., 1979). The purple color can be regenerated by the addition of a variety of cations (Kimura et al., 1984; Chang et al., 1985). Besides the importance to clarify the relationship between cation binding and the function of BR, one of our interests was to determine the effects of cation binding on the membrane structure. Our previous studies have shown the presence of five binding sites of high and medium affinity for Mn^{2+} and suggest a close interaction between retinal environment and some of the cation binding sites (Duñach et al., 1986, 1987, 1988a).

Infrared spectroscopy has proved to be a powerful technique to study protein conformation (Susi, 1972; Mendelsohn, 1984; Braiman & Rothschild, 1988). However, the spectra of proteins result from the superposition of several bands arising from the different structures and amino acids. Until the development of mathematical methods to narrow the component bands, such as Fourier self-deconvolution (Mantsch et al., 1986) or Fourier derivation (Cameron & Moffatt, 1987), only an overall picture of the protein conformation could be obtained. The application of these mathematical procedures to the IR spectra, together with band fitting programs, allows more detailed structural information to be obtained from the infrared spectra [for a recent review, see Surewicz and Mantsch (1988)].

The purple membrane exhibits an anomalous amide I band (Rothschild & Clark, 1979) with a maximum at 1660 cm^{-1} that does not correspond to the expected position for an α-helical structure (Henderson & Unwin, 1975). Theoretical studies have shown that this frequency is consistent with a distorted α-helical structure (α_{II}) in which there are changes in the dihedral angles with respect to the common α-helix (Krimm & Dwivedi, 1982). Infrared difference spectroscopy has previously been applied to study the cation-depleted membrane. In these studies, the involvement of carboxylic groups in the blue to purple transition (Gerwert et al., 1987) and a similarity between the deionized membrane and the M412 intermediate of the photocycle (Marrero & Rothschild, 1987) have been described.

In the present work, we have used resolution-enhanced FTIR to study structural changes induced in the deionized blue

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[1] Abbreviations: BR, bacteriorhodopsin; IR, infrared; FTIR, Fourier-transform infrared.

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membrane upon binding of stoichiometric amounts of Mn^{2+}, one of the cations known to regenerate the functional purple form. We also studied the effects of Hg^{2+} binding, a cation that is not able to restore the purple color (Ariki & Lanyi, 1986; Duñach et al., 1988a). In order to investigate further the relationship between the retinal moiety and the cation binding sites, the effect of cation binding on the $NaBH_4$-reduced membrane and on the bleached one has also been studied.

Materials and Methods

Membrane Preparations. Purple membrane was isolated from *Halobacterium halobium* strain S9 as described by Oesterhelt and Stoeckenius (1974). Reduction of the Schiff base was accomplished by illumination of the suspension in the presence of 0.2 M $NaBH_4$ at pH 8.0 with a slide projector equipped with a yellow filter. Bleaching of the purple membrane with hydroxylamine was effected as described (Duñach et al., 1987). Deionization of membrane suspensions was performed by passage through a cation-exchange column (Dowex 50W) as described (Duñach et al., 1987). The desired amount of cations [either $MnCl_2$ or $Hg(NO_3)_2$] was added to the deionized membrane samples (5 mg of BR/mL) and the pH readjusted to 5.0. Taking into account the affinities of the divalent cations (Duñach et al., 1987), the unbound cation concentration is negligible.

FTIR Spectroscopy. The membrane suspensions (about 100 μL) were deposited on a CaF_2 window, dried under vacuum, and rehydrated by exposing them to a 100% humidity atmosphere. Control experiments in the visible showed that the rehydrated samples had the same absorption maxima as the suspensions (Duñach et al., 1987), indicating that the film formation did not change the retinal conformation. The window was mounted in a Harrick cell (Harrick Scientific, Ossining, NY). IR spectra were acquired on a Nicolet 10 DX spectrometer. A total of 256 scans were averaged, apodized with a Happ–Genzel function, and Fourier-transformed with a spectral resolution better than 2 cm^{-1}. It has been described that sample orientation can influence the amide II/amide I ratio in rhodopsin (Rothschild et al., 1980). Therefore, in order to exclude artifacts, some experiments were performed in D_2O suspension. Cation-depleted and native purple membrane samples were lyophilized and resuspended in D_2O. The spectra were transferred to a personal computer, where Fourier self-deconvolution and Fourier third derivatives were performed in order to separate overlapping bands (Moffatt et al., 1986). The parameters used for deconvolution were a half-bandwidth of 15 cm^{-1} and a resolution enhancement factor of 1.9. The third derivatives in the Fourier space, equivalent to smoothed fourth derivatives (Cameron & Moffatt, 1987), were obtained with a breakpoint of 0.3.

