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Review

# Crystallographic analysis of protein conformational changes in the bacteriorhodopsin photocycle

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**Abstract**

A variety of neutron, X-ray and electron diffraction experiments have established that the transmembrane regions of bacteriorhodopsin undergo significant light-induced changes in conformation during the course of the photocycle. A recent comprehensive electron crystallographic analysis of light-driven structural changes in wild-type bacteriorhodopsin and a number of mutants has established that a single, large protein conformational change occurs within 1 ms after illumination, roughly coincident with the time scale of formation of the $M_2$ intermediate in the photocycle of wild-type bacteriorhodopsin. Minor differences in structural changes that are observed in mutants that display long-lived $M_2$, N or O intermediates are best described as variations of one fundamental type of conformational change, rather than representing structural changes that are unique to the optical intermediate that is accumulated. These observations support a model for the photocycle of wild-type bacteriorhodopsin in which the structures of the initial state and the early intermediates (K, L and $M_1$) are well approximated by one protein conformation in which the Schiff base has extracellular accessibility, while the structures of the later intermediates ($M_2$, N and O) are well approximated by the other protein conformation in which the Schiff base has cytoplasmic accessibility. 

*Keywords:* Electron microscopy; Time-resolved crystallography

## 1. Introduction

The active transport of ions by a membrane protein must involve, at a minimum, a mechanism that allows the protein to cycle through two distinct conformations in which the active site is exposed alternately to one or the other side of the membrane. In contrast to proteins that lack an internal chromophore, in bacteriorhodopsin (Fig. 1), the retinylidene moiety which is attached to the protein via a protonated Schiff base linkage provides a potentially powerful spectroscopic marker for following different stages in the proton transport process. It is now well established [1] that illumination results in the formation of at least five spectroscopically distinct intermediates: the K, L, M, N, and O intermediates. Retinal isomerization is essentially complete at the K intermediate stage, release of a proton from the Schiff base to the extracellular side occurs with formation of the M intermediate, reprotonation of the Schiff base from the cytoplasmic side of the membrane occurs with formation of the N intermediate,

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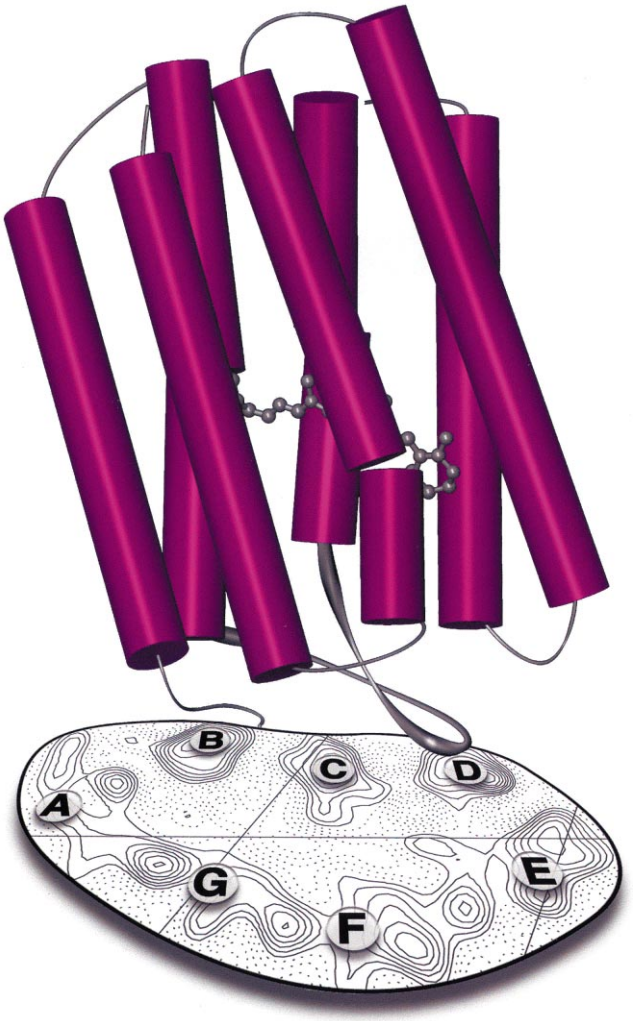


Fig. 1. A simplified schematic representation of bacteriorhodopsin and a projection view of the structure at 3.5 Å from the cytoplasmic side. The approximate positions of the helices, as well as the approximate boundary of the molecule are indicated.

and finally, proton uptake from the cytoplasmic medium and retinal re-isomerization occur over the timescale of the O intermediate, returning retinal and the protein to their starting states.

