Two Progressive Substates of the M-intermediate Can Be Identified in Glucose-Embedded, Wild-Type Bacteriorhodopsin

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ABSTRACT Glucose-embedded bacteriorhodopsin shows M-intermediates with different Amide I infrared bands when samples are illuminated at 240 or 260 K, in contrast with fully hydrated samples where a single M-intermediate is formed at all temperatures. In hydrated, but not in glucose-embedded specimens, the N intermediate is formed together with M at 260 K. Both Fourier transform infrared and electron diffraction data from glucose-embedded bacteriorhodopsin suggest that at 260 K a mixture is formed of the M-state that is trapped at 240 K, and a different M-intermediate (M_N) that is also formed by mutant forms of bacteriorhodopsin that lack a carboxyl group at the 96 position, necessary for the M to N transition. The fact that an M_N species is trapped in glucose-embedded, wild-type bacteriorhodopsin suggests that the glucose samples lack functionally important water molecules that are needed for the proton transfer from aspartate 96 to the Schiff base (and, thus, to form the N-intermediate); thus, aspartate 96 is rendered ineffective as a proton donor.

INTRODUCTION

Bacteriorhodopsin is a light-driven proton pump in *Halobacterium halobium*. It contains a retinal prosthetic group, which is linked to a lysine residue by a protonated Schiff base. *Trans-cis* isomerization of the retinal by light starts a photocycle that can be represented as bR→K→L→M→N→O→bR (Lozier et al., 1975). The essential steps are the release of the Schiff base proton to the external medium and subsequent reprotonation from the cytoplasmic side. The M intermediate is the only one that has a deprotonated Schiff base; the proton is released to Asp-85 in the L→M transition (Otto et al., 1990), and reprotonation from Asp-96 occurs upon formation of N (Otto et al., 1989). During the lifetime of M, a structural event is believed to occur that ensures that reprotonation of the Schiff base can only occur from the cytoplasmic side. Kinetic studies showed the existence of two substates of M, M_1, and M_2, linked by an irreversible step (Váró and Lanyi, 1991a), which was confirmed by the occurrence of a shift in the position of the absorption maximum of M in the photocycle of solubilized bR (Váró and Lanyi, 1991a) and some mutants (Zimányi et al., 1992; Váró et al., 1992). It is possible that this irreversible step involves a protein conformational change that ensures that reprotonation can only occur from the cytoplasmic side.

The protein structure of bacteriorhodopsin, which forms a highly ordered crystal in the halobacterial purple membrane, is known in the light-adapted, resting state from the electron crystallographic work of Henderson et al. (1990). In addition to confirming a great many structural inferences that had been drawn from spectroscopic experiments, site-directed mutagenesis, and functional studies, the crystallographic structure analysis revealed that the resting state conformation has an aqueous cavity or vestibule on the outward-facing (extracellular) side of the protein, in contact with Asp-85 and extending nearly to the protonated Schiff base.

The availability of the structure factor phases (Henderson et al., 1990) also makes it possible to study easily small structural changes in the photocycle intermediates by difference Fourier methods. Several studies of the changes in the projected protein structure upon formation of the M intermediate have been published, using electron diffraction (Glaeser et al., 1986; Subramaniam et al., 1993), x-ray diffraction (Koch et al., 1991; Nakasako et al., 1991), or neutron diffraction (Dencher et al., 1989). The highest resolution was obtained in the electron diffraction study of frozen hydrated purple membrane by Subramaniam et al. (1993). M-bR difference maps of the D96G mutant protein, which has a prolonged lifetime of M because of absence of a group to reprotonate the Schiff base, showed substantial protein changes. Some features of the structure are reproduced in the other studies. However, it is not clear which M-substate is trapped in each of these studies. The D96G mutant employed by Subramaniam et al. (1993) probably traps predominantly the last intermediate before the blocked transition to N, but it is not ruled out that a certain amount of an earlier M species is present. In the other studies, the situation is even less clear. For a structural study of the photocycle intermediates, it would be desirable to use a well characterized sample with minimum contributions of all but one intermediate. Perkins et al. (1992, 1993) have proposed a method to trap the two

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different M-substates in glucose-embedded samples suitable for electron crystallography.