Results

FTIR Spectra of Native and Deionized Membranes. Figure 1A shows the absorption spectrum of purple membrane in the region 1500–1900 cm^{-1} (lower trace), where the amide I and II bands are observed, and the same spectrum after being subjected to Fourier self-deconvolution (middle trace) and Fourier derivation (upper trace). The region 1800–1900 cm^{-1}, which does not exhibit bands either from the lipid or from the protein, is shown in order to evaluate the noise. In the absorption spectrum, two major bands with maxima at 1660 cm^{-1} (amide I) and 1545 cm^{-1} (amide II) and one weak band at 1736 cm^{-1} are observed. These bands are composed of several components distinguished in the deconvolved and derivative spectra. After data treatment, the amide I band exhibits

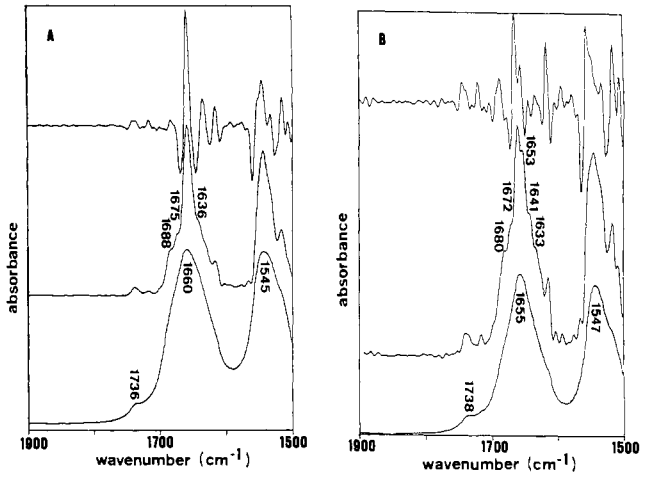

FIGURE 1: Infrared spectra of hydrated films (pH 5.0) of native purple membrane (A) and deionized blue membrane (B) in the 1500–1900 cm^{-1} region. The picture shows the original spectra (lower trace), the spectra after Fourier self-deconvolution using a Lorentzian bandshape of half-width 15 cm^{-1} and a resolution enhancement factor of 1.9 (middle trace), and the same spectra after Fourier derivation using a power of 3 and a breakpoint of 0.3 (upper trace). The apparent higher noise in the spectrum of (B) is due to a poorer removal of atmospheric water vapor.

protein peaks at 1636, 1660, 1675, and 1688 cm^{-1}. These bands can be assigned to protein structures (Krimm & Bandekar, 1986; see next section). The weak band at 1736 cm^{-1} is due to carbonyl vibrations of protonated aspartic and glutamic residues (Chirgadze et al., 1975). There are no carbonyl stretching bands due to the lipids in this spectral region because the lipids of purple membrane are ether-linked (Kushwaha et al., 1975).

Deionization of purple membrane suspensions changes the color to blue. Figure 1B shows the infrared absorption spectrum (bottom) together with the deconvolved and the derivative spectrum (middle and top traces, respectively) of the cation-depleted membrane. Comparison of these spectra with those of the native membrane (Figure 1A) reveals a shift from 1736 to 1738 cm^{-1} of the carboxyl major peak and several differences in the amide I and amide II bands. The major differences of the amide I band correspond to the appearance of a band at 1653 cm^{-1} and a shift of the other bands to 1633, 1641, 1662, 1672, and 1680 cm^{-1}. All these changes are observed on both the deconvolved and the derivative spectra. This rules out the possibility and side lobes as being the origin of some of the changes observed between the spectra of purple and deionized samples. It should be noted that the amide I band in the absorption spectrum of the deionized membrane is apparently narrower than in the native membrane. This effect might reflect the shift in band positions listed above, which in the deionized sample encompass a narrower interval than in the native sample. The major change in the amide II region corresponds to the appearance of a shoulder at 1554 cm^{-1} in the deconvolved spectrum, which is evidenced in the derivative spectrum as an increase in the 1554/1547 cm^{-1} ratio.

