Reversal of the Surface Charge Asymmetry in Purple Membrane Due to Single Amino Acid Substitutions

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ABSTRACT Twenty-seven mutant bacteriorhodopsin's were screened to determine the pK_a for reversal of the permanent electric dipole moment. The photoelectric response of an aqueous purple-membrane suspension was used to determine the direction of the purple-membrane dipole moment as a function of pH. The pK_a for the dipole reversal of wild-type bacteriorhodopsin is 4.5. Six of the 27 mutant bacteriorhodopsin's were found to have a pK_a for dipole reversal larger than that of wild-type bacteriorhodopsin. Two of these mutants, L93T and L93W, involve a neutral amino acid substitution in the interior of the protein. The direction of the purple-membrane permanent electric dipole moment is determined by the purple-membrane surface charge asymmetry. We conclude that these two substitutions, which do not involve charge replacement, alter the pK_a for the reversal of the purple-membrane surface charge asymmetry. We suggest that these changes to the pK_a are due to altered protein folding at the surface of the purple-membrane induced by single-site substitutions in the protein interior.

INTRODUCTION

Bacteriorhodopsin (BR) is a protein found in the cell membrane wall of *Halobacterium halobium* where it functions as a light driven proton pump (Henderson et al., 1990; Lanyi, 1993; Ebrey, 1993; Rothschild, 1992; Oesterhelt et al., 1992; Mathies et al., 1991). A single BR molecule, or monomer, consists of two parts: 1) the opsin, and 2) a retinal chromophore. Opsin is a seven-α helical array of 248 amino acids and has a molecular weight of 26,000. Each of the seven-α helices spans the membrane and is roughly perpendicular to it. The retinal chromophore is responsible for visible-light absorption by BR, and interaction between it and the opsin causes the absorption spectrum of the retinal to be strongly red shifted from that of free retinal. The electric dipole moment of the retinal lies 23° out of the plane of the membrane.

Within the cell membrane wall the BR molecules are arranged in a two-dimensional crystalline sheet called the purple membrane (PM). Each PM is ~ 1 μm in diameter, 45 Å thick and contains $\sim 10^5$ BR molecules. The BR molecules are vectorially oriented to pump protons from inside of the cell to the outside. BR molecules in the PM are located on a hexagonal lattice with three BR molecules—a trimer—at each lattice site. The region between trimers is filled with lipid, which accounts for $\sim 15\%$ of the PM by weight. The crystalline structure of the PM is retained after it is isolated from the bacteria, and the BR molecules are still functional in an aqueous suspension.

The PM has a net negative surface charge. This surface charge is due to charged amino acid residues at the surface, membrane lipids with acidic head groups, and the C-terminal and N-terminal ends of the polypeptide chain, which are on opposite sides of the membrane (Jonas et al., 1990; Szundi and Stoeckenius, 1989; Alexiev et al., 1994). The C-terminal is on the cytoplasmic side of the membrane and the N-terminal is on the extracellular side (Henderson et al., 1990). The C-terminal surface of the PM is more negatively charged than the N-terminal face at neutral pH (Jonas et al., 1990; Alexiev et al., 1994). This charge asymmetry of the PM results in a permanent dipole moment (Kimura et al., 1984; Barabas et al., 1983) that points from the C-terminal face to the N-terminal face.

Because the PM has an electric dipole moment, application of an electric field to an aqueous PM suspension causes the PM to orient in the direction of the field. Transient illumination of such an oriented PM sample results in a photoelectric response (Trissl, 1990). The polarity of the photoelectric response is consistent with the surface charge asymmetry (the C-terminal face points toward the anode), and proton pumping is from the C-terminal to the N-terminal side of the membrane (Liu and Ebrey, 1988; Liu et al., 1990).

Site-directed mutagenesis is a powerful tool for studying the molecular mechanisms involved in proton pumping by BR (Chang et al., 1992; Krebs and Khorana, 1993). In this approach active sites in the protein are identified by the appearance of functional changes after particular amino acid substitutions. Ideally, the single-site mutations chosen will have little distal effect on the structure of the protein so that the effect of the mutagenesis is restricted to the local region of mutagenesis. This allows the structural model of wild-type (WT) BR to be used in a rational approach to determine the effect of mutations on the proton pump mechanism. For some single-site mutations the effects are indeed local. For

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example, the mutation asp-96→gly-96 alters the kinetics of the photocycle but does not alter the BR tertiary structure to 1-Å resolution (Subramaniam et al., 1993). However, the number of single-site mutants examined to date is relatively small.