However, uncertainty has recently arisen about the possibility of trapping the two putative substates of M at low temperature. An initial FTIR study by Ormos (1991) suggested that M intermediates with different protein structures could be trapped at 240 and 260 K, although the 260 K intermediate was contaminated by a certain amount of N. Subsequent work by Perkins et al. (1992) showed virtually the same FTIR spectra in glucose-embedded purple membrane, hydrated sufficiently to allow formation of M at low temperature (Perkins et al., 1993) and, in addition, it was shown by visible spectroscopy that there was no significant contamination by other intermediates (L or N). However, Ormos et al. (1992) showed in an elegant experiment that the differences between the 240 and 260 K spectra in the earlier study, with fully hydrated specimens (Ormos, 1991), were entirely caused by contamination of a single M species by the N or L intermediate. Ormos et al. used blue light to selectively drive M (the only blue-absorbing intermediate), back to the ground state. Subtracting the resulting spectrum (containing ground-state bR and possibly non-M intermediates) from the spectrum recorded after only green illumination (containing M and the same contaminants) yields a spectrum of pure M. This spectrum was identical at all temperatures from 230 to 270 K and similar to the earlier 240 K M spectrum. In addition, there was a second component that remained after the blue-light photoreaction, which was identified as L at low temperatures and as N at higher temperatures. The 260 K spectrum could thus be explained as a mixture of this single M species and the N intermediate.

We repeated the blue-light reversal experiment of Ormos et al. (1992) in glucose-embedded, humidified samples to explain the finding by Perkins et al. (1992) that glucose-embedded samples at 240 or 260 K are not contaminated by non-M intermediates, while having the same FTIR spectra as mixtures that are produced in fully hydrated samples. We show that at 240 K a pure M is trapped with a spectrum similar to the one found by Ormos et al. (1992) at all temperatures, whereas at 260 K a mixture is obtained of this M and another M intermediate, which has the same FTIR spectrum as N. In the accompanying paper (Han et al., 1994), difference maps from electron diffraction studies of these two M samples are presented; by comparing the data reported there with the electron diffraction data reported by Subramaniam et al. (1993), we get additional evidence that the M-state intermediate found in glucose-embedded samples is a mixture of the M-state trapped at 240 K plus an M-intermediate whose protein structure is very similar to that of the N-state intermediate.

MATERIALS AND METHODS

Purple membranes were isolated and purified from *Halobacterium halobium* strain ET1001 according to the protocol of Oesterhelt and Stoeckenius (1971). Glucose-embedded samples, hydrated at 81% humidity, were prepared on CaF_2 windows as described by Perkins et al. (1992). FTIR spectroscopy was performed at 2 cm^{-1} resolution on a Mattson Galaxy 3000 FTIR spectrometer (Mattson Instruments, Madison, WI) equipped with a home-made cryostat, in which the sample temperature could be kept constant to within 0.5 K by purging with cold nitrogen gas. Light-adapted samples were cooled to 240 or 260 K in the dark, and bR background scans were taken. Then the sample was illuminated through a green filter (filter 59820, Oriel Corporation, Stratford, CT) for 20 s to produce M, and the resulting FTIR spectrum was collected after a 20 s waiting period in the dark, or after a 20-s illumination through a blue filter (filter 59850, Oriel Corporation). Spectra were collected for 30 s at 260 K or 150 s at 240 K, to allow for the faster decay at higher temperature (Ormos, 1991; Perkins et al., 1993). After this, the system was heated to room temperature to complete the photocycle before a next series of scans was started. The spectra are averages of about 3000 scans.

