Biochim. Biophys. Acta 687, 296-302.
Kaupp, U. B., & Schnetkamp, P. P. M. (1982) *Cell Calcium 3*, 83-112.
Krebs, W., & Kühn, H. (1977) *Exp. Eye Res. 25*, 511-526.
Laemmli, U. K. (1970) *Nature (London) 227*, 680-685.
Mason, W. T., Fager, R. S., & Abrahamson, E. W. (1974) *Nature (London) 247*, 562-563.
McCaslin, D. R., & Tanford, C. (1981) *Biochemistry 20*, 5212-5221.
Michaelis, E. K., Michaelis, M. L., Chang, H. H., & Kitos, T. E. (1983) *J. Biol. Chem. 258*, 6101-6108.
Miki, N., Kuo, C.-H., Hayashi, Y., & Akiyama, M. (1980) *Photochem. Photobiol. 32*, 503-508.
Mueller, P., & Pugh, E. N., Jr. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 1892-1896.
Neufeld, A. H., Miller, W. H., & Bitensky, M. W. (1972) *Biochim. Biophys. Acta. 266*, 67-71.
Nöll, G., Stieve, H., & Winterhager, J. (1979) *Biophys. Struct. Mech. 5*, 43-53.
O'Brien, D. F. (1979) *Photochem. Photobiol. 29*, 679-685.
Papazian, D. M., Rahamimoff, H., & Goldin, S. M. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 3708-3712.
Papazian, D. M., Rahamimoff, H., & Goldin, S. M. (1984) *J. Neurosci. 4*, 1933-1943.
Pasantes-Morales, H., & Ordonez, A. (1982) *Neurochem. Res. 7*, 317-328.
Rhoden, V. A., & Goldin, S. M. (1979) *J. Biol. Chem. 254*, 11199-11201.
Sack, R. A., & Harris, C. M. (1977) *Nature (London) 265*, 465-466.
Schatzman, H. J. (1981) in *Membrane Transport of Calcium* (Carafoli, E., Ed.) pp 41-108, Academic Press, London.
Schnetkamp, P. P. M. (1979) *Biochim. Biophys. Acta 554*, 441-459.
Schröder, W. H., & Fain, G. L. (1984) *Nature (London) 309*, 268-270.
Simon, W., & Carafoli, E. (1979) *Methods Enzymol. 56*, 439-448.
Simons, T. J. B. (1979) *Nature (London) 281*, 337-338.
Smith, H. G., Stubbs, G. W., & Litman, B. J. (1975) *Exp. Eye Res. 20*, 211-217.
Smith, H. G., Fager, R. S., & Litman, B. J. (1977) *Biochemistry 16*, 1399-1405.
Steinberg, R. H., Fisher, S. K., & Anderson, D. H. (1980) *J. Comp. Neurol. 190*, 501-518.
Szuts, E. Z. (1980) *J. Gen. Physiol. 76*, 253-286.
Szuts, E. Z., & Cone, R. A. (1977) *Biochim. Biophys. Acta 468*, 194-208.
Thacher, S. (1978) *Biochemistry 17*, 3005-3011.
Uhl, R., & Abrahamson, E. W. (1981) *Chem. Rev. 81*, 291-312.
Vasington, F. D., Gazzotti, P., Tiozzo, R., & Carafoli, E. (1972) *Biochim. Biophys. Acta 256*, 43-54.
Wormington, C., & Cone, R. A. (1978) *J. Gen. Physiol. 71*, 657-681.
Yonetani, T. (1967) *Methods Enzymol. 10*, 332-335.
Yoshikami, S., & Hagins, W. A. (1971) *Biophys. Soc. Abstr. 11*, 47a.
Yoshikami, S., George, J. S., & Hagins, W. A. (1980) *Nature (London) 286*, 395-398.

Light-Driven Protonation Changes of Internal Aspartic Acids of Bacteriorhodopsin: An Investigation by Static and Time-Resolved Infrared Difference Spectroscopy Using [4-^{13}C]Aspartic Acid Labeled Purple Membrane†

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ABSTRACT: The molecular events during the photocycle of bacteriorhodopsin have been studied by the method of time-resolved and static infrared difference spectroscopy. Characteristic spectral changes involving the C=O stretching vibration of protonated carboxylic groups were detected. To identify the corresponding groups with either glutamic or aspartic acid, BR was selectively labeled with [4-^{13}C]aspartic acid. An incorporation of ca. 70% was obtained. The comparison of the difference spectra in the region of the CO_2^- stretching vibrations of labeled and unlabeled BR indicates that ionized aspartic acids are influenced during the photocycle, the earliest effect being observed already at the K_{610} intermediate. Taken together, the results provide evidence that four internal aspartic acids undergo protonation changes and that one glutamic acid, remaining protonated, is disturbed. The results are discussed in relation to the various aspects of the proton pumping mechanism, such as retinal isomerization, charge separation, pK changes, and proton pathway.

The elucidation of the mechanism of bacteriorhodopsin (BR),[1] the light-driven proton pump of the halophilic bacterium *Halobacterium halobium* [for a review, see Stoeckenius & Bogomolni (1982)], still represents a challenge. Its chromophore, which is *all-trans*-retinal in its light-adapted func-

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[1] Abbreviations: BR, bacteriorhodopsin; ^{13}C-Asp-BR, [4-^{13}C]aspartic acid labeled bacteriorhodopsin; FTIR spectroscopy, Fourier transform infrared spectroscopy.

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tionally active form, BR_{568}, is bound to the protein via a protonated Schiff base. It undergoes a cyclic photoreaction involving the intermediates K_{610}, L_{550}, M_{412}, and O_{640} (in the order of their appearance). Many investigations have dealt with the light-induced molecular events in the chromophore. Extraction experiments indicate that the chromophore has isomerized at later stages of the photocycle, from all-trans to 13-cis (Pettei et al., 1977; Tsuda et al., 1980; Mowery & Stoeckenius, 1981). Investigations in the UV-spectral range (Kuschmitz & Hess, 1982) and resonance Raman experiments (Braiman & Mathies, 1982) provide some indications that this isomerization has, at least partially, already occurred at the first photoproduct, K_{610}. The Schiff base is deprotonated at the M_{412} intermediate. Most models of the proton pumping mechanism assume that this deprotonation step is more or less directly connected with the transfer of protons across the membranes (Schulten & Tavan, 1978; Honig et al., 1979 Warshel, 1979; Kalisky et al., 1981). However, a link between the molecular events in the chromophore and in the protein, which only jointly perform the function as proton pump, is missing.