We were interested to find out if some single-site mutations could cause distal changes in the protein. This question is important for two reasons: 1) for the study of the molecular mechanism involved in proton pumping, and 2) for genetic engineering of bacteriorhodopsin for device applications (Hampp et al., 1992). To answer this question we focused on site-directed mutagenesis at the interior of the protein and the effect of such mutagenesis on the surface charge of the purple membrane. Experiments were used to rapidly screen 27 single-site mutants for such unexpected behavior. This was done by comparing the spectroscopic and photoelectric properties of the BR mutants with WT BR.

In this paper we present data showing that near neutral pH, some uncharged single amino acid substitutions within the protein interior change the direction of the PM permanent electric dipole moment relative to WT BR. Photoelectric measurements were used to determine the direction of the dipole moment. Based on these experimental results and other data, we conclude that these single amino acid substitutions in the interior of the protein alter the surface charge asymmetry of the purple membrane.

MATERIALS AND METHODS

Our approach was to compare properties of BR mutants obtained by single-site mutagenesis with those of WT BR. To do this rapidly and accurately we choose to make spectroscopic and photoelectric measurements on aqueous PM suspensions (Keszthelyi and Ormos, 1983). This approach has the following advantages: 1) identical aqueous suspensions of different BR mutants and WT BR are easily and rapidly prepared. 2) Only a relatively small amount (a few milligrams) of purified mutant BR is required for the measurements. 3) Spectroscopic and photoelectric measurements can be made on the same sample. 4) The photoelectric measurements give information about both the surface charge asymmetry of the PM (Jonas et al., 1990; Barabas et al., 1983) and the proton pumping behavior (Trissl, 1990; Liu et al., 1990).

Mutants

The following mutants were used for these studies: D38R, T46V, V49A, V49 M, A53G, Y57F, D85E, D85N, T89A, L93E, L93F, L93 M, L93S, L93T, L93V, L93W, D96A, D96N, D115N, G155D, E166G, W182F, Y185F, D212N, F219L, R227Q. These mutants were made using a *bop* gene introduced into *Halobacterium halobium* (Chang et al., 1992) on a replicating plasmid. Purple membranes of WT or mutant BR were prepared by standard methods (Oesterhelt and Stoeckenius, 1974) and purified by repeated washing in 100 mM NaCl. Except as noted below, the PM was suspended in distilled water containing 5 mM buffer (MES, PIPES or Malic Acid depending on the pH).

Visible spectroscopy

A Shimadzu UV-2101PC (Shimadzu Scientific Instruments, Inc., Columbia, MD) was used to measure the absorbance spectrum of the mutant and WT BR. Peaks in the absorbance spectrum were identified by the position of zero slope in the absorbance data after fitting the absorbance curve near the peak to a Gaussian.

Photoelectric measurements

Aqueous PM suspension

The design of the apparatus used to measure the photoelectric response of an aqueous PM suspension followed that described earlier by Keszthelyi and Ormos (Keszthelyi and Ormos, 1983). An electric field, which interacts with the PM permanent electric dipole moment, is used to orient the PM before measuring the photoelectric response. The orienting voltage cycle consisted of +10 V applied for 3 s, followed by 0 V for 6 s and −10 V for 3 s. Approximately 50 ms are required for full alignment of the PM after application of the positive voltage. The orienting voltage was applied to two platinum electrodes separated by 5 mm suspended in a 1 cm plastic cuvette filled with the PM suspension. The photoelectric response is measured by the same two electrodes during the 3-s period of the positive phase of the voltage cycle. A laser light flash (532 nm, 7-ns pulse width) from a Q-switched Nd-YAG laser (Quanta Ray GCR-11; Spectra Physics, Mountain View, CA) was used to generate the photoelectric response.

The photoelectric response was detected two different ways. The first method used a differential input amplifier (Burr Brown INA 110; Tucson, AZ). The illuminated cell was attached to one input and the reference cell was attached to the other input of the amplifier. The output of the differential amplifier, which measures the photoelectric response, was displayed and recorded by a Tektronix 2332 oscilloscope (Beaverton, OR). The rise time of this apparatus was 0.5 μs. The second method used a LeCroy 9360 digital oscilloscope (Chestnut Ridge, NY) in place of the differential amplifier. The illuminated cell was attached to the high band input of the oscilloscope, but a reference cell was not used. The electric signal due to the alignment field was removed by using the input offset of the oscilloscope. Using this arrangement, photoelectric signals with rise times <0.5 ns could be resolved. Both methods gave the same value for the peak height of the initial negative component in the photoelectric response.