Determination of the nature, extent and relevance of protein conformational changes at different stages of the bacteriorhodopsin photocycle has remained a problem of long-standing and central interest in the bacteriorhodopsin field. Considerable progress has been made in the last few years towards this goal starting with the early neutron [2], X-ray [3–5] and electron diffraction [6–8] experiments which first established that significant light-induced protein conformational changes occurred during the photocycle. In this review, we summarize findings from more recent crystallographic studies on wild-type bacteriorhodopsin and a variety of mutants, which have provided new and definitive insights into structural changes in the photocycle and into the molecular basis of vectorial proton transport.

## 2. The K and L intermediates

The isomerization of retinal, and the formation of the K intermediate occur within 1 ps after illumination, and the formation of the L intermediate follows within approx. 1 μs. Since the K intermediate is formed following illumination even at liquid nitrogen temperatures, it is reasonable to expect that its formation would involve only small rearrangements in protein conformation. This expectation has been confirmed by electron crystallographic studies with two-dimensional crystals. A 3.5 Å map projection difference Fourier map [9] calculated using electron diffraction amplitudes obtained crystals illuminated at near liquid nitrogen temperatures (−170°C) shows no features above the noise level. A similar set of electron diffraction experiments aimed at determining the structural changes at the L intermediate stage [10] by trapping crystals following illumination at −100°C also showed no detectable structural changes compared to the unilluminated state.

While the electron diffraction experiments to study the K and L intermediates have only provided projection maps, a more detailed insight into the extent of the structural changes in the early stages of the photocycle has come from X-ray crystallographic analysis of three-dimensional crystals illuminated at liquid nitrogen temperatures. Edman et al. [11] have recently reported a model for the K intermediate using diffraction data at 2.0 Å resolution obtained from illuminated three-dimensional crystals. The largest feature in the three-dimensional difference map has been interpreted to correspond to the loss of a water molecule near the Schiff base, with minimal changes observed in either the protein or the retinylidene moiety. Although it is somewhat unlikely that a pure K intermediate (with no contamination from the L intermediate) was obtained under the conditions of these experiments, they nevertheless

provide an upper limit for the extent of protein conformational changes in the early (K and L) stages of the photocycle.

## 3. The M intermediate

Structural changes at the M intermediate have been the focus of a very large number of investigations. The first of these studies was reported in the early work of Glaeser et al. [12] who determined the structural changes associated with the formation of a yellow intermediate obtained by illumination of two-dimensional crystals. Since no significant protein conformational changes were observed, it was concluded that the formation of the M intermediate was not associated with measurable protein conformational changes. This conclusion was, however, in error. It is now clear that because of the low humidity in the glucose-embedded crystalline specimens, light-driven structural changes were inhibited [13–15] despite the fact that the spectrum of the trapped intermediate was identical to that of the M intermediate that is accumulated under physiological conditions. It has become common now to refer to the M intermediate that accumulates at low hydration levels and without displaying the large conformational change as the $M_1$ intermediate, and the M intermediate that accumulates at higher hydration levels with the large protein conformational change as the $M_2$ intermediate. In the normal course of the photocycle at physiological conditions, the $M_1$ intermediate is only transiently formed since the equilibrium is shifted almost completely in favor of the $M_2$ intermediate.