RESULTS AND DISCUSSION

To check the presence of non-M intermediates produced in our sample preparation method, we used the M-photoreversal method introduced by Ormos et al. (1992). First, bR background scans were taken. Then the photocycle was started with green light. Subsequently, the M-intermediates were removed with blue light. Scans of the green-illuminated

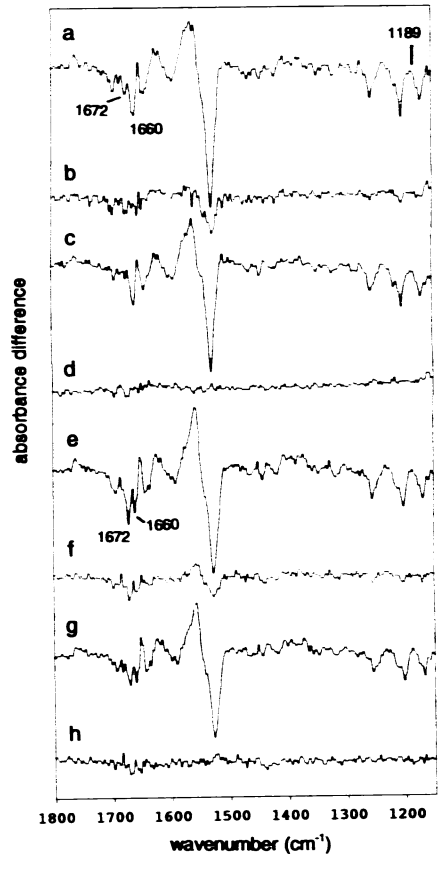

FIGURE 1 FTIR difference spectra that have been used to determine the presence of non-M intermediates in purple membrane-illuminated at 240 K (*a–d*) and 260 K (*e–h*). (*a, e*) Difference spectrum after green illumination to form M. (*b, f*) Difference spectrum after green illumination and blue illumination to photoreverse most of the M. (*c, g*) Pure M spectrum calculated from the difference between only green and green plus blue illuminated samples. (*d, h*) Spectrum of possible non-M intermediate, calculated by subtracting *c* from *a* (*d*) and *g* from *e* (*h*) in a ratio to minimize a typical M band (1762 cm^{-1}).

sample were taken after a waiting period equal to the blue illumination, to eliminate effects of decay.

The results at 240 K are presented in the top half of Fig. 1. After green illumination, a difference spectrum similar to published spectra (Ormos, 1991; Perkins et al., 1992) was obtained (Fig. 1 *a*), which we call M_{240K}. The difference spectrum after green and blue illumination had a much lower amplitude but showed the same peaks (Fig. 1 *b*). Because non-M intermediates are not affected by blue light, the difference between these two spectra will show a pure M spectrum. This spectrum, shown in Fig. 1 *c*, has again the same features and is similar to the pure M spectrum published by Ormos et al., characterized by a large negative peak at 1660 cm^{-1}, and a smaller negative peak at 1672 cm^{-1} in the Amide I region. The most characteristic peaks to distinguish L from M are a negative peak at 1742 cm^{-1}, representing Asp-96 (Braiman et al., 1988; Braiman et al., 1991; Maeda et al., 1992), and a positive peak at 1189 cm^{-1} (Braiman et al., 1991), which are present in L but not in M. There is no indication of these peaks in the possible mixture of Fig. 1 *a*. Subtracting Fig. 1 *c* (pure M) from Fig. 1 *a* (a mixture?) with a scaling factor to minimize typical M peaks should give a spectrum for a non-M intermediate, if it is present. All peaks disappear together in this process, however, and the resulting spectrum, shown in Fig. 1 *d*, consists of random noise without any indication of the peaks characterizing L. We conclude, therefore, that under our experimental conditions pure M is trapped at 240 K.

