A residue substitution near the β-ionone ring of the retinal affects the M substates of bacteriorhodopsin

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ABSTRACT The switch in the bacteriorhodopsin photocycle, which reorients access of the retinal Schiff base from the extracellular to the cytoplasmic side, was suggested to be an $M_1 \rightarrow M_2$ reaction (Váró and Lanyi. 1991. *Biochemistry.* 30:5008–5015, 5016–5022). Thus, in this light-driven proton pump it is the interconversion of proposed M substates that gives direction to the transport. We find that in monomeric, although not purple membrane-lattice immobilized, D115N bacteriorhodopsin, the absorption maximum of M changes during the photocycle: in the time domain between its rise and decay it shifts 15 nm to the blue relative to the spectrum at earlier times. This large shift strongly supports the existence of two M substates. Since D115 is located near the β-ionone ring of the retinal, the result raises questions about the possible involvement of the retinal chain or protein residues as far away as 10 Å from the Schiff base in the mechanism of the switching reaction.

INTRODUCTION

Bacteriorhodopsin is a small retinal protein that functions as a light-driven proton pump in the cytoplasmic membrane of halobacteria. A number of recent reports have described its reaction cycle after the initial photoreaction as a linear sequence of the intermediates J, K, L, M, N, O, containing reversible reactions (Váró and Lanyi, 1990, 1991a,d; Ames and Mathies, 1990; Gerwert et al., 1990; Milder et al., 1991). For proton translocation, the L ↔ M and M ↔ N steps are critical. After isomerization of the retinal from all-*trans* to 13-*cis*, the pK_a of the Schiff base is apparently lowered (as discussed by Váró and Lanyi, 1991d), making it an effective proton donor in the L → M reaction to D85 near the extracellular side (Braiman et al., 1988; Gerwert et al., 1989; Gerwert et al., 1990; Butt et al., 1989; Stern et al., 1989). This is followed by replacement of the proton on the Schiff base in the M → N reaction from D96 near the cytoplasmic side (Gerwert et al., 1989, 1990; Butt et al., 1989; Stern et al., 1989; Holz et al., 1989; Otto et al., 1989, 1990; Tittor et al., 1989). Kinetic and spectroscopic evidence have suggested that there are two sequential M substates, termed M_1 and M_2 (Váró and Lanyi, 1990, 1991a,d). The proposed $M_1 \rightarrow M_2$ reaction was linked to three essential functions in the transport: (*a*) reorientation of the Schiff base from D85 on the extracellular to D96 on the cytoplasmic side; (*b*) raising of the Schiff base pK_a, so it will become a proton acceptor in the following step; and (*c*) loss of part of the excess free energy in the chromophore to the proton gradient created, and enthalpy–entropy conversion which transfers the rest of the excess free energy from the chromophore to the protein. The latter implies that after M_2 some of the free energy is conserved, and as a conformational change of the protein; this will ensure the full recovery of the initial state of bacteriorhodopsin in the O → BR step.

Transient absorption changes near 400 nm in bacteriorhodopsin, immobilized in the crystalline lattice of the purple membrane, do not directly reveal the existence of the two proposed M states. Indeed, although a logical necessity for the Schiff base reorientation had been suggested (Nagle and Mille, 1981; Schulten et al., 1984; Henderson et al., 1990; Mathies et al., 1991), the existence of M substates was initially invoked in order to solve a kinetic anomaly in the decay of the L intermediate with an irreversible $M_1 \rightarrow M_2$ reaction (Váró and Lanyi, 1990). However, subsequent experiments with monomeric bacteriorhodopsin in detergent micelles revealed a splitting of the M spectrum into an early and a late component shifted by ~4 nm, coincident with the appearance of two M states as predicted by the kinetics (Váró and Lanyi, 1991a). This shift was confirmed in other recent reports (Milder et al., 1991; Subramaniam et al., 1991). Because the kinetic and spectroscopic data were consistent with one another, the two M spectra were assumed to be those of the proposed sequential M_1 and M_2 states rather than spectra of two kinetically independent M intermediates such as suggested to exist by several other investigators (e.g., Hanamoto et al., 1984; Dancshazy et al., 1988; Balashov et al., 1991). Importantly, whatever the differences that allow the

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wavelength shift in the monomers, they must be compatible with a functional switch because monomeric bacteriorhodopsin is competent in proton transport (Dencher and Heyn, 1979; Bamberg et al., 1981).