The recently developed technique of time-resolved (Siebert et al., 1981) and static (Rothschild et al., 1981; Rothschild & Marrero, 1982; Bagley et al., 1982; Siebert & Mäntele, 1983) infrared difference spectroscopy allows molecular changes of the chromophore as well as of the protein to be monitored. Thus, it could provide information on various aspects of the proton pumping mechanism such as energy transducing steps, stoichiometry [there are up to two protons pumped per photocycle (Bogomolni et al., 1980; Govindjee et al., 1980; Renard & Delmelle, 1980)], and proton conduction mechanism (Dunker & Marvin, 1978; Nagle & Morowitz, 1978; Knapp et al., 1980). Further, the allocation of molecular changes in the protein, shown to occur in the neighborhood of the chromophore, to specific amino acid side chains, could provide additional structural information.

Among the amino acid side chains potentially involved in the proton pump mechanism, carboxylic acid residues are thought to play an important role in regulating the spectral properties of bacteriorhodopsin (Nakanishi et al., 1980; Motto et al., 1980) and in the charge separation step (Honig et al., 1979; Warshel, 1979), which is assumed to store a large part of the light energy. Modification experiments indicate the presence of internal carboxylic side chains (Herz et al., 1983). Carboxylic groups representing proton donors and acceptors during the transfer process could also play a direct role in the proton pumping mechanism. Indeed, in a previous publication, we provided evidence by time-resolved infrared difference spectroscopy that two carboxylic groups become protonated and redeprotonated during the photocycle (Siebert et al., 1982).

In this paper, by the method of static and time-resolved infrared difference spectroscopy, we have investigated these carboxylic groups in greater detail using [4-^{13}C]aspartic acid labeled bacteriorhodopsin. This allowed them to be identified as aspartic acid. Since charge movements involving carboxylic groups could also occur at the earlier intermediates, the complete photocycle was investigated. Extending the spectral range to the region where ionized carboxylic groups have characteristic bands, their changes during the photocycle were investigated. Since many other bands are also present in this region, the use of labeled bacteriorhodopsin was essential. These results will be discussed in relation to current ideas of the molecular events in bacteriorhodopsin, such as isomerization of the chromophore (Honig et al., 1979; Warshel, 1979; Schulten & Tavan, 1978; Birge & Hubbard, 1981), charge separation, removal of the positively charged nitrogen of the protonated Schiff base from the counterion (Honig et al., 1979; Warshel, 1979; Birge & Cooper, 1983), and various pumping models, involving the protons of the protonated Schiff base and other groups (Schulten & Tavan, 1978; Honig, 1979; Warshel, 1979; Kalisky et al., 1981; Nagle & Mille, 1981). Finally, it will be shown that our data provide constraints for the assignments of the helices in the amino acid sequence, based on neutron scattering experiments (Trewhella et al., 1983).

Materials and Methods

Purple membranes from *H. halobium* strain R_1M_1 were isolated as described (Mäntele et al., 1980). The preparation of the hydrated film samples for the infrared investigations is given by Mäntele et al. (1982). ^2H_2O exchange was performed as described (Siebert & Mäntele, 1983). The same difference spectra were obtained for exchange times ranging from 2 to 24 h. The measurement of time-resolved infrared difference spectra is described by Siebert et al. (1981) and the measurement of static FTIR difference spectra by Siebert & Mäntele (1983), using a Bruker FTIR spectrophotometer, Model IFS 113v. To assess the pH in the hydrated films, a calibration curve was deduced from time-resolved measurements of the photocycle in the visible spectral range, using the biphasic decay of the M_{412} intermediate and the O_{640}/M_{412} ratio, measured at 705 and 412 nm, as internal indicators. pH was adjusted either by adding the appropriate buffer to the suspension of purple membranes from which by drying the hydrated film was formed or by adding a small amount of diluted NaOH or HCl to the hydrated films. The uncertainty of this pH estimation is about half a pH unit.

The static $BR_{568} - K_{610}$ difference spectrum was obtained as described (Siebert & Mäntele, 1983). The static $BR_{568} - L_{550}$ difference spectrum was obtained by illuminating the sample at 170 K for 10 min with light of wavelengths longer than 590 nm. The static $BR_{568} - M_{412}$ difference spectrum was obtained by illuminating the sample at 213 K and pH 9.5 for 10 min with light of wavelengths longer than 530 nm. In this way about 80% of BR_{568} could be converted to M_{412}, avoiding the branching of the photocycle at the L_{550} intermediate (Kalisky et al., 1981). Control measurements by illuminating the sample at pH 7 showed that the high pH did not influence the spectra. At pH 7, however, the yield of M_{412} was only 20%. In addition, a long relaxation time of more than 30 min was required to allow for the direct back-reaction of L_{550} to BR_{568}. The mixture of intermediates produced by illumination at low temperatures was controlled by measurements in the visible spectral range under otherwise identical conditions. For the $BR_{568} - L_{550}$ difference spectra, the presence of L_{550} was deduced from the shift of the absorption maximum to 550 nm with a concomitant reduction of its height. The short- and long-wavelength region did not provide any indication for the presence of M_{412} and K_{610}, respectively. Prolonged illumination did not change the infrared difference spectra, and shortening of the illumination yielded only reduced band intensities without altering the character of the spectra. Therefore, no indication for secondary photoproducts of L_{550} was obtained.

From measurement to measurement, the static infrared difference spectra showed small deviations in band position. This effect cannot be due to instrumental inaccuracies, since control measurements with small amounts of water vapor present in the spectrophotometer producing small bands also in the difference spectra showed that the frequency repro-

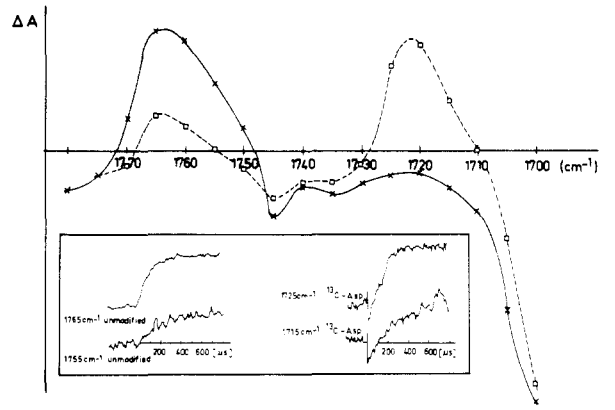

FIGURE 1: Time-resolved difference spectrum in the time range of the M_{412} intermediate. Typical signals from whose amplitude the spectrum was deduced are given in the insert. (×) Unmodified BR; (□) ^{13}C-Asp-BR. The two spectra were normalized to the amplitude at 1525 cm^{-1}, representing the C=C stretching vibration of the retinal in BR_{568}. The ordinate represents absorbance changes in arbitrary units. Spectral resolution, 6 cm^{-1}; temperature, 10 °C. For all the difference spectra in this paper, no indication was obtained of incorporation of ^{13}C into the retinal, which would have caused a change of the C=C stretching vibration.