Dencher et al. [2] first demonstrated the existence of significant light-induced conformational changes in fully hydrated bacteriorhodopsin by analyzing powder neutron diffraction patterns obtained from oriented stacks of two-dimensional crystals, at alkaline pH and proposed the potential involvement of movements of helix F in these conformational changes. Similar results were reported by Koch et al. [3], and Nakasako et al. [4] using powder X-ray diffraction analysis, of hydrated, oriented stacks of two-dimensional crystals, followed also later by Han et al. [7] using electron diffraction. These neutron and X-ray diffraction studies resulted in projection maps at 7 Å resolution of structural changes at the M intermediate stage of the photocycle. The conclusions from these studies were confirmed and extended by time-resolved electron diffraction studies of the M intermediate in fully hydrated two-dimensional crystals at physiological pH. The difference Fourier maps derived from the electron crystallographic studies [6], with a resolution of 3.5 Å resolution in the plane and less than 20 Å in the perpendicular direction, firmly establish that formation of the M intermediate (i.e. $M_2$ intermediate) involves significant structural rearrangements in helices F and G. An attempt to determine structural changes at the M intermediate stage from diffraction patterns collected from a yellow intermediate obtained by illumination of freeze-thawed three-dimensional crystals has also been recently reported [16]. As with the electron diffraction experiments of Glaeser et al. [12], no significant changes in protein conformation were observed, although Luecke et al. [16] report that helices F and G appear to be more disordered in the illuminated crystals. A likely explanation is that packing forces in the three-dimensional crystal inhibit the structural change which occurs under physiological conditions in the bilayer membrane.

## 4. Structural changes at the $M_2$ vs. N and O intermediate states

Are there further conformational changes associated with the N and O intermediates which follow the M intermediate? This question can be addressed in two different ways. One approach would be to obtain successive snapshots of structural changes in bacteriorhodopsin with a sufficiently high time resolution to establish whether distinct conformational changes are observed at different stages of the photocycle. Such an analysis has been carried out on wild-type bacteriorhodopsin. These studies, presented in Subramaniam et al. [17], unequivocally demonstrate that the large protein conformational change occurs within approx. 1 ms after illumination, and persists until the final stages associated with recovery of the starting protein conformation, making it unlikely that there are further large protein conformational changes on the time scales associated with formation of the N or O intermediates.