The molecular events that underlie the $M_1 \rightarrow M_2$ reaction determine energy coupling in this proton pump. Since the $M_1 \rightarrow M_2$ reaction has been resolved in very few experiments so far, the small wavelength shift between M_1 and M_2, and the fact that it occurs only in the more mobile monomeric protein, are the first and only direct clues that the environment of the retinal might change as a consequence of the switch step. We now report on M spectra in the site-specific mutated bacteriorhodopsin D115N. D115 is a buried aspartate that remains protonated throughout the photocycle (Braiman et al., 1988; Gerwert et al., 1989). It is located near the β-ionone ring end of the retinal (Henderson et al., 1990). Replacement of this residue with asparagine has a significant effect on the switch reaction: in the monomeric state the spectral shift between the two M substates is much larger than in wild-type. This provides considerable support to the model containing M substates, and suggests further that in the switch mechanism displacements of the retinal and/or the protein are not restricted to the Schiff base region.

METHODS

The clone containing the D115N bacteriorhodopsin was constructed from *Halobacterium halobium* strain L-33 by transformation with a shuttle vector containing the modified bop gene, as described elsewhere (Ni et al., 1990; Needleman et al., 1991). The mutated bacteriorhodopsin was prepared as purple membrane sheets (Oesterhelt and Stoeckenius, 1974). As will be described elsewhere, x-ray diffraction indicated that its principal lattice dimension was virtually identical to that of purple membrane containing the wild-type protein. All samples used for spectroscopy contained 100 mM NaCl, 50 mM phosphate at pH 7.0. The measurements were at 22°C. Solubilization was with 2% Triton X-100 for 2 d in the absence of salt (Dencher and Heyn, 1978); we have found that this procedure produced monomeric protein by several criteria (Váró and Lanyi, 1991b). Light adaptation was for 10 min with light filtered below 530 nm.

The measurement of stationary spectra was with a Shimadzu Scientific Instruments, Inc. UV-250 spectrophotometer (Columbia, MD); the time-resolved spectra were obtained with a gated optical multichannel analyzer described earlier (Zimányi et al., 1989). The spectra were smoothed by eliminating high frequency noise with FFT (Fast Fourier Transform) or with weighted binomial averaging with the program SPSERV (written by Csaba Bagyinka, Biological Research Center of the Hungarian Academy of Sciences, H-6701 Szeged, Hungary).

RESULTS

The absorption spectrum of wild-type bacteriorhodopsin shows a characteristic 14 nm blue-shift from 568 nm and a 20% amplitude decrease upon solubilization of the purple membranes with detergent. In the D115N protein these changes are much larger: in the purple membrane lattice the absorption maximum is at 566 nm, but in the monomeric form it is at 538 nm and the amplitude is 40% decreased. The spectra are shown in Fig. 1. Replacement of D115 with asparagine has only a minor effect on the absorption band in the visible when the protein is in the purple membrane, but causes a 27-nm blue-shift when it is monomeric. It appears, therefore, that this residue replacement does not, by itself, perturb retinal-protein interaction; the potential for a change in the retinal binding pocket is realized only when the protein is removed from the rigid two-dimensional crystalline lattice. Earlier reports on light-adapted *Escherichia coli*-expressed D115N bacteriorhodopsin showed a 17–28 nm blue-shift from wild-type (Mogi et al., 1988; Subramaniam et al., 1990); most likely the reason is that to varying degrees the protein was not organized into a purple membrane lattice. Also unlike these samples, we found that D115N in purple membrane patches showed normal light adaptation, i.e., a red-shift of 7 nm. The absorption maximum was not affected by pH between 4 and 9 (not shown).