One requirement of the PM suspension that was used for these photoelectric measurements is low electrical conductivity because of the orienting voltage that must be applied to the sample. At the same time it is not desirable to wash cations out of the membrane. Therefore, after washing in 100 mM NaCl the PM was suspended in distilled water containing 5 mM buffer. Three different buffers were used (MES, PIPES, and Malic Acid) depending on the pH of interest. The final pH was adjusted using NaOH or HCl.

PM-polyacrylamide gel

The photoelectric response of oriented PM in polyacrylamide gels was also measured. These oriented gels were formed following techniques described in the literature (Varo and Keszthelyi, 1983; Drachev et al., 1988) Briefly the procedure consists of first applying an electric field to an aqueous PM suspension containing acrylamide, bis-acrylamide, and initiators. The suspension is then polymerized while the electric field maintains orientation of the PM. The pH and salt concentration of the gel were adjusted after polymerization. Sections of the wet gel (a cube 5 mm on edge) were cut and placed between two platinized platinum electrodes for the photoelectric measurements. The laser described above was used to generate a photoelectric signal from the gel. No signals were obtained from gels without PM. The photoelectric signal was measured by a transconductance amplifier (Burr Brown OPA 101 with a 10^7 Ω feedback resistor; Tucson, AZ). Data was recorded and displayed on the Tektronix 2332 oscilloscope.

PM-Teflon films

PM were absorbed to a solid Teflon film to provide another system for measuring the photoelectric response. In the PM-Teflon film as in the gels

it is difficult to reproduce a sample, but once made the orientation of the PM is frozen in. Unlike the aqueous PM suspension and the PM-polyacrylamide gel, the photoelectric response of the PM-Teflon film can be measured on a long time scale (Trissl, 1990). The PM-Teflon films were formed following techniques previously described (Rayfield, 1983): BR vesicles were fused with a very thin lipid coated Teflon film which was immersed in bathing solution (100 mM NaCl, 5 mM buffer and 10 mM $CaCl_2$). Photoelectric signals were detected by a voltage follower (Burr Brown OPA 104; Tucson, AZ) connected to Ag-AgCl electrodes immersed in the electrolyte bathing solution and shielded from the actinic laser light flash.

RESULTS

Fig. 1a shows the photoelectric response from WT BR in an aqueous PM suspension at neutral pH (pH = 7.1). The photoelectric response contains three major components (Taneva et al., 1987; Liu et al., 1990; Trissl, 1990). A very fast negative polarity component (B1) is followed by two components (B2 and B3) of the opposite (positive) polarity. The polarity of the components B1, B2, and B3 in the photoelectric response is measured relative to the electric field that aligns the PM (positive polarity corresponds to the movement of positive charge in the direction of the alignment field). At this pH the permanent electric dipole moment of the PM points in the same direction as the alignment field and in the same direction as protons are pumped (i.e. from the C-terminal face to the N-terminal face). Consistent with this geometry it has been shown that the two positive polarity components, B2 and B3, are associated with proton pumping (Trissl, 1990). The fast negative polarity component is associated with the initial proton pumping event i.e., the light-driven *trans-cis* isomerization of the retinal (Liu and Ebrey, 1988; Trissl, 1990). Fig. 1 *b* shows the photoelectric response from mutant BR (L93T) in an aqueous PM suspension (pH = 7.1). The slower components, B2 and B3, are missing from the photoelectric response of this mutant, which is known to pump protons (Subramaniam et al., 1991). This is because the proton pumping is slowed in the L93T mutant (Cao et al., 1993), which results in a very small photocurrent signal; and such small signals cannot be resolved using the aqueous PM system (Trissl, 1990). However, both the PM-polyacrylamide gel and PM-Teflon film systems can be used to detect the slower components, B2 and B3, and verify their polarity relative to the alignment field (Trissl, 1990). For example, Fig. 2 shows the photoelectric response of L93T in the PM-polyacrylamide gel system at pH 7.1. The polarity of B1, B2, and B3 are easily detected. We found that B1 is always opposite in polarity to B2 and B3.