An alternative approach to investigate the confor-

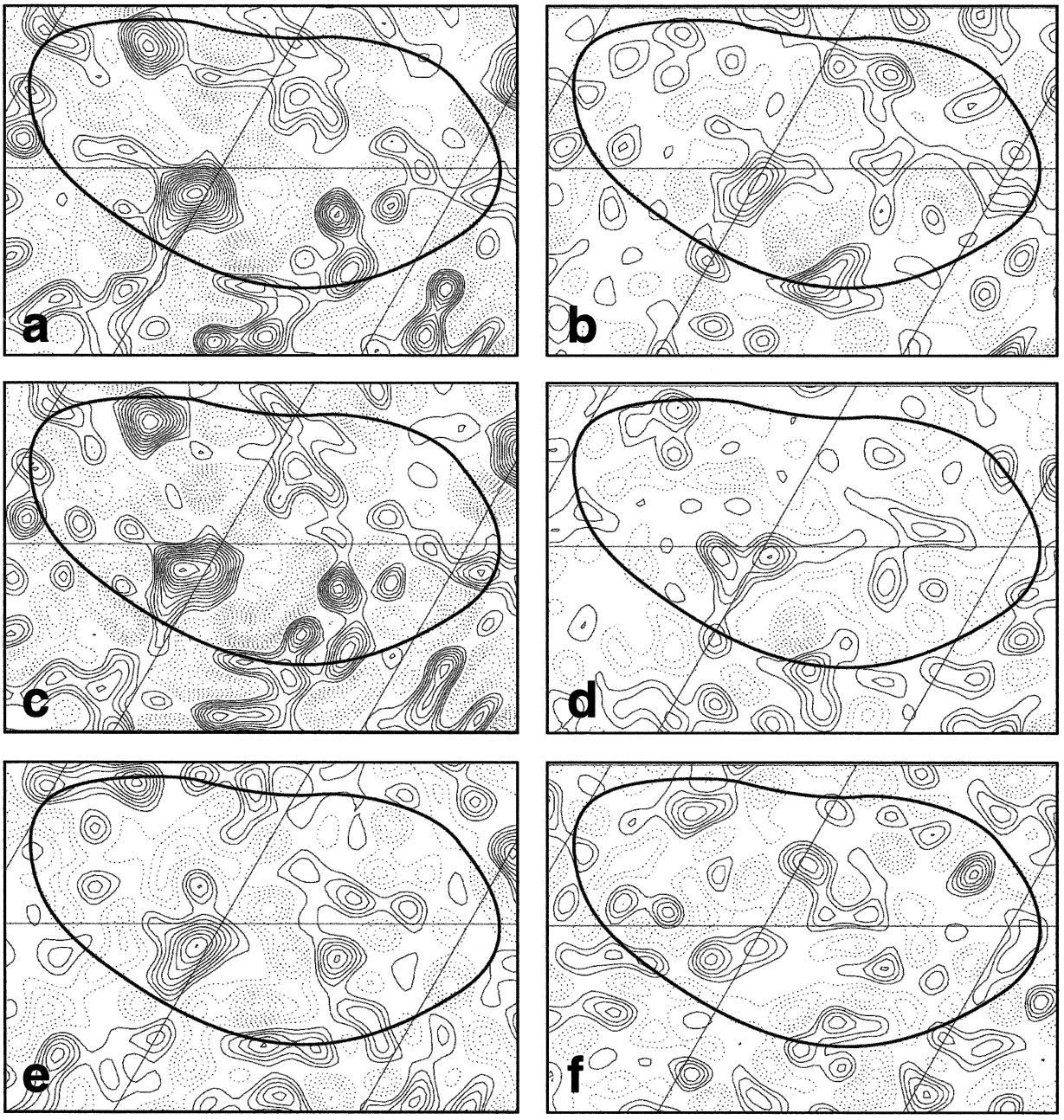


Fig. 2. (a) Difference map showing structural changes in crystals of wild-type bacteriorhodopsin 35 ms after illumination at pH 9.5 and at 5°C using unilluminated crystals of wild-type bacteriorhodopsin as a reference (from Subramaniam et al. [17]). (b) Difference map showing structural changes in crystals of the D96G mutant 20 ms after illumination at pH 8.5 and at 25°C, using unilluminated crystals of the D96G mutant as a reference. (c) Difference maps showing structural changes in crystals of the D96N mutant 10 ms after illumination at pH 6.0 at 5°C, using unilluminated crystals of wild-type bacteriorhodopsin as a reference. (d) Difference map showing structural changes in crystals of the F219L mutant 35 ms after illumination at pH 6.0 at 5°C, using unilluminated crystals of the F219L mutant as a reference. (e) Difference map (at 4.2 Å resolution) showing light-induced structural changes in the T46V mutant 10 ms after illumination at pH 6.0 using unilluminated crystals of the T46V mutant as a reference. (f) Difference map showing structural changes in crystals of the L93A mutant 5 s after illumination at pH 6.0 using unilluminated crystals of the L93A mutant as a reference. Maps are taken from data in [6,17,21].

mational changes at later stages of the photocycle is to use mutants in which decay of the N or O intermediates is the rate-limiting step of the photocycle. A selection of such projection difference Fourier maps is shown in Fig. 2, which includes the M/N intermediate mixture from wild-type bacteriorhodopsin (Fig. 2a), the M intermediate (referred to as the $M_N$ intermediate by Sasaki et al. [18]) from the D96G (Fig. 2b) and D96N (Fig. 2c) mutants, the N intermediate from the F219L (Fig. 2d) and T46V (Fig. 2e) mutants and the O intermediate from the L93A mutant (Fig. 2f). Comparison of changes in wild-type bacteriorhodopsin, the D96G and D96N mutants (Fig. 2a–c), shows that while the extent and nature of the changes are generally similar, some differences in peak locations can be detected. Inspection of the maps (Fig. 2d–f) under conditions where the N or the 13-*cis* O intermediate [19] is accumulated shows that while the peaks are in approximately the same place as in the map obtained for wild-type bacteriorhodopsin, the extents of the light-induced change vary considerably. An explanation for the origin of this variation comes from an analysis of the projection structures of the unilluminated state in these mutants. In these (T46V, F219L and L93A) mutants, the unilluminated state of the protein is already altered in conformation to varying extents as compared to wild-type bacteriorhodopsin. The extent of the light-induced change in these mutants is therefore smaller than that observed in wild-type or the Asp96 mutants. Interestingly, the structural change in the unilluminated state appears to be in the same direction as the change that occurs upon illumination; thus, the total structural change with respect to the unilluminated state of wild-type bacteriorhodopsin is very similar to the changes seen for wild-type bacteriorhodopsin.