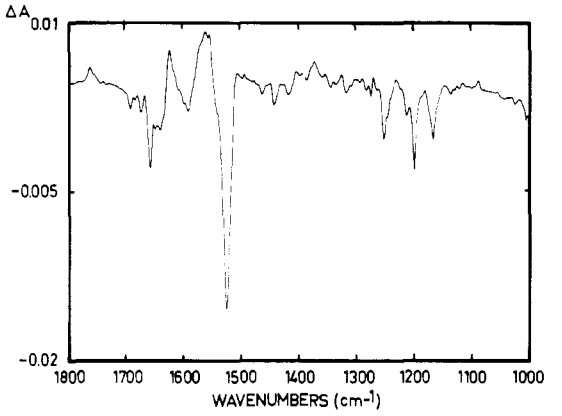

FIGURE 2: Static BR_{568} – M_{412} difference spectrum of unmodified BR. The ordinate represents absorbance changes. Measuring conditions are given under Materials and Methods. Spectral resolution, 2 cm^{-1}.

ducibility is better than 10^{-2} cm^{-1}. This set some limitations to the tracing of small effects caused by isotopic labeling in spectral regions where many other bands are present.

H. halobium (mutant R_1M_1) was grown in a synthetic basal salt medium using basically the procedure of Onishi et al. (1965) with an increased amount of L-Lys-HCl (1.59 g), L-Met (0.38 g), L-Thr (1.3 g), and L-Glu (1.3 g). After 24 h of growth, 0.5 g of α-ketoglutarate, 1.5 g of DL-[4-^{13}C]Asp (90.3% ^{13}C) (Amersham, Braunschweig), and as tracer 100 μCi of L-[4-^{14}C]Asp (Amersham, Braunschweig) were added to the suspension. Forty-eight hours later the bacteria were harvested and the modified purple membrane was isolated according to Oesterhelt & Stoeckenius (1974).

The extent and location of the ^{13}C incorporation into the protein were analyzed radioactively: L-[4-^{14}C]Asp was mixed with DL-[4-^{13}C]Asp, and the specific activity was determined by amino acid analysis and by counting the effluent directly after the ion-exchange column. After hydrolysis of the protein (24 h, 6 N HCl, 120 °C), the incorporation and specific activity of ^{14}C were measured similarly for the various amino acid fractions.

Results

Incorporation of [4-^{13}C]Asp into Bacteriorhodopsin. Aspartic acid, a precursor of a biosynthetic family with, e.g., Met, Thr, and Lys as members, can also replenish the tricarboxylic acid (TCA) cycle via a transamination. Therefore, it was expected that the yield of specifically labeled aspartate incorporated into bacteriorhodopsin would be relatively low. Indeed, first experiments showed an isotope enrichment of only 10%. Furthermore, the label was spread to other amino acids such as Glu, Thr, Pro, and Lys. Variation of the amount of added α-ketoglutarate, Lys, Met, Thr, Glu, and Asp yielded bacteriorhodopsin with 12.9 cpm/nmol Asp, 0.7 cpm/nmol Thr, and 3.1 cpm/nmol Glu (average of two experiments). With an original specific activity of 30.2 cpm/nmol of L-Asp (76% of theory), 43% of aspartic acid is incorporated into the protein. This adds up to an ^{13}C isotope enrichment of 39%.

A considerable amount of the label went into glutamic acid. Since the stereospecificity of the TCA cycle directs the label into the α-carboxyl group of Glu, this side product does not interfere with the measurements. There is as yet no proof that the TCA cycle operates in *H. halobium*. However, since our results provide evidence of the presence of a C=O stretching vibration of a protonated glutamic acid that is not shifted by this labeling (see below), the supposition that the label of the glutamic acid does not interfere is confirmed.

C=O Stretching Vibration of Protonated Carboxylic Groups. In Figure 1 the time-resolved difference spectrum in the time range of M_{412} between 1780 and 1700 cm^{-1} of labeled and unlabeled bacteriorhodopsin is shown. Representative signals, from whose maxima the spectrum was deduced, are given in the insert. As was discussed previously (Siebert et al., 1982), the positive band around 1760 cm^{-1} (unmodified bacteriorhodopsin) represents two carboxylic groups, one becoming protonated during the L_{550} – M_{412} transition and the other with a slower time course. Both deprotonate with the re-formation of BR_{568}. The band pattern is shifted 40 cm^{-1} to lower wavenumbers in [4-^{13}C]aspartic acid labeled bacteriorhodopsin (^{13}C-Asp-BR). This is the expected isotope shift for an isolated C=O stretching vibration, based on the increased reduced mass. The two signals at 1725 and 1715 cm^{-1} of ^{13}C-Asp-BR show, respectively, within the limits imposed by the noise, the same half-times as the corresponding signals at 1765 and 1755 cm^{-1} of the unmodified BR sample. Therefore, it is clear that both bands are shifted and that they correspond to aspartic acid side chains. (The unresolved absorbance decrease is caused by a band in the time-resolved BR_{568} – L_{550} difference spectrum of unmodified BR, as can be seen in Figure 4. It is not present in the static difference spectrum.) Furthermore, the remaining band at 1760 cm^{-1} in the spectrum of ^{13}C-Asp-BR demonstrates that the labeling amounts to about 70%, which is approximately double the value determined by the tracer method. We assume that this discrepancy is due to the presence of a racemase in the bacteria converting D-amino acids into L-amino acids.

We also measured the effect of [^{13}C]Asp labeling by static FTIR difference spectroscopy. Figure 2 shows the BR_{568} – M_{412} difference spectrum of unmodified BR in H_2O (for measuring conditions, see Materials and Methods). It is in agreement with the time-resolved difference spectrum (Siebert et al., 1981; Siebert & Mäntele, 1983). The part of this spectrum corresponding to the spectral region of Figure 1 is presented in Figure 3a. It reproduces essentially the time-resolved spectrum of Figure 1. The deviations are due to a continuous absorbance decrease in the time-resolved difference spectra, extending to about 2000 cm^{-1}. It could be caused by a change in the water–membrane interaction, since the con-

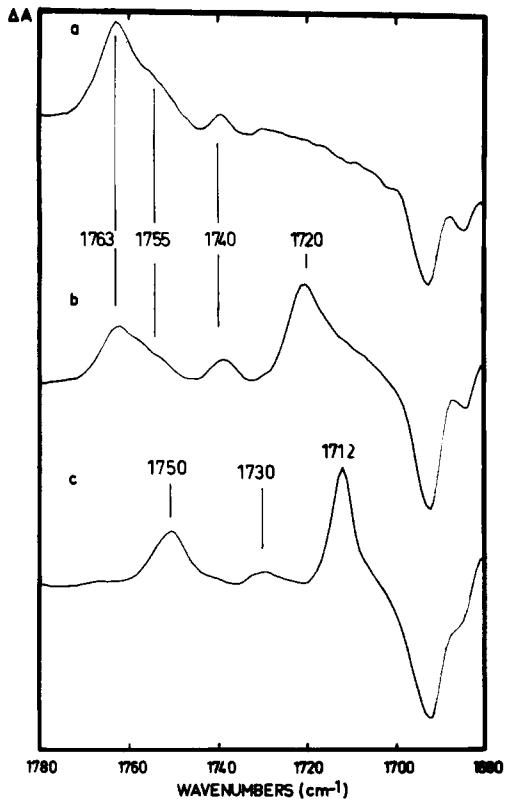

FIGURE 3: Static $BR_{568} - M_{412}$ difference spectrum of unmodified BR (a), ^{13}C-Asp-BR (b), and ^{13}C-Asp-BR in ^2H_2O (c). The ordinate represents absorbance changes in arbitrary units. The spectra were normalized to the C=C band of the retinal in BR_{568} at 1528 cm^{-1} (Figure 2). Spectral resolution, 2 cm^{-1}.