The polarity of B1 is used to determine the polarity of the photoelectric response. When B1 has negative polarity, the photoresponse has normal polarity and proton pumping is in the direction of the alignment field i.e., in the same direction as the permanent electric dipole moment of the PM. The photoelectric response from WT BR in an aqueous PM suspension exhibits a reversal in the polarity of the photoelectric response below pH 4.5 i.e., the fast component, B1, has positive polarity below pH 4.5. This means that proton pumping is opposite to the alignment field below pH 4.5. Because the permanent electric dipole moment always points in the direction of the alignment field, the permanent electric dipole moment and proton pumping are in opposite directions at this pH, and the dipole moment points from the N-terminal face to the C-terminal face of the PM i.e., the permanent dipole moment is reversed below pH 4.5 (Barabas et al., 1983). When BR is immobilized in a PM-polyacrylamide gel or in a PM-Teflon membrane at pH 7 there is no polarity reversal when the pH is lowered. This verifies that the reversal of the polarity of the photoelectric response

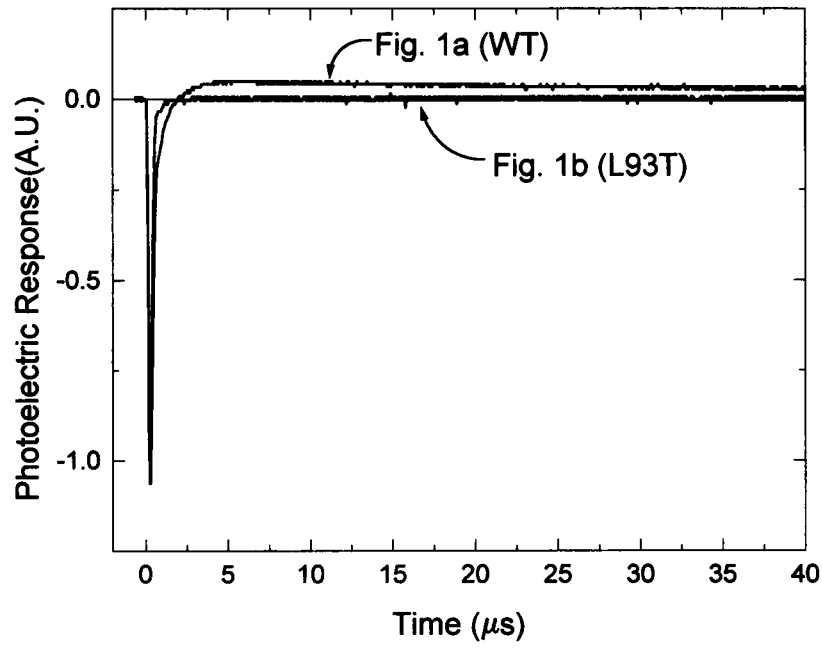

FIGURE 1 The photoelectric response of WT BR (*a*) and a mutant (L93T) BR (*b*) in an aqueous PM suspension (pH 7.1) is shown. The positive phase in the WT BR photoelectric response indicates proton pumping. The positive phase in *b* is too small to be detected because the kinetics of proton pumping are slow in the mutant. The initial negative phase, which is associated with the initial photoevent, is easily detected in both the WT and mutant BR.

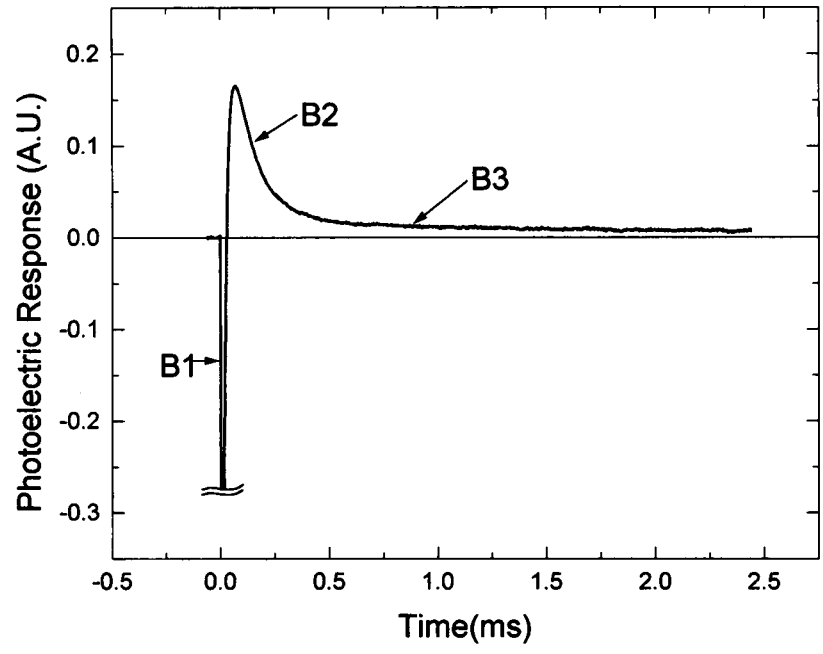

FIGURE 2 The photoelectric response from a PM-polyacrylamide gel of mutant (L93T) BR at pH 7.1 is shown. The positive phases, B2 and B3, in the photoelectric response are clearly measurable in this system. For all the mutants studied, we found that the initial component, B1, is always opposite in polarity to B2 and B3.