In Fig. 2 measured time-resolved difference spectra are shown for D115N bacteriorhodopsin in the μs to ms time region after photoexcitation. The spectra are lined up with respect to the difference maximum near 400 nm, which represents the absorption band of M. When the protein is in purple membrane patches the maximum of M is unchanged with delay time except in amplitude

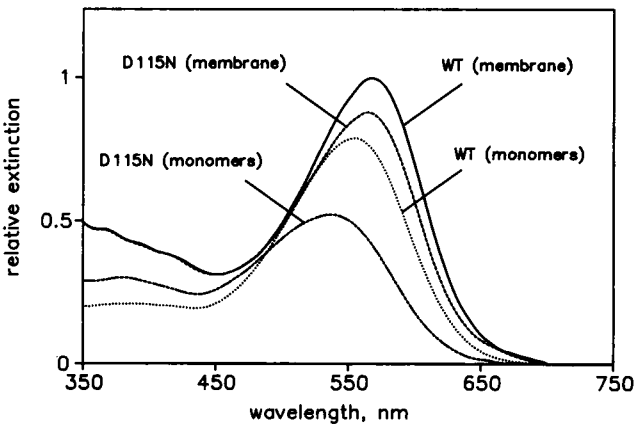

FIGURE 1 Absorption spectra for light-adapted bacteriorhodopsin (wild-type, WT; and the site-specific mutant D115N), in the purple membrane and monomeric forms. WT in purple membrane (——). WT solubilized (·····). D115N in purple membrane (- - - - -). D115N solubilized (-- ·· -- ·· -- ·· --). The relative amplitudes of the spectra for WT and D115N purple membranes were estimated by normalizing absorption at 280 nm.

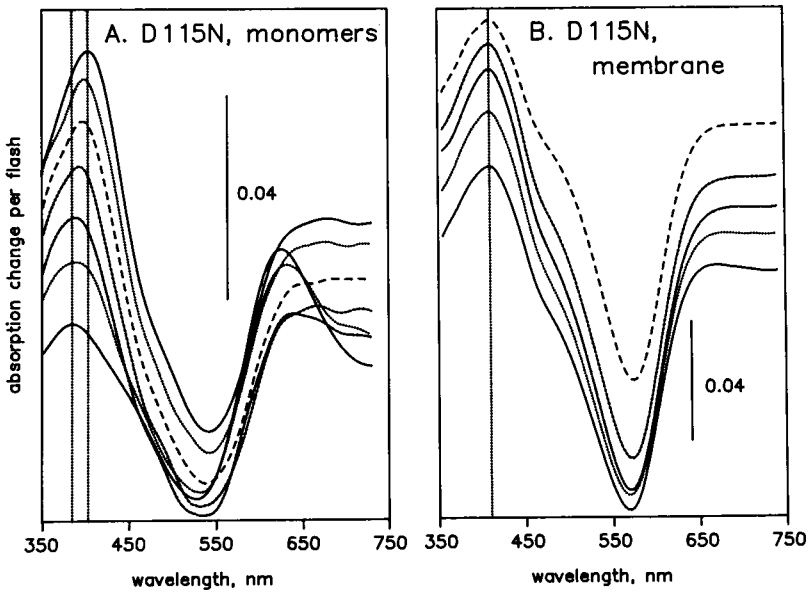

FIGURE 2 Time-resolved difference spectra measured after photoexcitation of D115N bacteriorhodopsin in the solubilized state (*A*), and in purple membranes (*B*). The delay times, beginning with the spectra of the highest amplitudes, are as follows. (*A*) 40 μs (———); 100 μs (·····); 250 μs (— — — — — —); 600 μs (- - - - - -); 1.5 ms (— ·· — ·· — ··—); 4 ms (·····); 10 ms (————). (*B*) 170 μs (— — — — —); 360 μs (- - - - - - - -); 520 μs (— · — · — · —); 1.1 ms (·····); 1.6 ms (————). In *A*, the vertical lines are at 384 and 402 nm; in *B*, the line is at 409 nm.

(Fig. 2 *B*), but when it is solubilized the maximum of M is clearly resolved into two time-dependent species (Fig. 2 *A*). The maximum of M is so far removed from the maxima of all other species present that these difference spectra identify its position without much error. The dotted lines, drawn in Fig. 2 *A* through the early and late peak positions, indicate therefore that the two M maxima are at 402 and 387 nm. The half-width of the peak near 400 nm was about the same in the monomeric protein as in purple membranes, both before and after the wavelength shift. The approximate time constant for the interconversion of the two M states is 200 μs. In wild-type monomeric bacteriorhodopsin this time constant was 160 μs (Váró and Lanyi, 1991a). The M produced from the same protein but in purple membrane had a maximum at 409 nm (Fig. 2 *B*).