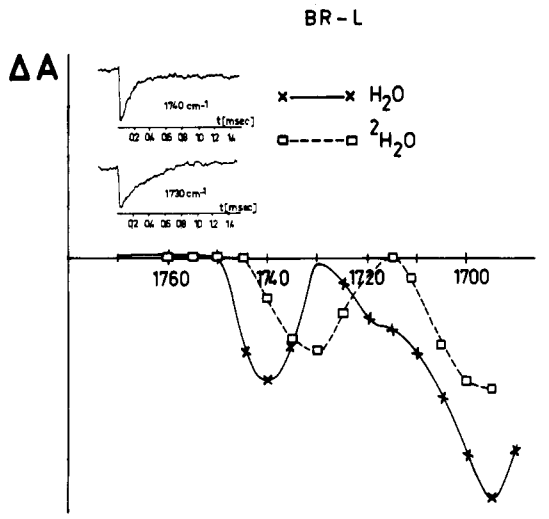

FIGURE 4: Time-resolved $BR_{568} - L_{550}$ difference spectrum. (×) BR in H_2O; (□) BR in ^2H_2O. The insert shows the corresponding two signals of BR in H_2O (1740 cm^{-1}) and ^2H_2O (1730 cm^{-1}). The ordinate represents absorbance changes in arbitrary units. The two spectra were normalized to the amplitude at 1525 cm^{-1}, representing the C=C stretching vibration of the retinal in BR_{568}. Spectral resolution, 6 cm^{-1}; temperature, 10 °C.

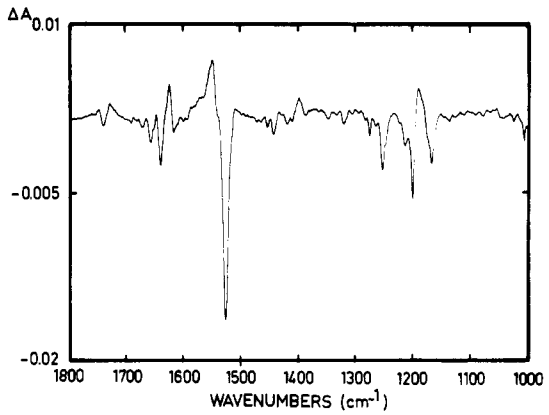

FIGURE 5: Static $BR_{568} - L_{550}$ difference spectrum of unmodified BR. The ordinate represents absorbance changes. Measuring conditions are given under Materials and Methods. Spectral resolution, 2 cm^{-1}; temperature, 170 K.

tinuous absorbance of water is sensitive to changes in the water structure. In addition, the negative band around 1700 cm^{-1} is more pronounced in the time-resolved difference spectra. Since the shoulder at 1755 cm^{-1} can be discerned, the "slow" aspartic acid also accepts the proton under the measuring conditions of the static difference spectrum, i.e., at 213 K and pH 9.5. The corresponding spectra of ^{13}C-Asp-BR in H_2O and ^2H_2O (parts b and c of Figure 3) demonstrate the expected shift caused by [^{13}C]Asp labeling, in accordance with Figure 1, and the additional effect of ^2H_2O. For unmodified BR and ^{13}C-Asp-BR (parts a and b of Figure 3) a small band is visible at 1740 cm^{-1}. It is shifted 10 cm^{-1} to lower wavenumbers by ^2H_2O. This provides evidence that it represents a protonated carboxylic group of a glutamic acid, although, for a definitive proof, corresponding ^{13}C labeling of the glutamic acids would be required. This band can also be discerned in Figure 1, where, however, the small negative continuous absorbance change is superimposed.

Since it is of great importance to know whether carboxylic groups are influenced at the earlier intermediates, we measured the $BR_{568} - L_{550}$ difference spectrum. Figure 4 shows the time-resolved difference spectrum, for H_2O and ^2H_2O. It was first described by Mäntele (1982). A negative band at 1740 cm^{-1} that is shifted 10 cm^{-1} to lower wavenumbers by ^2H_2O is clearly present. The corresponding signals measured at the maximum of the bands, i.e., at 1740 and 1730 cm^{-1}, are shown in the insert. In a separate measurement (data not shown), it was verified that the rise time of the two signals is identical and corresponds to the K_{610}–L_{550} transition. The decay of the two signals corresponds to the L_{550}–M_{412} transition, which is especially evident from the kinetic isotope effect: in ^2H_2O the decay is slowed down by a factor of 4. These spectral changes indicate that a carboxylic group becomes deprotonated during the K_{610}–L_{550} transition and reprotonated during the L_{550}–M_{412} transition.

Again, we measured the static $BR_{568} - L_{550}$ difference spectra of unmodified BR, ^{13}C-Asp-BR and ^{13}C-Asp-BR in ^2H_2O. The complete spectrum of the unmodified sample is given in Figure 5. It is noteworthy that, as in the $BR_{568} - M_{412}$ difference spectrum, mostly negative bands are present. A detailed discussion of this will be published elsewhere. The enlarged part of the spectrum for the three samples is shown in Figure 6. As in the time-resolved spectra, a negative band is located at 1740 cm^{-1} for unmodified BR (trace a). For ^{13}C-Asp-BR, part of this band is shifted 40 cm^{-1} to lower wavenumbers. In ^2H_2O both bands are shifted 10 cm^{-1} to lower wavenumbers. Therefore, this band originates from an aspartic acid side chain. Since this group is protonated in BR_{568} it should be deprotonated at high pH if it were exposed to the aqueous phase. But measurements of the $BR_{568} - L_{550}$ difference spectrum at pH 4, 7, and 9 showed no decrease of the band at 1740 cm^{-1}. As in the case of the glutamic acid seen in the $BR_{568} - M_{412}$ difference spectrum, this aspartic acid must be buried but still susceptible to ^1H–^2H exchange. For samples in ^2H_2O, the bands are weaker. Although no plausible