The results of the same experiment at 260 K are shown in the bottom part of Fig. 1. Just as at 240 K, the difference spectrum after only green illumination, which we call M_{260K} (Fig. 1 *e*), after green and blue illumination (Fig. 1 *f*), and the difference between these two (Fig. 1 *g*) all show similar features. This spectrum is characterized by a large negative peak at 1672 cm^{-1} and a smaller one at 1660 cm^{-1}, like the 260 K spectra published by Ormos (1991) and Perkins et al. (1992). Again, the mixture minus pure M (Fig. 1 *h*) shows noise only without any indication of typical N peaks, like the positive peak at 1186 cm^{-1} (Fodor et al., 1988; Gerwert et al., 1990; Pfefferlé et al., 1991). This finding confirms the visible spectroscopy data from Perkins et al. (1992), which also did not show significant amounts of non-M intermediates, but it is strikingly different from the result of Ormos et al. (1992), who could separate their 260 K spectrum into an M and an N fraction, with the M spectrum indistinguishable from the 240 K spectrum.

The explanation for this difference must lie in the use of different sample preparation conditions. Although Ormos' samples are fully hydrated, our glucose-embedded samples are only partially hydrated (Perkins et al., 1993). It is well known that the hydration level has a profound influence on the photocycle. Specifically, the M_1 to M_2 step as well as the M to N step are slowed in partially dehydrated samples (Pfefferlé et al., 1991; Váró and Lanyi, 1991b; Cao et al., 1991), whereas glucose-embedded samples do not form M at all at low temperature without taking precautions against dehydration (Perkins et al., 1993). In our case, the partial dehydration seems to inhibit the M to N step at 260 K, because in otherwise similar conditions the fully hydrated samples used by Ormos et al. (1992) form N, whereas the humidified, glucose-embedded samples form only M.

Sasaki et al. (1992) recently showed the existence of an intermediate (which they called M_N) that has the protein structure (i.e., FTIR Amide I difference spectrum) of N but a deprotonated Schiff base, indicative of M. This intermediate was trapped in the D96N mutant at high pH. Apparently, the protein structural change giving rise to the N intermediate had taken place in this sample, but because of a lack of a proton to reprotonate the Schiff base, the retinal remained in the deprotonated configuration specific for M.

If we assume that this M_N intermediate is trapped in our humidified, glucose-embedded samples as well, we can now reconcile our results quite easily with those of Ormos et al. (1992). The only difference would be that our 260 K samples contain a mixture of M and M_N, whereas the samples in Ormos' experiments contained a mixture of M and N. In our case, then, unlike that of Ormos et al. (1992), all bR molecules could be photoreversed by blue light, which is absorbed by the retinal containing a deprotonated Schiff base.

To get an estimate of the relative contributions of M and M_N in our 260 K spectra, we made linear combinations of the "separated" M and N spectra from Ormos et al. (1992) and compared the combinations with our 260 K data. The main difference between the M_{260K} data and the M/N combinations is the much reduced separation of the 1660 and 1672 cm^{-1} Amide I bands in the latter. This can probably be attributed to the fact that Ormos's spectra are averages of spectra taken at different temperatures and whose peak positions, therefore, are shifted because of the difference in temperature. The averaging of such slightly different spectra might have blurred some high resolution features. This apparent blurring makes it impossible to estimate the relative contributions of M and M_N accurately. "Smoothing" of the original data (Fig. 2 *d*) by averaging over several data point tends to reduce the separation of the two peaks and makes the comparison easier (Fig. 2 *e*). The best fit seems then to be at 60% M, 40% N (Fig. 2 *a*), when the relative heights of the 1660 and 1672 cm^{-1} Amide I bands are compared. Differences exist, as expected, in the absence of a positive 1186 cm^{-1} peak and a 1742 cm^{-1} negative peak in our data, which are caused by the protonated Schiff base and deprotonated Asp-96 in N, respectively, and so should occur in an M/N but not in an M/M_N mixture. On the basis of the data above, we believe that in illuminated, glucose-embedded samples at 240 K all molecules are in an M state similar to the one that Ormos et al. (1992) have shown to be the only M intermediate that could be detected between 230 and 270 K, whereas at 260 K approximately half of the molecules are still in this state, but the other half have progressed to M_N.