Effect of Mn^{2+} Binding to the Deionized Membrane. The recovery of the purple color is followed by monitoring the effect of each bound Mn^{2+} to the five high- and medium-affinity binding sites. Figure 2A and Figure 2B show the deconvolved and the Fourier derivative spectra, respectively, of the deionized blue membrane films supplemented with increasing quantities of Mn^{2+} (pH 5.0) in the amide I spectral region. Cations appear to have a strong influence on the main peaks at 1653 and 1662 cm^{-1}. Figure 2C shows the derivative spectra corresponding to the same samples in D_2O suspension. The three

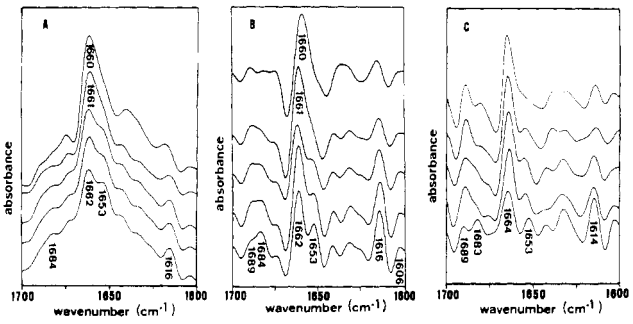

FIGURE 2: (A) Fourier self-deconvolved spectra (from bottom to top) of films of the deionized blue membrane, the blue membrane with two, three, and five bound Mn^{2+} per BR, and the native purple membrane (pH 5.0), in the amide I region, using the conditions of Figure 1. (B) Fourier derivative spectra of the same samples of (A). (C) Fourier derivative spectra of the same samples of (A), in D_2O suspension (20 mg/mL, pD 5.0). The derivative spectra were obtained with a power of 3 and a breakpoint of 0.3 and interpolated by using a zero filling factor of 2. In this and the following figures, the spectra of each panel have been scaled to the same maximum size.

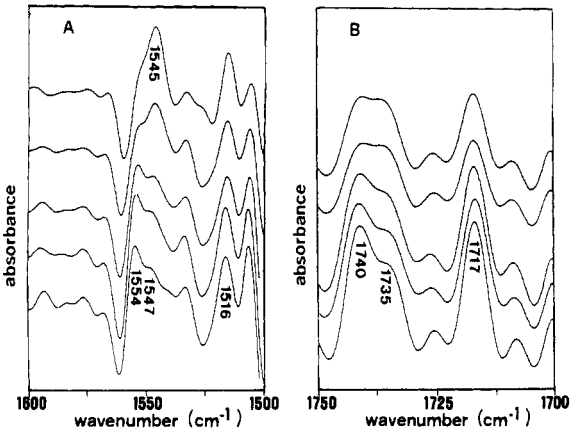

FIGURE 3: Fourier derivative spectra (from bottom to top) of films of the deionized blue membrane, the blue membrane with two, three, and five bound Mn^{2+} per BR, and the native purple membrane (pH 5.0). (A) Amide II region. (B) Region corresponding to protonated carboxyl groups. Data treatment was performed as in Figure 2.