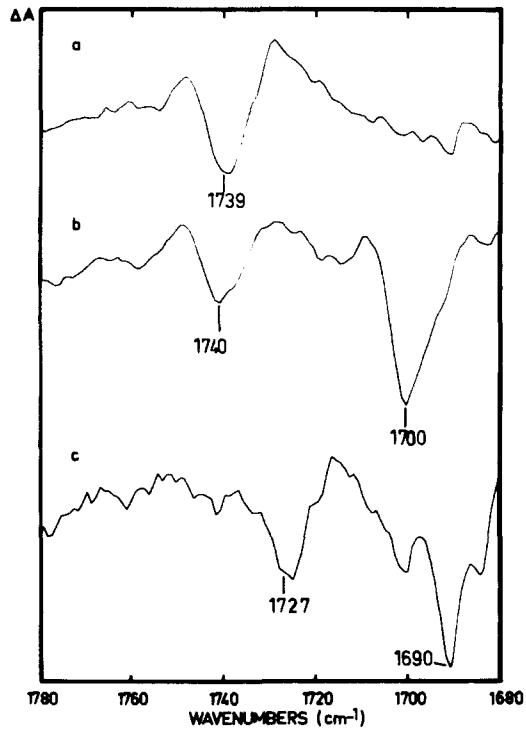

FIGURE 6: Static $BR_{568} - L_{550}$ difference spectrum of unmodified BR (a), ^{13}C-Asp-BR (b), and ^{13}C-Asp-BR in ^2H_2O (c). The ordinate represents absorbance changes in arbitrary units. The spectra were not normalized to the C=C band of the retinal in BR_{568} at 1528 cm^{-1}. Spectral resolution, 2 cm^{-1}; temperature, 170 K.

explanation can be given for this effect, intensity changes upon isotopic substitution are not unusual. The spectral feature around 1740 cm^{-1} in Figure 6a cannot be caused by a single band. It can be interpreted as if a broader positive band is superimposed on a stronger and narrower negative band. The apparent deviation from the time-resolved spectrum can be explained by the higher spectral resolution. Since this band pattern is shifted by [^{13}C]Asp labeling, both bands must be due to protonated aspartic acids. The positive band indicates that an aspartic acid also becomes protonated during the K_{610}–L_{550} transition.

Figure 7 shows the static $BR_{568} - K_{610}$ difference spectra for BR, ^{13}C-Asp-BR and BR in ^2H_2O. A small differential band can be detected at 1740 cm^{-1}, which is shifted 10 cm^{-1} to lower wavenumbers by ^2H_2O but not by [^{13}C]Asp labeling. Therefore, it reflects a glutamic acid residue in its protonated form. Since at the same wavenumber a band was discerned in the $BR_{568} - M_{412}$ difference spectrum, originating from a glutamic acid, the same residue may be involved. Also this band was observed up to pH 9.5. The small differential band indicates that a protonation or deprotonation has not occurred but rather that the environment of this group has changed, thus lowering the strength of the carbonyl bond.

Symmetric and Antisymmetric Stretching Vibrations of CO_2^-. So far we have identified one glutamic acid and four aspartic acids that are influenced during the photocycle. The glutamic acid is slightly influenced at the K_{610} and M_{412} intermediates but appears to remain protonated. For the four aspartic acids, however, evidence has been provided that they undergo protonation changes.

An unequivocal proof of the deprotonation or protonation of carboxylic groups would be the demonstration of the corresponding appearance or disappearance of the ionized form. In addition, monitoring the CO_2^- group could help to provide some insight into the first molecular events of the photocycle: if the first step involved isomerization and concomitant charge

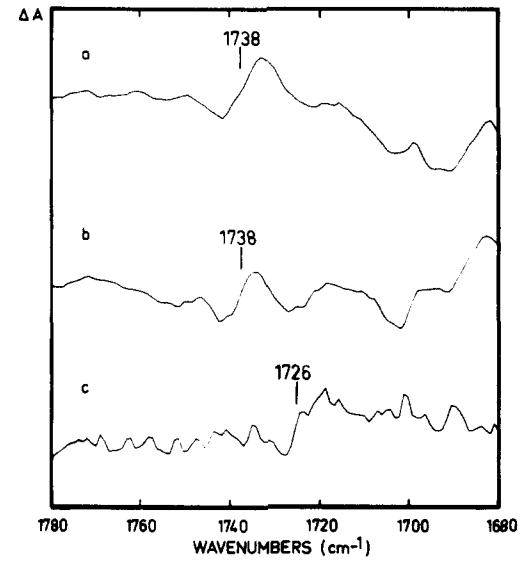

FIGURE 7: Static $BR_{568} - K_{610}$ difference spectrum of unmodified BR (a), ^{13}C-Asp-BR (b), and ^{13}C-Asp-BR in ^2H_2O (c). The ordinate represents absorbance changes. The spectra were normalized to the C=C band of the retinal in BR_{568} at 1529 cm^{-1}. Scale is expanded by a factor of approximately 2 as compared to the absorbance scale of Figure 6, based on the band at 1250 cm^{-1}, equally present in the $BR_{568} - K_{610}$ difference spectrum. Spectral resolution, 2 cm^{-1}; temperature, 77 K.

separation of the protonated Schiff base from the counterion, and if this counterion is an ionized carboxylic group, then this step should have an influence on the CO_2^- stretching vibrations. It is, therefore, of interest whether or not ionized carboxylic groups are influenced during the BR_{568}–K_{610} transition.

The bands caused by the CO_2^- groups are located in spectral regions where many additional bands are present. A direct identification is, therefore, not possible, and the use of ^{13}C-labeled carboxylic acids is essential. For the band positions a wide variability is found: 1550–1610 cm^{-1} for the antisymmetric vibration and 1300–1420 cm^{-1} for the symmetric vibration (Bellamy, 1957). In ^{13}C-labeled sodium formate the former is shifted from 1602 to 1559 cm^{-1}, and the latter is shifted from 1363 to 1336 cm^{-1}, which is in good agreement with theoretical calculations (Pinchas & Laulicht, 1971). The analysis is based on the general observation that in salts of carboxylic acids the two oxygens are equivalent, since the cations are placed symmetrically. In proteins, however, the position of the counterion is mainly determined by the protein structure. Therefore, it is possible that the two oxygens are no longer equivalent, causing a still wider variability of band positions. The band intensities are considerably weaker than that of the carbonyl band of the protonated carboxylic group. The antisymmetric stretching vibration, located in a region where large and broad bands are present (Figures 2 and 5), whose shape is in addition influenced by base-line distortions caused by the amide II band (Siebert & Mäntele, 1983), is especially difficult to detect. In the region of the symmetric vibration, base-line distortions, a general problem in static difference spectroscopy, make it difficult to decide whether small bands in the difference spectrum are due to absorbance increase or decrease. In principle, it should be possible to substract the difference spectra of labeled and unlabeled BR from each other. However, control experiments show that the difference spectra are not reproducible enough. As discussed under Materials and Methods, small deviations in band positions and band intensities produce artificial bands in the difference, which mask the small effects due to [^{13}C]Asp labeling. We chose, therefore, another procedure. Each