in the aqueous PM suspension at low pH is because of a reversal of the PM permanent electric dipole moment.

Fig. 3 shows the photoelectric response from WT BR in an aqueous PM suspension at pH 6. Only the initial fast component, B1, in the photoelectric response is shown. Also shown in Fig. 3 are the photoelectric responses of three mutant BR's: V49A, D212N, and L93T. We have observed that the B1 component is present in the photoresponse of all the mutants so far studied, regardless of whether or not they pump protons. At this pH, B1 has negative polarity for WT BR and V49A, but positive polarity for L93T and D212N. The polarity of B1 determines the polarity of the photoelectric response. When B1 has negative polarity, proton pumping is in the direction of the alignment field and the permanent electric dipole moment points from the C-terminal face to the N-terminal face of the PM. When the polarity of B1 is positive it indicates that the direction of the PM permanent electric dipole moment is reversed. As previously reported for WT BR we find that the polarity of B1 is pH dependent. Above pH 7, the polarity of B1 is negative for WT BR and all three mutants.

Fig. 4 shows the peak amplitude (and polarity) of B1 as a function of pH for WT and 3 mutant BR's:, L93T, L93W, and F208R. The pH for the reversal of the polarity of the

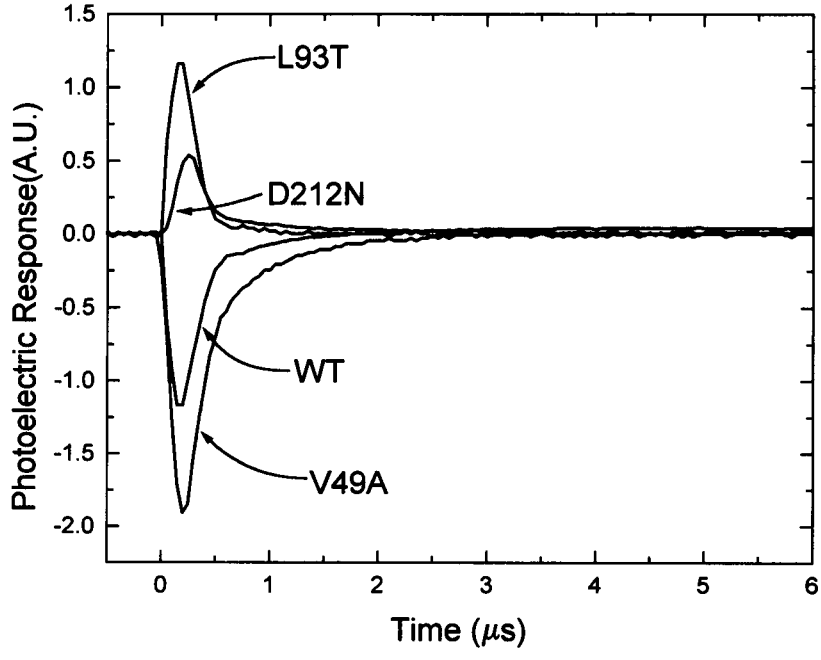

FIGURE 3 This figure shows the initial component, B1, in the photoelectric response from four different aqueous PM suspensions at pH 6: WT BR, V49A, D212N, and L93T. The permanent electric dipole moment of the PM for the BR mutants D212N and L93T is reversed from that of WT BR at pH 6 (see text).

photoelectric signal was found using similar data for each of the 27 mutants and determined the pK_a for reversal of the permanent electric dipole moment for each BR mutant. This data is summarized in Table 1, which shows the pK_a for reversal of the electric dipole moment of each BR mutant studied. The polarity of the photoresponse of D85N is still defined by the polarity of B1 even though this mutant does not pump protons.