The measured spectra for purple membranes containing the D115N protein were deconvoluted into component spectra as in several earlier publications (Váró and Lanyi, 1991a,c); the results will be described in detail elsewhere. The spectra were very similar to the spectra caculated before for wild-type bacteriorhodopsin, and the kinetics gave a good fit to the photocycle model BR $-h\nu \rightarrow K \leftrightarrow L \leftrightarrow M_1 \rightarrow M_2 \leftrightarrow N \leftrightarrow O \rightarrow BR$. The main differences from wild-type under the same conditions were the greater accumulations of L and N, caused by accelerated $K \rightarrow L$ and $M_2 \rightarrow N$ reactions, respectively.

The spectra between 4 and 10 ms from monomeric D115N (Fig. 2 *A*) show the development of an intermediate with considerable absorption between 600 and 650 nm. This was noted before with *E. coli*-expressed D115N bacteriorhodopsin and ascribed to the O state (Stern et al., 1989; Otto et al., 1990). However, we could not achieve a self-consistent deconvolution of the measured spectra for solubilized D115N into component spectra and thus calculate the kinetics. We suspect that the problem is all-*trans*/13-*cis* heterogeneity even in the light-adapted samples; such a mixture would produce parallel photoreactions and the measured spectra would not be described by a single set of intermediates and a single kinetic scheme. It should be noted, however, that the photocycle of 13-*cis* bacteriorhodopsin does not include an M intermediate.

Fig. 3 *A* shows the time course of absorption changes in monomeric D115N bacteriorhodopsin at 384, 402, and 540 nm. The first two of these wavelengths were selected because they roughly correlated with the splitting in the M spectra in Fig. 2 *A*. As described earlier for monomeric wild-type bacteriorhodopsin (Váró and Lanyi, 1991b), the rise kinetics for M is roughly exponential in the 1 to 10 μs time range, while the decay is described by two exponential components of about equal amplitudes, with time constants of ~2 and 12 ms (*lines* in Fig. 3 *A*). This was true both at 384 and 402 nm. The wide separation of the rise and decay lifetimes of M resulted in a broad time domain, from ~10 to ~500 μs, where

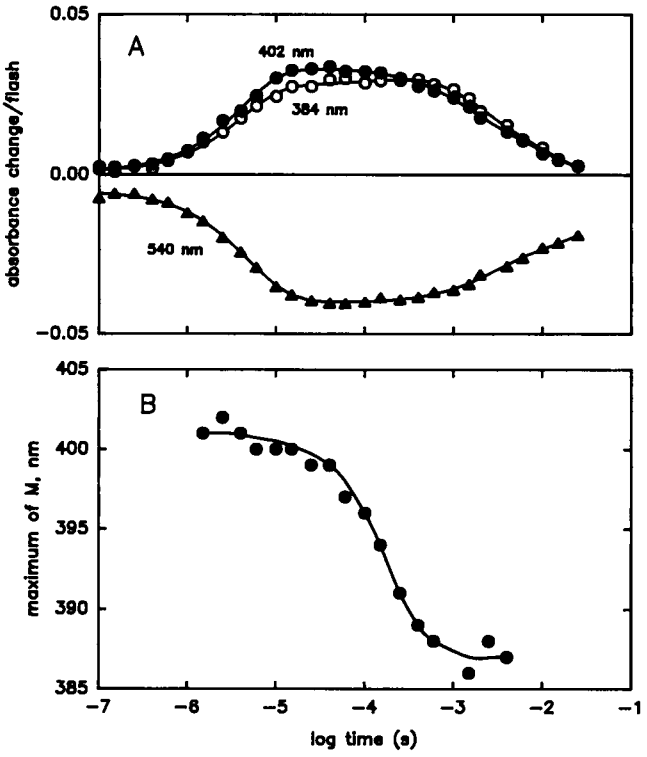

FIGURE 3 Time course of absorption changes in monomeric D115N bacteriorhodopsin. (*A*) At 384 nm (averaged amplitudes between 379 and 389 nm) (○); at 402 nm (averaged amplitudes between 396 and 406 nm) (●); and at 540 nm (averaged amplitudes between 531 and 551 nm) (▲). (*B*) Wavelength maximum of the peak near 400 nm.