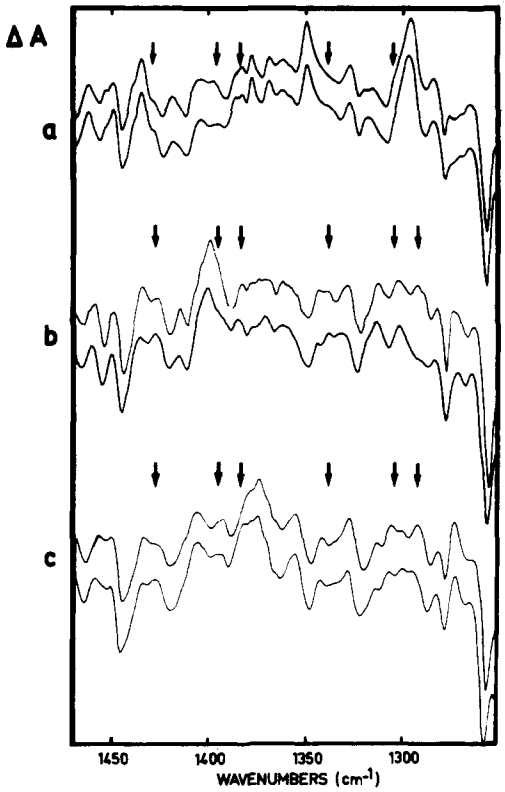

FIGURE 8: Effect of [^{13}C]Asp labeling in the region of the symmetric CO_2^- stretching vibration. (a) Static $BR_{568} - K_{610}$ difference spectrum; (b) static $BR_{568} - L_{550}$ difference spectrum; (c) static $BR_{568} - M_{412}$ difference spectrum. In each pair of difference spectra the upper trace corresponds to unmodified BR and the lower trace to ^{13}C-Asp-BR. Measuring conditions were as for the previous difference spectra. Differences caused by [^{13}C]Asp labeling according to the criteria described in the text are marked by arrows. Spectral resolution, 2 cm^{-1}.

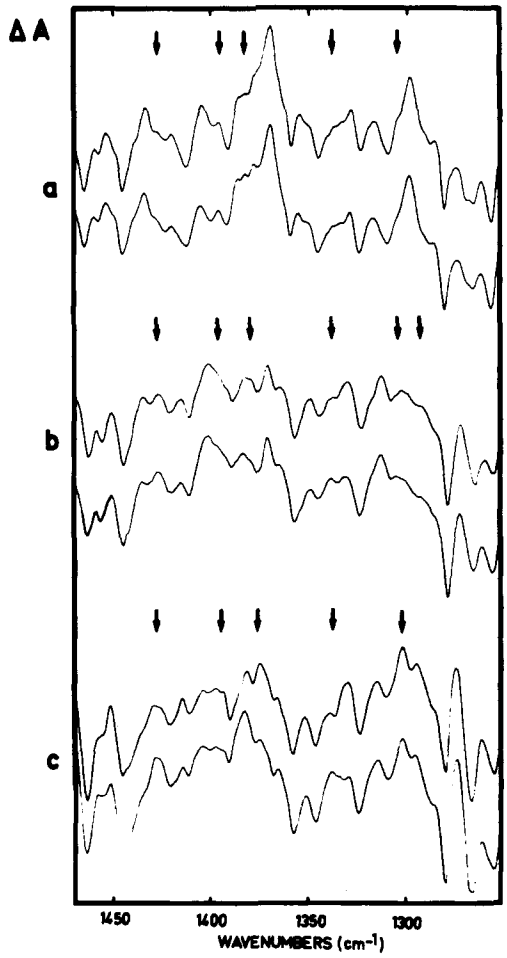

FIGURE 9: Same as Figure 8 but for samples in ^2H_2O.

difference spectrum of labeled and unlabeled BR was measured several times (ca. 5) and each difference spectrum of labeled BR compared with each difference spectrum of unlabeled BR by visual inspection. The equivalent spectra were coadded. Only those spectral changes that were observed for each comparison of the individual spectra and of the coadded spectra were considered as real effects caused by [^{13}C]Asp labeling. Measurements in ^2H_2O provided an additional test. Whereas ^2H_2O causes many changes in this spectral region, ionized carboxylic groups are not influenced. Therefore, a comparison of the difference spectra of labeled and unlabeled BR in ^2H_2O should unveil the effects due to [^{13}C]Asp labeling, though the difference spectra themselves are altered by ^2H_2O.

In applying these criteria to the evaluation of the $BR_{568} - K_{610}$, $BR_{568} - L_{550}$, and $BR_{568} - M_{412}$ difference spectra, we are unable to identify the antisymmetric stretching vibration with a high degree of confidence. We have some indication of an effect of [^{13}C]Asp labeling around 1570 cm^{-1}, but the difficulties outlined above impede a reliable identification. Figure 8 shows an enlarged part of the $BR_{568} - K_{610}$, $BR_{568} - L_{550}$, and $BR_{568} - M_{412}$ difference spectra of BR and ^{13}C-Asp-BR, between 1470 and 1250 cm^{-1}, the spectral region of the symmetric stretching vibration. Figure 9 gives the corresponding spectra of the samples in ^2H_2O. Differences between the spectra of modified and unmodified BR according to the criteria developed above are marked by arrows. Although the spectral changes caused by [^{13}C]Asp labeling are small, their consistency, regardless of whether measured in H_2O or ^2H_2O, provides confidence that deprotonated aspartic acids are reflected. It is remarkable that most positions of effects coincide in the various difference spectra.