Subramaniam et al. (Subramaniam et al., 1991) studied three mutant BR's, L93A, L93T, and L93V, obtained using an *Escherichia coli* expression system. When the WT bop gene is expressed in this system the resulting BR has an absorbance maximum, λ_{max}, shifted 10 nm to the blue from WT BR. We measured the peak spectral absorbance at pH 7 for the leu-93 mutants, both light and dark adapted. These values are given in Table 2.

The λ_{max} values of the mutants, L93T and L93V expressed in the *Escherichia coli* expression system, are shifted ~10 nm toward the blue from those obtained using the *Halobacterium halobium* expression system (Subramaniam et al., 1991). Such differences may reflect abnormal folding in the *Escherichia coli* expression system (Lanyi, 1993). The λ_{max} of L93V expressed in the *Halobacterium halobium* system is near that of WT BR, both light and dark adapted. All the other leu-93 mutants have a blue-shifted λ_{max}. Like the L93T mutant obtained from the *Escherichia coli* expression system, L93T has a red shift in the peak absorbance after dark adaptation. Subramaniam et al. (1991) propose that the effect of the substitution leu-93→thr-93 on the absorbance spectrum is either due to changes in the leu-93 interaction with the C-13 methyl group or to perturbations of the protein structure in the vicinity of the retinal.

TABLE 1 pK_a for B1 polarity reversal*

Mutant	pK_a
WT, D38R, T46V, V49A, V49M, A53G, Y57F, D85E, T89A, L93E, L93M, L93S, L93V, D96A, D96N, D115N, G155D, E166G, W182F, Y185F, F219L, R227Q	4.5 ± 0.5
L93F	5.5 ± 0.5
L93T	6.7 ± 0.1
D85N	6.8 ± 0.1
D212N	6.9 ± 0.1
L93W	7.2 ± 0.1
F208R	7.4 ± 0.1

*All the samples were washed in 100 mM NaCl solution with 10 mM HEPES buffered at pH 7.

DISCUSSION

Our results indicate that the polarity reversal of the photoelectric response of the mutants D85N, D212N, F208R, and L93T relative to WT BR at pH 6 is due not to a fundamental change in the photoelectric behavior but rather to a reversal in orientation of the PM. This conclusion is based on our observation that the polarity of the photoelectric response does not vary with pH if the orientation of the PM is fixed by immobilization in gel or absorption to a Teflon film. The reversal of the orientation of the PM in an alignment field indicates a reversal of the permanent electric dipole moment in these mutants.

What is the origin of the dipole moment? In the experiments described here the torque acting on the PM in a static electric field defines the electric dipole moment of the PM. Therefore, the permanent electric dipole moment is due to