spectral series of Figure 2 indicate that addition of Mn^{2+} leads to a progressive increase of the 1662 cm^{-1} peak intensity and a shift to 1661 cm^{-1}, at the expense of the 1653 cm^{-1} band. When the membrane possesses five bound Mn^{2+} per BR, which corresponds to completely fill the high- and medium-affinity binding sites (Duñach et al., 1987), the peak at 1653 cm^{-1} has almost disappeared. Thus, a spectrum similar to that of the native purple membrane is obtained, although a shift of 1 cm^{-1} is still observed in the major amide I peak. These bands are generally attributed to "normal" or α_I-helices (1653 cm^{-1}) and "distorted" or α_{II}-helices (1662 cm^{-1}) (Krimm & Dwivedi, 1982). Thus, binding of Mn^{2+} to the deionized membrane induces a gradual increment in the α_{II} content of BR, at the expense of the normal α_I-helix, i.e., restores the native conformation of BR. This could arise from a reorientation of peptide bonds to more distorted positions along one or several transmembrane α-helical segments. A weak peak arising from arginine at 1606 cm^{-1} and another at 1616 cm^{-1} due to vibrations of the aromatic ring of tyrosine residues (Chirgadze et al., 1975) reflect no appreciable changes upon cation binding. The peaks appearing in the 1630–1645 cm^{-1} region, due mainly to β-sheet structures (Susi, 1969), show no major changes compared to the deionized blue membrane. The 1670–1700 cm^{-1} region, attributed mainly to vibrations of β-turns (Krimm & Bandekar, 1986), shows a gradual decrease in the intensity ratio between the two peaks at 1684 and 1689 cm^{-1} upon cation binding.

Derivative spectra are often difficult to interpret since side lobes can appear on performing the Fourier transform. The conditions used in this work (power = 3; breakpoint = 0.3) have been shown to minimize these effects (Cameron & Moffat, 1987). However, to confirm the results obtained with derivatives, the same spectra were also subjected to deconvolution. With this mathematical treatment, band narrowing is smaller than with derivation, but there is also less band distortion. Comparison of Figure 2A and Figure 2B shows clearly that the deconvolved spectra follow the same pattern as the derivative ones, thus validating the use of derivatives.

The amide II region is expected to be sensitive to protein conformational changes (Lee & Chapman, 1986). Whereas a good relationship has been established between protein conformation and the amide I region, very few reports have established a similar correlation with the amide II bands. Figure 3A shows that cation binding mainly affects the two peaks at 1554 and 1547 cm^{-1}. When cations are added to the deionized blue membrane, no changes are seen in this spectral region up to three Mn^{2+} per BR. With five bound Mn^{2+} per BR, the spectrum recovers the pattern of the purple membrane. No changes in the frequency of the C–C stretching band of the tyrosine aromatic ring at 1516 cm^{-1} are evidenced between the blue and purple forms. There are also few small changes in the 1570–1590 cm^{-1} region, in which the vibrations of deprotonated carboxyls should appear, among others.

As previously indicated, the bands above 1700 cm^{-1} arise from protonated carboxylic groups of glutamic and aspartic amino acid side chains. Figure 3B shows the spectra of the deionized blue membrane and the sequence obtained when Mn^{2+} was added progressively, keeping the pH value at 5.0. No changes are seen in the 1717 cm^{-1} peak, whereas the intensity ratio 1735/1740 cm^{-1} increases upon cation binding. Resolution-enhanced spectra clearly reveal that the most important increase occurs between three and five bound Mn^{2+}, giving a final spectrum identical with that obtained for the purple membrane.

The use of films for examining changes in protein structure can be influenced by sample orientation (Rothschild et al., 1980). Preparation of the samples in D_2O suspensions avoids these problems and may provide additional information. Figure 2C shows the Fourier derivative spectra of the same samples as those of Figure 2A,B, in D_2O suspensions. The amide I components are revealed at 1689, 1684, 1664, and 1653 cm^{-1}. α_I and α_{II} bands reflect changes similar to those evidenced in the H_2O films, including the progressive reduction of the 1653 cm^{-1} band. Thus, possible artifacts due to sample orientation in films do not influence the changes observed in these samples. Down-shifted bands are expected in D_2O on the basis of the isotopic exchange (Siebert et al., 1982). However, in our measurements, we have observed that not all the hydrogens in glutamic and aspartic acids have been substituted by deuterium. Thus, several carboxylic acid bands are obtained (data not shown), making it difficult for their interpretation. It might be possible that this exchange is dependent on the depth of the residues in the lipid bilayer.