DISCUSSION

Whereas clear results have been obtained for the protonated carboxylic groups, difficultiesd arise for the interpretation of the data obtained for the ionized carboxylic acids. The smallness of the effects impedes an unequivocal description in terms of absorbance increase/decrease and band shifts. Further, for the prediction of the isotopic shift caused by [^{13}C]Asp labeling, the degree of asymmetry of the two oxygens resulting from the asymmetric position of the counterion should be known. In the extreme, the shift could be as low as 10 cm^{-1} for the former symmetric vibration (one oxygen bound by a single bond and the other by a double bond). Shifts caused by environmental effects such as displacement of the counterion could encompass the range between 1420 and 1250 cm^{-1}. Therefore, only a qualitative evaluation of the data is presented. Our results clearly provide evidence that ionized aspartic acids are influenced already at the K_{610} intermediate, indicating that they are located in the neighborhood of the chromophore. Four aspartic acids that are protonated at least at some of the intermediates could be identified on the basis of their C=O band. According to the arrangement of the polypeptide chain spanning the membrane (Engelman et al., 1980), there are just four internal aspartic acids. Thus, it is clear that at least one of the aspartic acids undergoes protonation and deprotonation steps. Asp(1), Asp(2), and Asp(3) exhibit carbonyl bands of comparable strength. A mere environmental influence on a protonated carboxylic group would cause a smaller band than a protonation change. Therefore, the result that for one of the aspartic acids protonation changes are observed leads to the conclusion that all three aspartic acids undergo protonation and deprotonation reactions. Asp(1*)

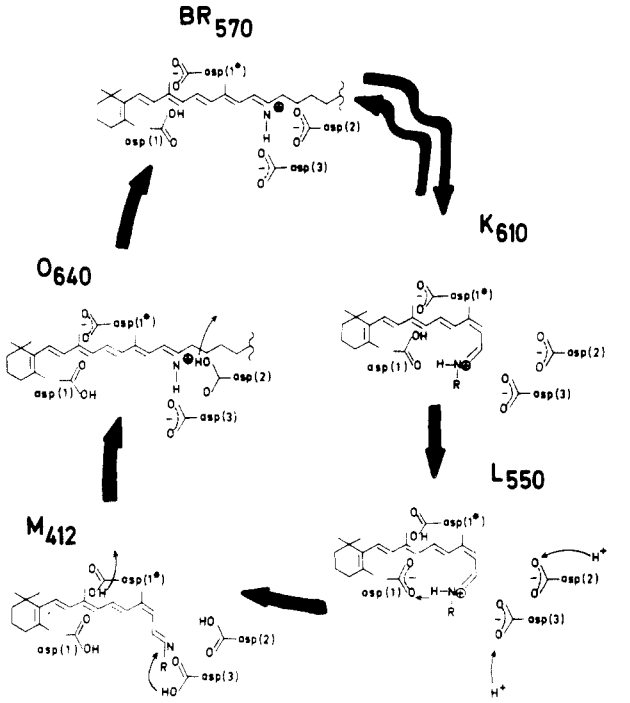

FIGURE 10: Model of the photocycle with the protonation changes of the four aspartic acids. Arrows indicate changes leading to the next intermediate.

causes a smaller but broader band. The integral intensity is of comparable size to that of the other aspartic acids. This would indicate that this group also undergoes protonation changes.

An important result, which may be of relevance for the elucidation of the structure of BR, can be deduced from our measurements: there are at least two protonated carboxylic acids in BR_{568}. These groups must be buried, since they cannot be deprotonated up to pH, 9.5. However, these groups are accessible for protons from the medium, since they can be deuterated by ^2H_2O. The position of the C=O band is indicative of non-hydrogen-bonded carboxylic groups (Bellamy, 1957). In the models for the fit of the amino acid sequence into the seven helices, it is generally assumed that carboxylic acids are deprotonated and, therefore, require a counterion. The importance of polar forces, such as ion pairs and hydrogen bonds, for the structure of membrane proteins has especially been stressed (Engelman, 1982). Our results indicate that these two carboxylic groups are not involved in such a type of structure stabilization.

A model for the molecular events during the photocycle leading to proton pump activity is shown in Figure 10. It is largely based on a model proposed by Schulten & Tavan (1978) and by Schulten et al. (1984). Since ionized aspartic acids are influenced at the BR_{568}–K_{610} transition, which involves the isomerization of the retinal and the concomitant removal of the protonated Schiff base from the counterion, it will be assumed that an aspartic acid represents the counterion. As in the model by Schulten and Tavan, an isomerization about the C13=C14 double bond and the C14—C15 single bond has been assumed. In our model the isomerization has moved the Schiff base in close proximity of the protonated aspartic acid Asp(1). The charge separation without the formation of a new salt bridge explains the red-shifted absorption maximum of K_{610}. However, the proton of Asp(1) is destabilized, leading to the deprotonation in L_{550}. Thus, a new salt bridge is formed, resulting in the blue-shifted absorption maximum of L_{550}. This can partly explain the reduced infrared band intensities of chromophore vibrations in L_{550}, although such a drastic decrease would not have been expected. Therefore, an interaction different from that in BR_{568} has to be assumed. The proton of Asp(1) is transferred to Asp(1*). These proton movements are in agreement with measurements of photoelectric signals (Trissl, 1983; Drachev et al., 1981). A component of the signal was described whose rise time corresponds approximately to that of the L_{550} intermediate, although it was measured in an artificial system. This was confirmed recently by measuring the signal in a suspension of purple membranes (H.-W. Trissl, personal communication).

According to Schulten and co-workers (Schulten & Tavan, 1978; Schulten, 1978; Orlandi & Schulten, 1979; Schulten et al., 1984), the isomerization about the C14–C15 single bond is not complete, thus leaving the bond twisted. This results in a large decrease of the pK of the Schiff base. Thus, it donates the proton to Asp(1) in M_{412}. Since the previous counterion would now represent an isolated negative charge, it also becomes protonated with the deprotonation of the Schiff base. Therefore, Asp(2) is the counterion in BR_{568}. We assume that Asp(2) accepts the proton from the cell interior. This is not in conflict with the observation that the protonation of Asp(2) is observed up to pH 9.5: an isolated ionized carboxylic group without water for solvation and without a counterion represents a state of high energy, which will be lowered by protonation from the aqueous phase even at pH 9.5.

The deprotonation of the Schiff base renders the C14–C15 single bond flexible, thus allowing for the back-isomerization about this bond. In this way the pK of the Schiff base is increased, leading to its protonation with the formation of O_{640}. Since the counterion, Asp(2), is still protonated, the absorption maximum of O_{640} is red-shifted. Resonance Raman experiments provide evidence that the chromophore in O_{640} is all-trans (Smith et al., 1983). Therefore, simultaneously with the protonation of the Schiff base, lowering the rotation barrier of the retinal about the C13=C14 double bond, back-isomerization is occurring. This destabilizes the proton of Asp(2), and with the formation of BR_{568} Asp(2) becomes deprotonated and the original salt bridge between the protonated Schiff base and Asp(2) is reformed.

In our model, the Schiff base accepts the proton from Asp(3), which became protonated somewhat later than Asp(2). Asp(3) is deprotonated in BR_{568} and, thus, requires a counterion. However, since the protonation is observed even at pH 9.5, the pK of Asp(3) has to increase correspondingly. This could be accomplished by a conformational change of the protein, removing the counterion from Asp(3). This change of the protein has to occur before the formation of M_{412}, since no corresponding delay in the protonation of Asp(3) is observed (Siebert et al., 1982).