The possibility that our M_{260K} sample is a mixture of M and M_N substates is further supported by electron diffraction data. If we assume that some M_N intermediate is trapped in our glucose-embedded samples at 260 K, and that the M-state of the D96G sample used by Subramaniam et al. (1993) is a pure

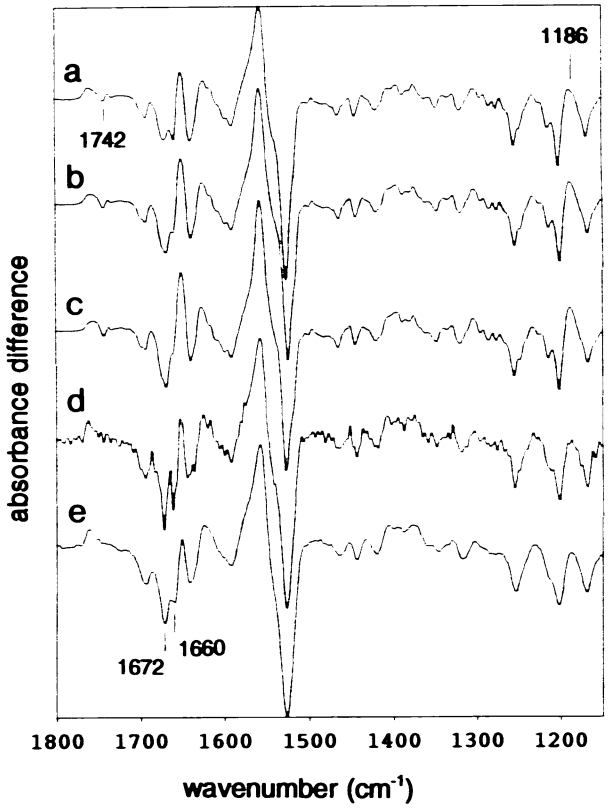

FIGURE 2 Comparison of M_{260K} FTIR data of glucose-embedded samples with mixtures of M and N difference spectra. Linear combinations of the absorbance differences of pure M-bR and N-bR from Ormos et al. (1992) are shown in ratios M/N = 80/20 (*a*), 60/40 (*b*), 40/60 (*c*). *d* represents the M-bR at 260 K from Fig. 1 *e*. *e* is the same data as *d*, after boxcar averaging over 9 data points.

M_N (like that of D96N), then we should expect that a linear combination of the diffraction data of Han et al. (1994) for the M_{240K} sample plus the diffraction data for the mutant bR M-state should correspond well with the diffraction data for the M_{260K} sample. Fig. 3 shows that the correlation coefficient between the M_{260K} diffraction data, and the linear combination described above takes on a maximum value of 0.78 at a ratio of 48% mutant M and 52% M_{240K}. This maximum is much higher than the correlation coefficients of pure M_{mutant} and M_{240K} with M_{260K}, which are 0.69 and 0.58, respectively. This indicates a good correspondence, as we had expected. The optimal ratio is furthermore similar to the ratio that best accounts for the M_{260K} FTIR difference spectrum as a mixture of M and N FTIR spectra, confirming the previous conclusion that our M_{260K} sample is a mixture of M_{240K} and M_N.