the concentration of M was constant. The kinetics of 540 nm confirmed this, because M has virtually no absorption at this wavelength and BR depletion indeed did not change here (Fig. 3 *A*). Significantly, it was during this plateau that the 15 nm shift in the maximum of M took place (Fig. 3 *B*). Thus, the shift is well separated in time from the rise kinetics and from the biphasic decay. For these reasons we interpret the observed wavelength shift as an interconversion between two M states, rather than two M states that are produced in parallel photocycles with different time-constants, perhaps from two subpopulations of bacteriorhodopsin. The latter alternative would require that the rise in kinetics for M from a second BR substate near 200 μs exactly match the decay kinetics for M from the first substate, and that the amplitudes of these two M intermediates be the same.

DISCUSSION

The results strongly support the existence of M substates in the bacteriorhodopsin photocycle. It has been somewhat disconcerting that in the purple membrane the proposed two M states have indistinguishable absorption spectra,[1] but not unexpected because a deprotonated Schiff base is much less sensitive to its environment than the protonated form. The influence of protein residues on the chromophore spectrum are ultimately traced to their effect on the electron density of the Schiff base nitrogen, and therefore to the extent of the delocalization of the π-orbitals over the retinal chain (Kakitani et al., 1985; Nakanishi et al., 1980; Warshel, 1978). Whatever the underlying molecular events in the $M_1 \rightarrow M_2$ reaction, they evidently do not result in a sufficiently changed protein–retinal interaction to produce an observable effect on the spectrum. On the other hand, in the motionally less restricted monomeric bacteriorhodopsin, the maximum of the M_2 substrate does shift a few nanometers to the blue relative to the maximum of M_1 (Váró and Lanyi, 1991a; Milder et al., 1991; Subramaniam et al., 1991). We now find that this wavelength shift is much greater in monomeric D115N than in the monomeric wild-type. It is not obvious why a residue substitution at position 115 should affect the spectrum of M. D115 remains protonated throughout the photocycle (Braiman et al., 1988; Gerwert et al., 1989). The FTIR spectra did suggest an alteration in its hydrogen-bonding during the photocycle. However, T89 is the only residue that is a reasonable candidate for hydrogen bonding with D115, and replacement of T89 with valine did not produce an unusual photocycle (Marti et al., 1991). Replacement of D115 with asparagine is virtually isomorphic, and would be expected to cause more subtle effects than the replacements of the functionally more directly involved D96, D85, and D212. According to the highest resolution structure of bacteriorhodopsin to date (Henderson et al., 1990), D115 is located in the neighborhood of the C-9 methyl group, i.e., much nearer to the β-ionone ring than to the Schiff base of the retinal. Fig. 4 illustrates the position of D115 relative to the Schiff base and D85 and D96. Henderson et al. (1990) pointed out that the vertical position of the helix containing D115 was particularly difficult to determine accurately (an estimate [R. Henderson, personal communication] of the vertical uncertainty would be about ±3 Å). Given these stipulations, the distance of D115 from the Schiff base nitrogen seems to be ≥10 Å. We suggest on the basis of the differential effect of D115 on the spectra of the two M substates, and this structure, that the molecular events in the $M_1 \rightarrow M_2$ reaction cannot be exclusively at the Schiff base. Thus, some basis must be found for

[1]We have been unable to reproduce results in an earlier report on the splitting of the M spectrum of bacteriorhodopsin after deionization and treatment with organometallic cations (Mathew et al., 1985).