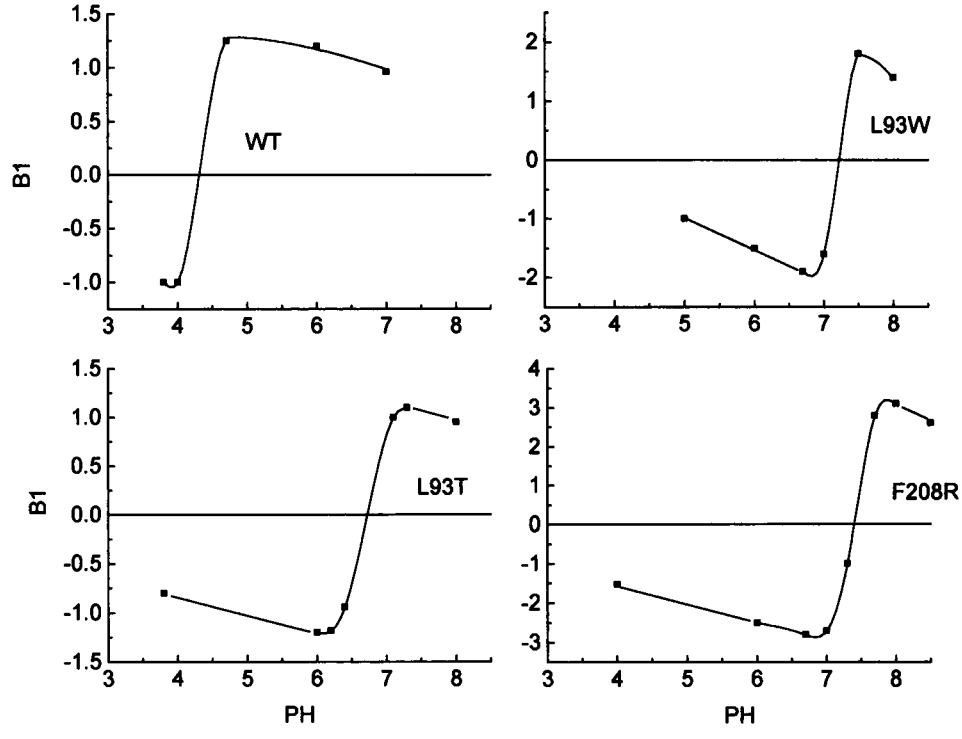

FIGURE 4 The peak amplitude and polarity of B1 as a function of pH is shown for WT BR and the BR mutants L93T, L93W, and F208R. The pK_a for reversal of the PM permanent dipole moment was found from these curves.

TABLE 2 Absorption maximum of light-dark adaptation of WT and 93 mutants*

Mutants	Absorption maximum (nm) of dark adaptation	Absorption maximum (nm) of light adaptation
WT	560	568
L93V	556	564
L93E	548	549
L93M	550	550
L93F	547	547
L93S	541	541
L93T	541	537
L93W	540	537

*All the samples were in 100 mM NaCl solution with 10 mM HEPES buffered at pH 7.

charge asymmetry about the center of mass of the PM. We take the center of mass of the PM to lie at the center of the membrane. The electric dipole is then $\Sigma_i q_i x_i$ where the sum i is over all ionic charges in the PM, x_i is the distance of the charge from the center plane of the PM and q_i is the charge at a site i. Charges within the PM have little effect on the dipole moment for two reasons: first, a charge located near the center of the PM has only a small contribution to the dipole moment because x is small; second, there is a large Born solvation energy for isolated charges in the interior of the BR molecule (Bashford and Gerwert, 1992). This energetically favors ion-counterion pairs (with negligible contribution to the dipole moment) or a shift in the pK_a of internal ionizable groups to favor a noncharged state (Bashford and Gerwert, 1992). Experiments support these calculations and indicate that only charges at the surface of the PM make significant contribution to the dipole moment (Jonas et al., 1990; Trissl, 1990). As only charges on the PM surface contribute to the dipole moment, the permanent electric dipole moment of the PM is given by $t(Q_c - Q_n)/2$ where t is the PM thickness, Q_c is the charge on the cytoplasmic surface, Q_n is the charge on the extracellular surface and $(Q_c - Q_n)$ is the PM surface charge asymmetry (Otomo et al., 1986).

The PM has a net negative surface charge; both surfaces of the membrane are negatively charged, and the cytoplasmic side is more negative than the extracellular side. This surface charge is due to ionizable amino acid residues at the surface, membrane lipids with acidic head groups, and the C-terminal and N-terminal of the polypeptide chain, which are on opposite sides of the membrane (Jonas et al., 1990; Szundi and Stoeckenius, 1989; Alexiev et al., 1994). The C-terminal is on the cytoplasmic side of the membrane and the N-terminal is on the extracellular side of the membrane (Henderson et al., 1990). A comprehensive review article by Jonas et al. (1990) discusses the experimental evidence for the surface charge asymmetry of PM. The results indicate that the C-terminal side of the membrane is more negatively charged than the N-terminal side. Recent results obtained by Alexiev et al. (1994) using covalently attached pH indicator dyes verify this surface charge asymmetry. The polarity of the photoelectric response measured by us is consistent with an electric dipole moment that points from the C-terminal side to the N-terminal side of the PM. Other photoelectric measurements, discussed in Trissl's review (1990), support this result i.e., the C-terminal side of the PM is more negative than the N-terminal side. When the pH is reduced below pH 4.5, the polarity of the photoelectric response of an aqueous PM suspension is reversed (Barabas et al., 1983), which indicates a reversal in the surface charge asymmetry at low pH. Consistent with this is the surface charge asymmetry determined from adsorption of PM to poly-L-lysine-treated glass (Fisher et al., 1978), which shows that the C-terminal face is more negative than the N-terminal face at pH 7.4 and that this charge asymmetry is reversed at pH 3. All of these experiments are evidence that permanent electric dipole moment of the PM is due to the surface charge asymmetry of the PM. We used the photoelectric response of an aqueous PM suspension to measure the reversal of the PM surface charge asymmetry of mutant BR relative to WT BR.