Although there are many possible reasons why our humidified, glucose-embedded samples are not able to reprotonate the Schiff base (at 260 K), once the M_N intermediate has been formed, we would like to mention two alternative models that seem to be especially worth considering. The first model proposes that the cytoplasmic half of the proton-transport channel normally contains a string of continuously hydrogen-bonded water molecules, which serve as the "proton wire" to transfer the proton from Asp-96 to the deprotonated Schiff base (Nagle, 1987; Deamer and Nichols, 1989). If as few as one of these water molecules would be removed in the glucose-embedded state, reprotonation of the Schiff base would no longer be possible. However, it has been shown by Cao et al. (1991), in an analysis of osmotic effects on the photocycle, that much more water is essential for the reprotonation step than the amount that would form a single, 12 Å long line of water molecules. The second model is inspired by the suggestion of Subramaniam et al. (1993) that a widening of the structure occurs on the cytoplasmic surface in the M-state of the D96G mutant, which we now believe to be the N-like (i.e., M_N) protein conformation. In this model, the M to M_N transition comprises the opening of a cleft or channel where water can flow in and make the proton transfer possible from Asp-96 to the Schiff base. The opening of a cleft on the cytoplasmic side, to admit access to water molecules, has also been proposed on the basis of neutron scattering data (Dencher et al., 1992). If no free water is available when the M_N state is formed, as might

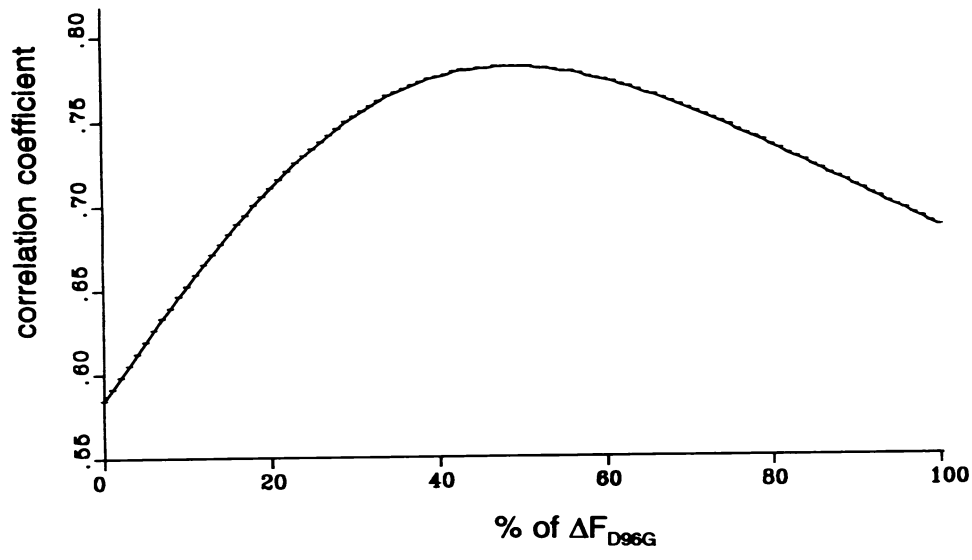

FIGURE 3 Correlation coefficients of M-bR electron diffraction amplitude data. Linear combinations were made of the M-bR difference amplitudes, ΔF_{M240K} (Han et al., 1994) and ΔF_{MD96G} (Subramaniam et al., 1993). Correlation coefficients were calculated between the mixtures and ΔF_{M260K} (Han et al., 1994).

be the case in our glucose-embedded samples, proton transfer from Asp-96 to the Schiff base could not occur. This second model is attractive in that: (1) The aqueous hydration of Asp-96 that would occur provides a natural explanation for the large decrease in pK_a, which causes the carboxylic acid group of Asp-96 to dissociate; (2) direct, aqueous communication between the cytoplasmic side of the protein and the Schiff base, postulated in this model, explains how the Schiff base can be reprotonated in the D96N mutant, and why the rate of that reprotonation depends upon the bulk pH (Holz et al., 1989; Cao et al., 1991); (3) creation of a water-filled channel on the cytoplasmic side in the N-state can be easily pictured as allowing vectorial release of Cl^- to the cytoplasm in the halorhodopsin photocycle.