The protonation of Asp(1*) and Asp(2), which initiate in our model the proton release from the membrane, occurs earlier than the acidification of the medium. Therefore, additional groups mediating between the carboxylic groups and the aqueous phase have to be present. The carboxylic groups involved in the photocycle do not form hydrogen bonds. Protonation and deprotonation of these groups are better described by a hopping mechanism involving rotation of the groups rather than by a model composed of a hydrogen-bonded network (Dunker & Marvin, 1978; Nagle & Morowitz, 1978; Knapp et al., 1980).

The observed effects on the carboxylic groups can only be explained if they are located in the neighborhood of the terminal part of the retinal since most of the charge movements

occur in this region. This provides considerably constraints on the models proposed by Trewhella et al. (1983). Further constraints are obtained if the effects of modification of tyrosine-26 and tyrosine-64 (Lemke & Oesterhelt, 1981; Lemke et al., 1982) are taken into consideration and if lysine-41 is assumed to be in the neighborhood of the retinal binding site.

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Registry No. Asp, 56-84-8.

REFERENCES

Bagley, K., Dollinger, G., Eisenstein, L., Singh, A. K., & Zimanyi, L. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 4972–4976.

Bellamy, L. J. (1957) *The Infrared Spectra of Complex Molecules*, 2nd ed., Methuen, London.

Birge, R. R., & Hubbard, L. M. (1981) *Biophys. J. 34*, 517–534.

Birge, R. R., & Cooper, T. M. (1983) *Biophys. J. 42*, 61–69.

Bogomolni, R. A., Baker, R. A., Lozier, R. H., & Stoeckenius, W. (1980) *Biochemistry 19*, 2152–2159.

Braiman, M., & Mathies, R. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 403–407.

Drachev, L. A., Kaulen, A. D., Khitrina, L. V., & Skulachev, V. P. (1981) *Eur. J. Biochem. 117*, 461–470.

Dunker, A. K., & Marvin, D. A. (1978) *J. Theor. Biol. 72*, 9–16.

Engelman, D. M. (1982) *Biophys. J. 37*, 187–188.

Engelman, D. M., Henderson, R., McLachland, A. D., & Wallace, B. A. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 2023–2027.

Govindjee, R., Ebrey, T. G., & Crofts, R. (1980) *Biophys. J. 30*, 231–242.

Herz, J. M., Mehlhorn, R. J., & Packer, L. (1983) *J. Biol. Chem. 258*, 9899–9907.

Honig, B. (1978) in *Energetics and Structure of Halophilic Microorganisms* (Caplan, S. R., & Ginzburg, M., Eds.) pp 109–121, Elsevier/North-Holland Biomedical Press, New York.

Honig, B., Ebrey, T. G., Callender, R. H., Dinur, U., & Ottolenghi, M. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 2503–2507.

Kalisky, O., Ottolenghi, M., Honig, B., & Korenstein, R. (1981) *Biochemistry 20*, 649–655.

Knapp, E.-W., Schulten, K., & Schulten, Z. (1980) *Chem. Phys. 46*, 215–229.

Kuschmitz, D., & Hess, B. (1982) *FEBS Lett. 138*, 137–140.

Lemke, H.-D., & Oesterhelt, D. (1981) *Eur. J. Biochem. 115*, 595–604.

Lemke, H.-D., Bergmeyer, J., Straub, J., & Oesterhelt, D. (1982) *J. Biol. Chem. 257*, 9384–9388.

Mäntele, W. (1982) Thesis, University of Freiburg, FRG.

Mäntele, W., Siebert, F., & Kreutz, W. (1980) *FEBS Lett. 128*, 249–254.

Mäntele, W., Siebert, F., & Kreutz, W. (1982) *Methods Enzymol. 88*, 729–740.

Motto, M. G., Sheves, M., Tsajimoto, K., Balogh-Nair, V., & Nakanishi, K. (1980) *J. Am. Chem. Soc. 102*, 7945–7947.

Mowery, P. C., & Stoeckenius, W. (1981) *Biochemistry 20*, 2302–2306.

Nagle, J. F., & Morowitz, H. J. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 295–302.

Nagle, J. F., & Mille, M. (1981) *J. Chem. Phys. 74*, 1367–1372.

Nakanishi, K., Balogh-Nair, V., Arnaboldi, M., Tsajimoto, K., & Honig, B. (1980) *J. Am. Chem. Soc. 102*, 7947–7949.

Oesterhelt, D., & Stoeckenius, W. (1974) *Methods Enzymol. 31A*, 667–678.

Onishi, H., McCance, M. E., & Gibbons, N. E. (1965) *Can. J. Microbiol. 11*, 365–373.

Orlandi, G., & Schulten, K. (1979) *Chem. Phys. Lett. 64*, 370–374.

Pettei, M. J., Yudd, A. P., Nakanishi, K., Henselman, R., & Stoeckenius, W. (1977) *Biochemistry 16*, 1955–1958.

Pinchas, S., & Laulicht, I. (1971) *Infrared Spectra of Labeled Compounds*, Academic Press, London and New York.

Renard, M., & Delmelle, M. (1980) *Biophys. J. 32*, 993–1006.

Rothschild, K. J., & Marrero, H. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 4045–4049.

Rothschild, K. J., Zagaseki, M., & Cantore, W. (1981) *Biochem. Biophys. Res. Commun. 103*, 483–489.

Schulten, K. (1979) in *Energetics and Structure of Halophilic Microorganisms* (Caplan, S. R., & Ginzburg, M., Eds.) pp 331–334, Elsevier/North-Holland Biomedical Press, New York.

Schulten, K., & Tavan, P. (1978) *Nature (London) 272*, 85–86.

Schulten, K., Schulten, Z., & Tavan, P. (1984) in Proceedings of the 10th International Conference on Biological Membranes, Information and Energy Transduction in Biological Membranes, Allan R. Liss, New York (in press).

Siebert, F., & Mäntele, W. (1980) *Biophys. Struct. Mech. 6*, 147–164.

Siebert, F., & Mäntele, W. (1983) *Eur. J. Biochem. 130*, 565–573.

Siebert, F., Mäntele, W., & Kreutz, W. (1981) *Can. J. Spectrosc. 26*, 119–125.

Siebert, F., Mäntele, W., & Kreutz, W. (1982) *FEBS Lett. 141*, 82–87.

Smith, S. O., Pardoen, J. A., Mulder, P. P. J., Curry, B., Lugtenburg, J., & Mathies, R. (1983) *Biochemistry 22*, 6141–6148.

Stoeckenius, W., & Bogomolni, R. A. (1982) *Annu. Rev. Biochem. 51*, 587–616.

Trewhella, J., Anderson, S., Fox, R., Gogol, E., Khan, S., Engelman, D., & Zaccai, G. (1983) *Biophys. J. 42*, 233–241.

Tsuda, M., Glaccum, M., Nelson, B., & Ebrey, T. G. (1980) *Nature (London) 287*, 351–353.

Warshel, A. (1979) *Photochem. Photobiol. 30*, 285–290.