The surface charge asymmetry at pH 6 was reversed from that of WT BR in the BR mutants D85N, L93T, F208R, and D212N. Asp-85 and asp-212 are in the retinal pocket, form part of the active site in BR and are charged at pH 6 (Lanyi, 1993; Ebrey, 1993). These results show that these single-site mutations in the interior of BR have a distal effect. The substitution asp→asn in the mutants D85N and D212N involves replacement of the charged acidic side chain with an uncharged derivative. Phe-208 is located in the proton channel toward the extracellular surface of the PM (Henderson et al., 1990). The mutant F208R replaces a neutral aromatic hydrophobic side chain with a side chain that is ionizable. Leu-93 lies in the retinal pocket near the center of the PM. The substitution leu→thr replaces an aliphatic side chain that is hydrophobic with an aliphatic side chain containing a hydroxyl group making the group hydrophilic. This substitution does not involve an ionizable group so that changes to the PM surface charge by electrostatic interactions is ruled out. Therefore, the reversal of surface charge asymmetry in this mutant, L93T, is evidence that the substitution alters protein folding at the surface and changes the pK_a of ionizable groups on the surface.

To determine the effect of different side chains, six different amino acid substitutions at leu-93 were tested in addition to leu→thr. Thr is the hydroxylated form of val. We found that absorbance spectrum of L93V differed only slightly from WT BR and the reversal of surface charge asymmetry occurred at the same pH as WT BR. This suggested that the hydroxyl group in the side chain of the amino acid was an important factor so leu-93 was substituted with ser-93. We expected L93S to behave like L93T but it didn't. The reversal of surface charge asymmetry for L93S occurred at the same pH as WT BR. Like all the leu-93 substitutions, except leu→val, there was a blue shift in λmax relative to WT BR. Two other substitutions leu→met and leu→glu behaved like leu→ser. Met is larger than ser and hydrophobic rather than hydrophilic, whereas

glu is an amino acid with an ionizable side chain. The final two substitutions to leu-93 that we investigated, leu-93→phe-93 and leu-93→trp-93, involve amino acids with aromatic side chains that are very hydrophobic. In spite of this both of the BR mutants L93F and L93W behave like L93T i.e., the pK_a for reversal of the PM permanent electric dipole moment is greater than that of WT BR.

Other investigators have proposed that altering charged amino acids within the interior of BR could alter the protein's secondary structure distal to the site of mutation (Engelhard et al., 1990; Alexiev et al., 1994). Using covalently bound pH indicator dyes, Alexiev et al.(1994) measured a change in the PM surface charge due to a single-site mutation at asp-96 (asp→asn). They suggested that single-site mutations can alter the PM surface charge either by changing the protein folding at the surface, which would change the pK_a of ionizable groups at the surface (Bashford and Gerwert, 1992), or through direct or indirect electrostatic interactions. Alexiev et al. concluded that the changes in surface charge were not due to protein structural changes, because such changes were not observed by Subramaniam et al. (1993) in D96G. However, calculations indicate that the required structural changes are smaller than the structural resolution (Bashford and Gerwert, 1992). In other studies, Engelhard et al. (1990) used NMR to detect and study structural changes accompanying three different single-site substitutions (asn, gly, and glu) at asp-96; three single-site mutants, D96N, D96G, and D85E, were studied. They concluded that these amino acid substitutions perturbed not only their immediate environment but also that of more distant amino acids.

CONCLUSIONS

Our conclusion is that some single-site mutations can cause unexpected distal changes in the protein. This conclusion is based on the nonlocal effect of several single-site mutants on a static property of the purple-membrane i.e., the electric dipole moment. In particular, we found that the electric dipole moment of PM is reversed at pH 6 for the single-site mutants D85N, L93T, L93W, F208R, and D212N. These mutations are in the interior of the protein, whereas the electric dipole moment has its origin in charge groups at the membrane surface. Both charged and uncharged residue replacement cause these distal changes in the protein. We propose that changes in protein folding at the PM surface, distal to the site of the substitution, are responsible for the nonlocality of the effect.

This result has two important implications: first for mechanistic studies of BR in which site-directed mutagenesis is used as a probe to identify and study an active site—the possibility of nonlocal effects of residue replacement must be considered in developing structure-function relations; second for the use of BR in device applications in which mutagenesis is used to enhance the material properties. In this case the nonlocal effect of a mutation can have important advantages in developing a modified BR material with improved optical or electrical properties. Such modified BR can be found by developing a library of random mutants, which is then screened by simple and rapid physical measurements. A great spectrum of enhanced material properties can be obtained by such an approach. In other work we have identified mutants with improved material properties that were not expected based on the structure of unmodified BR. This work will be described elsewhere.

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