Effect of Hg^{2+} Addition. Previous work has established the special nature of Hg^{2+} interaction with the deionized blue membrane, in the sense it does not restore the purple form (Ariki & Lanyi, 1986; Duñach et al., 1988a). Therefore, it is interesting to compare the changes induced by Hg^{2+} addition with those already described for Mn^{2+}. As shown in Figure 4A, addition of five Hg^{2+} per BR produces alterations in the amide I components at 1653 and 1662 cm^{-1}, which are com-

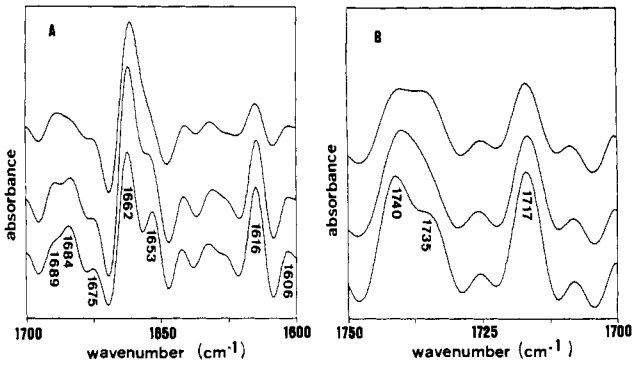

FIGURE 4: Fourier derivative spectra of the deionized blue membrane (lower trace), the blue membrane plus five Hg^{2+} per BR (middle trace), and five Mn^{2+} per BR (upper trace) in the amide I region (A) and in the region corresponding to protonated carboxyl groups (B). Data treatment was performed as in Figure 2.

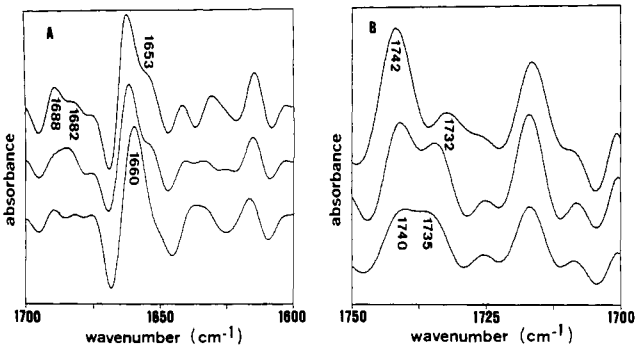

FIGURE 5: Fourier derivative spectra of the purple membrane (lower trace), the reduced membrane (middle trace), and the bleached membrane (upper trace) in the amide I region (A) and in the region corresponding to the protonated carboxyl groups (B). Fourier derivative and interpolation parameters as in Figure 2.

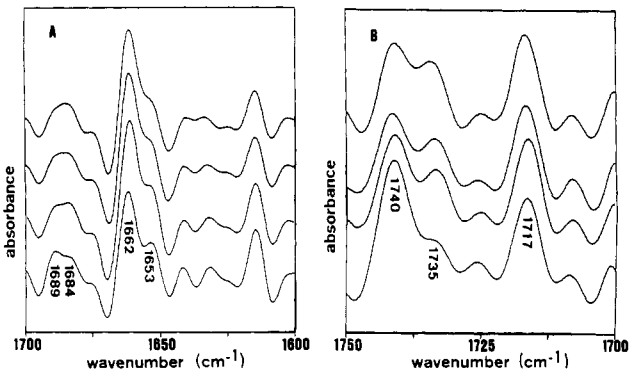

FIGURE 6: Fourier derivative spectra (from bottom to top) of the reduced deionized membrane, the reduced deionized membrane plus three and five added Mn^{2+} per BR, and the reduced membrane. (A) Amide I region. (B) Region corresponding to the protonated carboxyl groups. Data treatment as in Figure 2.

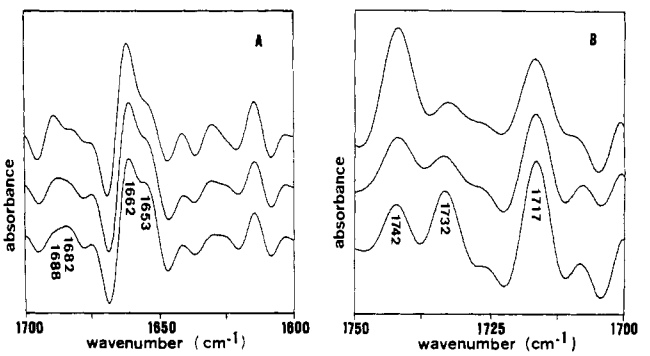

FIGURE 7: Fourier derivative spectra of bleached deionized membrane (lower trace), bleached deionized membrane plus 100 Mn^{2+} per BR (middle trace), and bleached purple membrane (upper trace) in the amide I region (A) and in the protonated carboxyl region (B). Data treatment as in Figure 2.