Together, these experiments lead to the conclusion that in wild-type bacteriorhodopsin, and in all of the mutants studied so far, there is a single large protein conformational change that occurs with formation of the $M_2$ intermediate, and persists until the very late

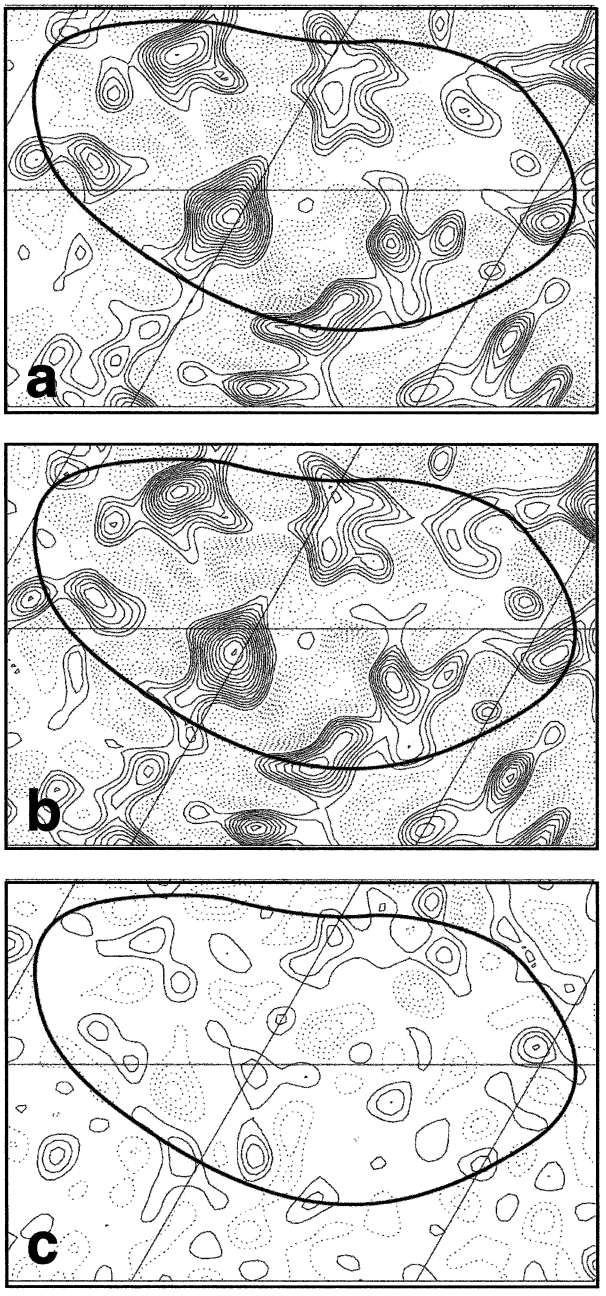


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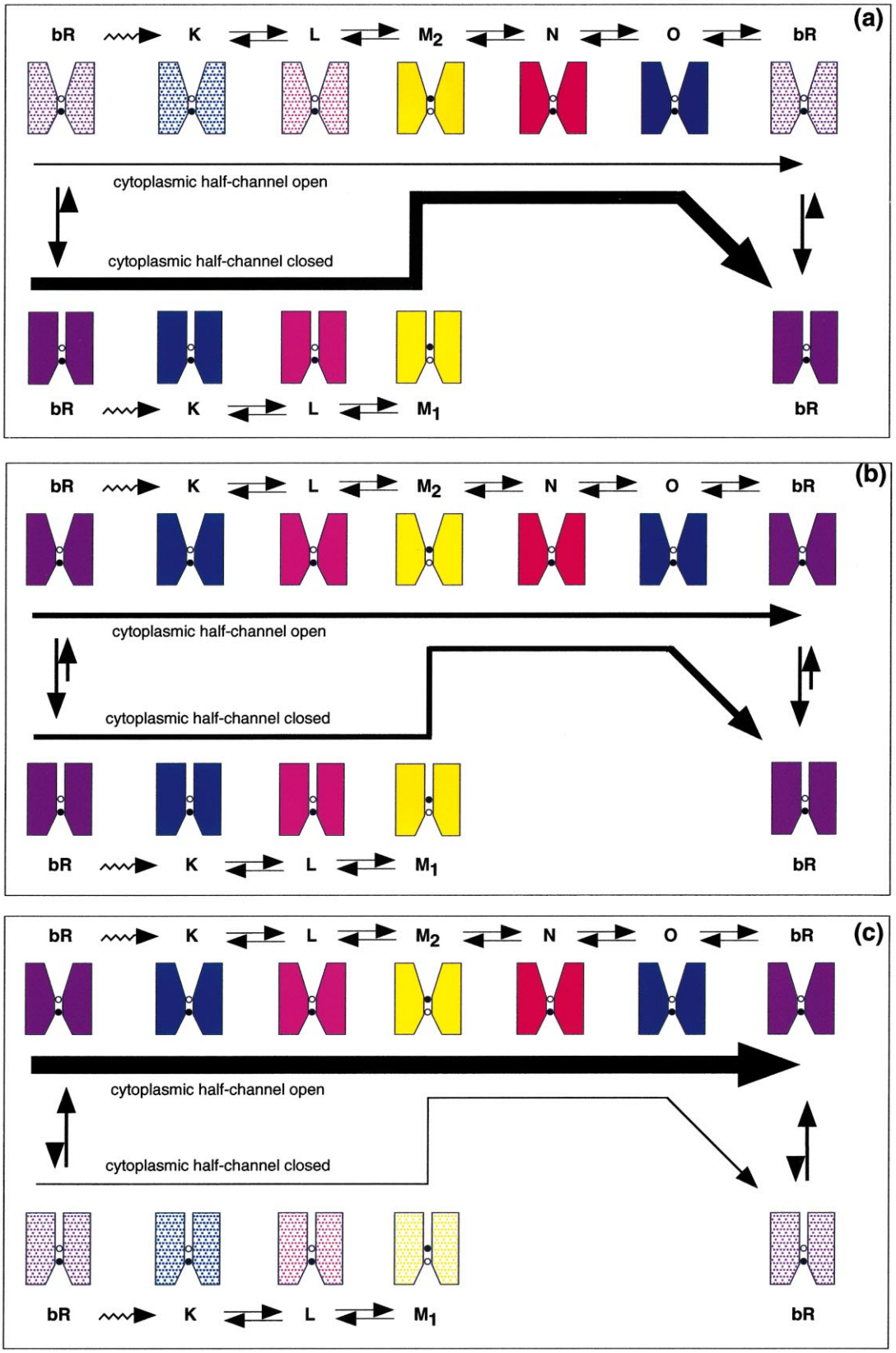
Fig. 3. (a) Difference map showing structural changes in unilluminated crystals of the D96G/F171C/F219L triple mutant at pH 6 and at 5°C using unilluminated crystals of wild-type bacteriorhodopsin as a reference. (b) Difference map showing light-induced structural changes in the D96G/F171C/F219L triple mutant 10 ms after illumination using unilluminated crystals of the wild-type bacteriorhodopsin as a reference. (c) Double difference map showing the extent of light-induced structural changes in the D96G/F171C/F219L triple mutant 10 ms after illumination at pH 6 using unilluminated crystals of the same mutant as a reference. The features in this double difference map are near the noise level, implying that there are no significant light-induced structural changes that are detectable at 3.5 Å resolution. Maps are taken from data in [17].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stages of the photocycle. There is no evidence that the formation of the N or the 13-*cis* O intermediates require any further large structural changes other than those already present at the M intermediate stage.

## 5. Structural changes in the D96G/F171C/F219L triple mutant

A dramatic example of conformational changes induced by mutations in the absence of illumination is observed in the D96G/F171C/F219L triple mutant. The three mutations, all involving residues in the upper cytoplasmic region, destabilize the structure sufficiently to shift the equilibrium in the unilluminated state almost completely towards the conformation that is only observed transiently in wild-type bacteriorhodopsin during the photocycle (Fig. 3a). The structure of the illuminated state (Fig. 3b) is essentially indistinguishable from that of the unilluminated state, as confirmed by the double difference map (Fig. 3c) which shows no significant light-driven changes in this mutant. An example similar to the D96G/F171C/F219L triple mutant comes from the work of Kataoka et al. [20], who have previously reported that mutations D85N and D85N/D96N also show structural changes in the unilluminated state that are comparable to those observed upon illumination of wild-type bacteriorhodopsin.