What is the relationship of the M intermediates found in our experiments to the kinetically and spectroscopically identified M_1 and M_2 intermediates in the photocycle? Sasaki et al. (1992) suggest that the M intermediate that occurs together with M_N represents the M_2 intermediate and M_N is a normally unstable intermediate between M_2 and N. Evidence for this interpretation is the apparent equilibrium between M and M_N as seen in the decay of the D96N sample (Sasaki et al., 1992). If this interpretation is correct, the photocycle around the M-intermediate can be described as $L \leftrightarrows M_1 \rightarrow M_2 \leftrightarrows M_N \leftrightarrows N$. The M state that is normally seen in FTIR spectra would then be M_2. This is supported by the observation that in the few experiments in which different M substates are resolved, M_1 appears as a transient intermediate during the rise of M (Váró and Lanyi, 1991a; Zimányi et al., 1992; Váró et al., 1992).

One can speculate on the possibility that M_1 (which has never been resolved in FTIR experiments) has a similar protein structure as L, essentially only differing in the protonation states of Asp-85 and the Schiff base, and could be named "M_L" analogous with M_N in the nomenclature of Sasaki et al. (1992). The resulting photocycle can be represented as $bR \rightarrow K \leftrightarrows L \leftrightarrows M_L \rightarrow M \leftrightarrows M_N \leftrightarrows N \leftrightarrows O \rightarrow bR$. During the light-induced $bR \rightarrow K$ reaction, the retinal isomerizes from the all-*trans* to the 13-*cis* form (Braiman and Mathies, 1982). In $K \rightarrow L$, the protein relaxes to accommodate the changed geometry of the retinal, in a way that changes the pK_a of the Schiff base and/or Asp-85 (Váró and Lanyi, 1991c). Subsequently, in the $L \rightarrow M_L$ step, the Schiff base proton is transferred to Asp-85. This is followed by the $M_L \rightarrow M$ (or in the conventional nomenclature $M_1 \rightarrow M_2$) conformational change which, in our model, closes the outward facing proton channel. This involves withdrawal of Asp-85 from the aqueous compartment, thus stabilizing the neutral, protonated carboxyl group. In M, the deprotonated Schiff base is consequently not accessible from either side, but in the $M \rightarrow M_N$ step an inward facing channel opens and the inflowing water allows transport of the now destabilized Asp-96-proton to the Schiff base. In $N \rightarrow O$ the retinal reisomerizes (Smith et al., 1983) and the inward facing channel closes again, reprotonating Asp-96 (Heßling et al., 1993) and in $O \rightarrow bR$ the outward facing channel reopens, Asp-85 deprotonates and the protein relaxes to the ground state (Souvignier and Gerwert, 1992; Heßling et al., 1993). The idea that active transport (and exchange transport) membrane proteins should cycle between inward facing and outward facing conformations is, of course, well established and, indeed, the first models that postulate cyclic access of the Schiff base of bR to opposite sides of the membrane have been proposed several years ago (Stoeckenius, 1979; Nagle and Mille, 1981).

The possibilities to study the part of the photocycle around the M-intermediate and test the model described above by direct structural methods are promising. We have shown here that at 240 K it is possible to trap a pure M-intermediate (which may be M_2). The results of an electron diffraction study in projection using these conditions are presented in the companion paper (Han et al., 1994). Subramaniam et al. (1993) have used the D96G mutant (which accumulates M because of the absence of a proton donor for the Schiff base) to study the M intermediate by electron diffraction. It seems likely that they, like Sasaki et al. (1992) with the D96N mutant protein, have trapped predominantly the M_N intermediate, which is N-like in protein structure (see Fig. 2 *b*). If the mutant M-state, indeed, is not significantly contaminated by M, it will be more useful than our M_{260K} mixture for getting diffraction data on the N-like protein conformation, provided that the problem of getting adequate specimen flatness can be solved. The data presented by Han et al. (1994) show the existence of a substantial conformational change in the M to M_N step. If it can be shown that M_1 has a protein structure similar to L, trapping of L might be a way to study the changes associated with the M_1 to M_2 transition.

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