parable to the effect produced by binding of two to three Mn^{2+} per BR (cf. Figure 2). Only small changes are detected in the peaks at 1684 and 1689 cm^{-1} or in the remaining peaks of the amide I region. The amide II region also undergoes some changes upon Hg^{2+} addition, comparable to those promoted by binding of two to three Mn^{2+} (data not shown). Figure 4B shows that, analogous to the binding of Mn^{2+}, there is no change in the carboxylic band at 1717 cm^{-1} but in the 1735 and 1740 cm^{-1} peaks the effect of Hg^{2+} binding appears to be different from that elicited by Mn^{2+} binding. In this latter case, the intensity ratio 1735/1740 cm^{-1} increases, whereas Hg^{2+} binding has an opposite effect.

Effect of Schiff Base Reduction and of Bleaching. As we have previously reported, there is a close relationship between the retinal moiety and some cation binding sites (Duñach et al., 1986, 1988a). Thus, it was interesting to study the changes induced by reduction or by bleaching of purple membrane samples and to follow the effect of cation removal. Figure 5 shows the Fourier derivative spectra corresponding to purple, reduced, and bleached membranes. From the amide I region (Figure 5A), it is apparent that both Schiff base reduction and bleaching induce a decrease in the 1660/1653 cm^{-1} ratio, indicating a diminution in α_{II} content with respect to the purple membrane. The region 1670–1700 cm^{-1} also shows changes. In particular, Schiff base reduction increases the 1682/1688 cm^{-1} ratio as compared to the purple membrane, whereas bleaching slightly decreases it. Above 1700 cm^{-1} (Figure 5B), an important increase in the intensity ratio 1740/1735 cm^{-1} is observed in the reduced and in the bleached membranes, with a concomitant shift of the 1735 and 1740 cm^{-1} bands in the purple membrane to 1732 and 1742 cm^{-1}, respectively, in the bleached membrane. In this latter sample, this shift leads to the complete separation of the two peaks.

Effect of Mn^{2+} Binding to the Reduced and the Bleached Deionized Membranes. Modification of the retinal moiety is accompanied by changes in cation binding. After Schiff base reduction, only four divalent cation binding sites are found, whereas bleaching the membrane decreases the divalent cation binding sites to only one of low affinity (Duñach et al., 1986, 1988a). Hence, it should be expected that cation binding to these modified membranes would affect the protein conformation in a different way than in the native membrane. Figure 6 shows the dependence of the reduced deionized membrane on Mn^{2+} binding. In the amide I region (Figure 6A), deionization induces an increase of the 1653 cm^{-1} band that is reversed as Mn^{2+} cations are bound. However, the changes in the ratio of peak intensities at 1684 and 1689 cm^{-1}, which are completed after addition of three Mn^{2+} per BR, are opposed to those observed for the native membrane (see Figure 2B). The alterations detected in the amide II region (not shown) and also above 1700 cm^{-1} are similar to those observed upon cation binding to the native membrane, except for a major change between the cation-depleted and the three bound Mn^{2+} per BR sample (Figure 6B).

Due to the low affinity of the unique cation binding site of the bleached membrane, high Mn^{2+}/bacterioopsin ratios are required in order to study cation binding effects. As shown in Figure 7, small changes occur in the amide I region upon Mn^{2+} binding to the bleached deionized membrane. Above 1700 cm^{-1}, an interesting change occurs (Figure 7B). Opposite to that observed for the native and the reduced membranes, cation binding leads to a decrease in the 1732/1742 cm^{-1}

intensity ratio. However, addition of 100 Mn^{2+} per bacterioopsin does not induce a complete reversal of the bleached conformation.