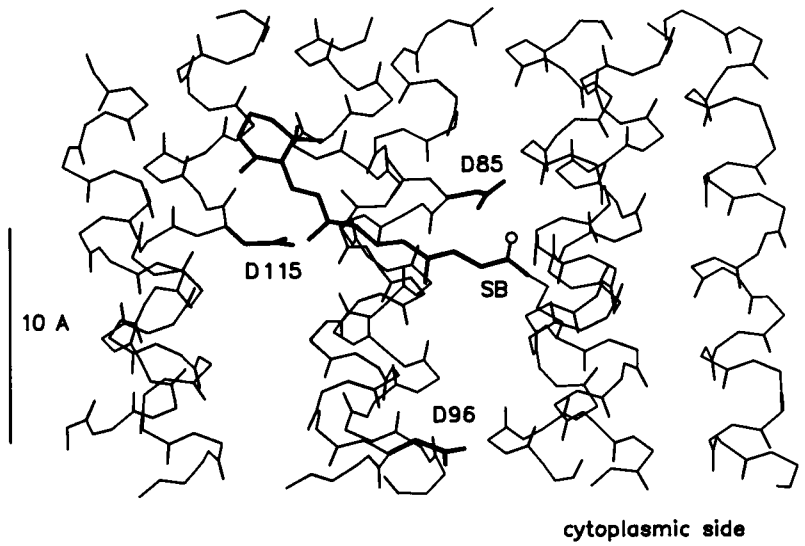

FIGURE 4 Structural model for bacteriorhodopsin, based on diffraction data from electron cryo-microscopy (Henderson et al., 1990). A side view of the most of the polypeptide chain of the seven transmembrane helical segments is shown, with D85, D96, D115, and the all-*trans* retinal (attached to K216) outlined in bold. The Schiff base proton is shown as a circle. The structure was rotated to best illustrate the disposition of D115 relative to the Schiff base; from this viewpoint three pairs of helices overlap one another. Note that Henderson et al. (1990) pointed out that the vertical position of the helix containing D115 was particularly difficult to determine accurately. An estimate of the vertical uncertainty would be about ±3 Å (Henderson, personal communication).

the participation of the retinal chain or the protein near the β-ionone ring in this reaction.

Two mechanisms have been proposed for the bacteriorhodopsin switch. In one, photoexcitation of the chromophore causes simultaneous *trans* to *cis* isomerization reactions around both the C13–C14 double bond and the C14–C15 single bond, and in the switch reaction at M, the C14–C15 single bond, but not the C13–C14 double bond, reassumes its *trans* configuration (Gerwert and Siebert, 1986). This would reorient the electron pair of the Schiff base nitrogen and change access from the extracellular to the cytoplasmic side between the deprotonation and reprotonation steps. However, the existence of the C14–C15-*cis* configuration during the photocycle has been disputed on the basis of a different vibrational frequency assignment (Fodor et al., 1988b). In the second suggested mechanism (Fodor et al., 1988a) the conformation of the protein, i.e., the shape of the retinal-binding pocket, changes instead. In this "C-T model" access of the Schiff base is changed from the extracellular to the cytoplasmic side through switching from one proton-conducting chain of residues to another, at either the N → O (Fodor et al., 1988a) or the $M_1 \rightarrow M_2$ (Mathies et al., 1991) reaction. Protein conformational changes in the second half of the photocycle are indeed suggested by shifting of amide bands (Braiman et al., 1987; Ormos, 1991) and time-resolved diffraction (Koch et al., 1991). Although direct evidence for this is lacking, we assume that they consist of reversal of the conformation changes in the $M_1 \rightarrow M_2$ reaction. The nature of the detected changes is not yet clear; difference electron density maps between a stabilized M and the initial bacteriorhodopsin states are consistent with slight tilting of one or more of the transmembrane helices (Glaeser et al., 1986; Dencher et al., 1989).

Since the idea of the switch refers to the orientation of the Schiff base, these schemes concentrated on the possible events near this location. That a residue replacement at a distance of 10 Å from the Schiff base has a differential influence on retinal-protein interaction before and after the switch step suggests that the switch is a more global reaction. Thus, there might be coupling of structural changes of the protein near the β-ionone ring and the Schiff base regions of the retinal. Another possibility is that during the switch reaction the conformational change of the protein causes rotation of the entire retinal chain around its long axis, thereby providing the required reorientation of the Schiff base.

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