## 6. The molecular mechanism of vectorial proton transport

Taken together, the above crystallographic investigations lead to the conclusion that the structures of the initial unilluminated state and those of the early intermediates (K, L and $M_1$) are well approximated by one conformation, in which the Schiff base is preferentially accessible to the extracellular side (Fig. 4). The structures of the other intermediates ($M_2$, N, and the 13-*cis* O intermediate) are well approximated by the other conformation, in which the Schiff base is preferentially accessible to the cytoplasmic side. In wild-type bacteriorhodopsin, and in several mutants, this conformational change occurs with the formation of the $M_2$ intermediate, and is reversed in the final stage of the photocycle, corresponding to the decay of the N and O intermediates. In selected mutants, a partial conformational change is already present before illumination, and the full extent of the conformational change is realized upon illumination. In the special case of the D96G/F171C/F219L triple mutant, the conformation of the unilluminated protein is almost completely shifted in favor of the conformation that is only observed upon illumination in wild-type bacteriorhodopsin.

How is it possible that proton pumping can occur with such a wide range in the extent of the light-driven protein conformational changes in the different mutants? In all of the mutants that we have studied, the conformational change is always present

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Fig. 4. Schematic description of conformational changes in wild-type bacteriorhodopsin and the various mutants studied by electron crystallography (figure is taken from [17]). The unilluminated state of the protein is represented as an equilibrium mixture between two conformations. In one, the Schiff base is primarily accessible to the extracellular side, and in the other, primarily to the cytoplasmic side. In each panel, the predominant conformation of each photocycle intermediate is shown in solid color, whereas the minor conformation is shown with dotted coloring. (a) In unilluminated wild-type bacteriorhodopsin, the equilibrium is shifted largely towards the conformation which has accessibility to the extracellular side. Upon illumination, retinal is isomerized, followed by deprotonation of the Schiff base, and the equilibrium is then shifted to favor the conformation that has accessibility to the cytoplasmic side. The protein component of the accessibility change is illustrated by the schematic opening of the cytoplasmic channel, and the retinal component of the accessibility change is indicated by the change in 'fill' pattern in the pair of circles in the center of the protein. Following re-protonation of the Schiff base, and the thermal re-isomerization of retinal, the equilibrium is shifted back in favor of the conformation that has accessibility to the extracellular side. (b) In mutants such as T46V, L93A, or F219L, the equilibrium is partially shifted even in the unilluminated state towards the conformation that has accessibility to the cytoplasmic side. Structural changes in both retinal and the protein contribute to the 'switch' in accessibility that is required for light-driven proton transport, but the relative contribution of the protein conformational change is less than in wild-type bacteriorhodopsin. (c) In the D96G/F171C/F219L triple mutant, the equilibrium is shifted fully towards the conformation that has accessibility of the cytoplasmic side. In this mutant, structural changes in retinal provide most of the contribution to the light-induced switch in accessibility.

in the M intermediate state that is accumulated in the illuminated state, consistent with the increased access of the Schiff base to the cytoplasmic side from the expected opening of the cytoplasmic proton channel. The accumulated evidence thus strongly argues that the large protein conformational change plays a central role in ensuring access of the Schiff base to protons in the cytoplasmic medium. The studies with the different mutants, however, also show that the contribution of the conformational change to the overall light-driven switching mechanism can vary. In particular, since the triple mutant can function as a proton pump, it follows that a switch in accessibility of the Schiff base can occur even in the absence of a significant light-induced protein conformational change. We have proposed [17] that the origin of the switching mechanism in the triple mutant must lie almost completely in the reduction of the curvature of retinal that occurs upon deprotonation of the Schiff base. The reduced curvature results in displacement of the Schiff base nitrogen towards the cytoplasm, while simultaneously breaking accessibility to the extracellular medium. In mutants such as T46V, F219L and L93A, where a partial protein conformational change is already observed prior to illumination, it is likely that there is a smaller relative contribution from the protein conformational change to the switch mechanism as compared to wild-type bacteriorhodopsin. Finally in wild-type bacteriorhodopsin, both the protein conformational changes and the change in retinal curvature triggered by Schiff base deprotonation must contribute to the overall 'switch' mechanism that is at the heart of light-driven proton pumping. A more thorough understanding of the detailed molecular mechanism now awaits determination of atomic structure of the conformation with cytoplasmic accessibility.

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