Discussion

The structure of the native purple membrane deduced from our IR spectra is in close agreement to those reported earlier (Rothschild & Clark, 1979; Cortijo et al., 1982; Lee et al., 1987). The major feature is an α_{II}-helix in which steric constraints induce a tilting of the peptide group with respect to the helical axis (Krimm & Dwivedi, 1982). Contributions of β-turns (1670–1700 cm^{-1}) and bands in the 1630–1645 cm^{-1} region due to β-sheet structure are also present. The absence of bound cations appears to be responsible for a protein conformational change between the purple and the deionized blue forms, in which the α_{II}-helical content decreases in the deionized species. These changes also involve some differences in the bands arising from β-turns, whereas the β-sheet region shows two bands in the deionized membrane instead of one in the native membrane.

The conformational change (from native to deionized) was reversed progressively upon binding of cations to the five functionally important sites of the membrane and suggests that each bound cation contributes partially to this change, allowing the nearly complete recovering of the native conformation when all five high- and medium-affinity sites are occupied. Simultaneously to this conformational change, the purple color of the membrane is restored. A gradual change is also found in the bands assigned to β-turns (1670–1700 cm^{-1}; Krimm & Bandekar, 1986). These structures are expected to be mainly located at the outer part of the protein (Rose et al., 1985), that is, in the loops connecting the transmembrane segments. Therefore, a change in the major protein structure would also affect these loops. It is interesting to note that, upon deionization, the $\alpha_{II} \rightarrow \alpha_I$ transition is not complete. Cation removal seems to "loose" some of the forces that constrain the α_{II}-helical segments located close to the surface, allowing them to return to their most favored conformation, whereas the remaining segment forming α_{II} must be buried. It is possible that such conformational changes associated with cation binding might be the consequence of a decrease in the negative membrane surface potential. Indeed, it has been proposed that the purple to blue transition of BR is mainly due to protonation of one or more control group(s) of the protein. Binding of cations decreases the apparent pK of the group(s) responsible for the transition by lowering the negative surface charge density due to membrane lipids.

Binding of Hg^{2+} to the deionized blue membrane (pH 5.0) does not restore the purple color (Ariki & Lanyi, 1986; Duñach et al., 1988a).[2] On the other hand, the conformational changes induced by $Hg(NO_3)_2$ addition are similar to those produced by two to three bound Mn^{2+} per BR. The similarity refers to global properties of the protein like the content of α_I, α_{II}, β-turn, or β-sheet structures. However, the effects produced by Hg^{2+} and Mn^{2+} addition in the bands arising from the C=O stretching vibration of protonated carboxyl groups appear to be opposite. The smaller changes induced by Hg^{2+} in the global structural properties, as compared to those elicited by Mn^{2+}, could be associated with Hg^{2+} binding to its four specific sites, because Hg^{2+} binding to these sites decreases the negative surface potential.[2] However, Hg^{2+} binding to the three cytoplasmic Ca^{2+}-sensitive sites does not influence the surface potential (no effect on the amide I band) but has an effect on the carboxyl bands. This might reflect the fact that Hg^{2+} forms different coordination complexes than Mn^{2+} or Ca^{2+}, so that binding to these sites does not necessarily involve identical groups. The opposite behavior observed in the carboxyl bands upon Hg^{2+} binding could be associated with a different hydrogen-bonding interaction or with the fact that Hg^{2+} sites do not share the same carboxyl groups.

The conformational changes observed in the amide I and amide II regions for the reduced and the bleached samples are similar to the aforementioned alterations observed upon deionization of native purple membrane. Namely, in all these samples, our FTIR data detect a certain decrease in the α_{II} content and other minor changes corresponding mainly to β-turns. No extensive changes appear to be produced by Schiff base modification or retinal elimination, other than those already induced by deionization. Indeed, comparison between IR spectra of the deionized blue membrane and the deionized forms of the reduced and the bleached membranes (thus circumventing the effects of the different amounts of bound cations in these samples) supports this conclusion. However, the carboxyl region (above 1700 cm^{-1}) shows considerable differences between the effects induced upon native membrane deionization and the effects of retinal extraction or Schiff base reduction. Thus, it seems that retinal alteration affects mainly its immediate environment and that several carboxyl groups may be involved in this environment.

The involvement of carboxylic groups in both the BR photocycle (Rothschild et al., 1981; Bagley et al., 1982; Siebert et al., 1982; Engelhard et al., 1985; Roepe et al., 1987) and the blue to purple transition (Chang et al., 1985; Duñach et al., 1987) has been proposed on the basis of several findings. Recently, Gerwert et al. (1987) used FTIR difference spectroscopy to study the blue to purple transition induced by exposing films of the blue membrane to NH_3 vapor. They observed a negative peak at 1723 cm^{-1} and a positive one at 1571 cm^{-1} which arise from deprotonation of external carboxylic amino acids. However, these changes may be due to a pH increase, and thus it is difficult to separate the blue to purple transition from the pH effect. Our present results have been obtained at the same pH value for the deionized blue and the purple forms, and whereas clear changes are obtained in the 1700–1760 cm^{-1} region (C=O stretch of COOH) no changes are seen in the 1560–1600 cm^{-1} region (COO^- stretching) where unprotonated carboxyl vibrations should appear. From previous studies, it seems reasonable to assign to the membrane surface a high negative potential (due to the presence of acidic groups of the lipids and the protein) at low ionic strength (Chang et al., 1986; Szundi & Stoeckenius, 1987). Thus, at a bulk pH value near 5, the effective pH at the membrane surface should be lower than 3, and nearly all surface carboxyl groups should be protonated. However, even if some carboxyl groups deprotonate during the blue to purple transition, its detection using resolution-enhanced methods would face several difficulties (Engelhard et al., 1985): First, COO^- bands appear in spectral regions crowded with bands from other groups, and thus are difficult to identify. Second, the band intensities are lower than those corresponding to the protonated carboxyls. Third, in COO^- groups of proteins, the two oxygens may not be fully equivalent, thus giving rise to

[2] We have directly confirmed this binding using radioactive Hg^{2+}. Our data indicate competitive binding of Hg^{2+} for three Ca^{2+} sites and noncompetitive binding for the other four sites (i.e., sites that bear no relation with the Ca^{2+} sites). Hg^{2+} binding to the latter sites—which are probably located at the external surface—affects the surface potential, whereas Hg^{2+} binding to the three Ca^{2+}-sensitive binding sites—which appear to be located at the cytoplasmic surface—does not modify the surface potential (Duñach et al., 1988b).

vibrations in spectral regions different from those corresponding to simple carboxylate salts. Therefore, the absence of changes in the 1560–1600 cm^{-1} region, where the vibrations of deprotonated carboxyls normally appear, does not preclude the possibility of one or more deprotonations to exist. Thus, we cannot assure whether a carboxyl deprotonation (corresponding to the Schiff base counterion, for example) occurs in parallel to the blue to purple transition.

Concerning the carbonyl bands of protonated carboxylic acids found above 1700 cm^{-1}, only those at 1735 and 1740 cm^{-1} show changes in the blue to purple transition. The lower frequency (1735 cm^{-1}) would correspond to carboxylic side chains involved in stronger hydrogen bonds, whereas the band at 1740 cm^{-1} would correspond to weaker hydrogen bonds (Bellamy, 1980). In the blue membrane, the dominant carboxyl population seems to be involved in weak hydrogen bonds, and this situation is kept until more than three Mn^{2+} per BR are bound; a transition is then seen, and both populations are in a distribution coincident to that of the native purple membrane. This might indicate a more localized conformational change induced upon binding of the fourth or fifth Mn^{2+} cation, or indeed a direct binding of metal ions to these carboxyls.

However, our results do not completely rule out the possibility of a protonation of carboxyl groups, rather than a change in hydrogen bonding, since as it has been pointed out above, the use of derivatives can mask changes in the intensity of the peaks; therefore, both the 1735 and 1740 cm^{-1} bands can grow substantially upon deionization. If this is the case, a protonation of carboxyl groups cannot be left out as an interpretation rather than changes in the intensity of hydrogen bonds.

These results suggest the presence of some carboxyl groups structurally close to the retinal Schiff base. These groups may be involved in cation binding and would also be affected by retinal modification.

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Registry No. Mn, 7439-96-5; Hg, 7439-97-6; retinal, 116-31-4.

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