JOURNAL OF BACTERIOLOGY, Mar. 1993, p. 1555–1560
0021-9193/93/061555-06$02.00/0

Vol. 175, No. 6

MINIREVIEW

Mechanism of Light-Dependent Proton Translocation by Bacteriorhodopsin

MARK P. KREBS AND H. GOBIND KHORANA*

Departments of Biology and Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

INTRODUCTION

How do membrane proteins transport ions against a concentration gradient? The clearest answer to this question comes from studies of the light-dependent proton pump, bacteriorhodopsin (bR) (23, 50). bR is located in the cellular membrane of the archaeon *Halobacterium halobium*, where it pumps protons from the cell interior to the external medium. To drive proton translocation, bR absorbs energy from light via a covalently bound retinal chromophore. The primary goals in studying bR have been to determine what changes occur in the protein following light absorption and how those changes are coupled to proton transport. Here, we discuss the contributions of site-specific mutagenesis and biophysical techniques toward this goal. Several recent reviews focus on biophysical aspects of bR studies (27, 32, 44, 56).

PROPERTIES OF bR

bR is one of several integral membrane proteins whose structure is known in detail. The sequence of its 248-amino-acid polypeptide chain (Fig. 1) has been determined at both the protein (24, 43) and nucleotide (11) level. Its tertiary structure has been modeled from electron diffraction measurements of the purple membrane (19), a two-dimensional crystalline lattice of bR that forms in *H. halobium*. At the 3.5-Å (0.35-nm) resolution obtained by this approach, the protein appears as seven transmembrane α-helices (boxed sequences in Fig. 1), with bulky hydrophobic residues clearly visible. The α-helices form a bundle that provides a binding pocket for retinal, which is linked as a protonated Schiff base (PSB) to the ε-amino group of Lys-216 (Fig. 2).

Much of what is known about the response of bR to light has been obtained from spectroscopic studies. Typically, bR is activated by a nanosecond pulse of laser light and the ensuing structural changes are observed spectroscopically on a time scale of nanoseconds to milliseconds. Time-resolved UV-visible spectroscopy detects changes in the chromophore and its environment. Resonance Raman vibrational spectroscopy probes with greater resolution the retinal structure and the Schiff base protonation state. Time-resolved Fourier transform infrared (FTIR) spectroscopy provides information on the chromophore and on protein residues. This technique has been essential for monitoring the protonation state of amino acid side chains. Finally, pH-sensitive dyes have been used to follow proton release and uptake.

These methods have led to a description of events occurring in bR upon illumination (28, 32, 44, 50). Photon absorption causes the bound retinal to isomerize from an all-*trans* retinal to a 13-*cis* configuration (Fig. 2). The change in configuration moves the PSB into a new protein environment and results in a metastable state, which relaxes thermally to the initial bR_{568} form in a pathway consisting of at least five intermediates (Fig. 2). This photochemical reaction pathway, or photocycle, can be divided into (i) the release phase, during which the PSB gives up its proton to a nearby acceptor, resulting in release of a proton from the extracellular side of the protein, and (ii) the uptake phase, during which the Schiff base picks up a proton from a nearby donor, resulting in uptake of a proton from the cytoplasmic side. Ultimately, the retinal is reisomerized to the all-*trans* configuration and the protein returns to its initial state. Site-specific mutagenesis has helped to define these events and to identify amino acid residues that participate in proton translocation.

PREPARATION OF bR MUTANTS

Mutants prepared by site-specific techniques can be expressed in the native organism or in a heterologous system. Since methods for *H. halobium* transformation were not initially available, bR mutants were first expressed in *Escherichia coli* (23). The study of mutants was made possible by several achievements: (i) construction of a synthetic gene containing restriction enzyme sites for efficient cassette mutagenesis (38) and its expression in *E. coli* (22), (ii) extraction and purification of the apoprotein from *E. coli* membranes (8), and (iii) refolding of the apoprotein and its regeneration with all-*trans* retinal in lipid-detergent micelles (21). Mutant proteins prepared in this way are studied spectroscopically and reconstituted in lipid vesicles to measure proton translocation.

Methods for expressing bR mutants in *H. halobium* have recently been developed. This complementary approach allows bR mutants to be studied in their native form and enables their structure to be determined by X-ray, neutron, or electron diffraction. In early experiments, mutants were isolated by random mutagenesis in vivo and an enrichment scheme that selected against cells growing under phototrophic conditions (46, 47). More recently, transformation and expression methods have become available, allowing site-specific mutagenesis to be used in *H. halobium* (9, 10, 25, 26, 39, 40, 55). So far, all of the bR mutants expressed in *H. halobium* form a crystalline lattice identical to the purple membrane (5, 39). Their properties are generally similar to

* Corresponding author.

those of mutants expressed in *E. coli* (26), although distinct spectral behavior has been noted in a few cases (26, 39, 55).

Most bR mutants have been expressed in *E. coli*. Figure 1 shows the locations of the single amino acid substitutions studied, roughly 140 changes at about 90 different sites. Initial mutagenesis targets were amino acid residues with side chains that might participate in proton transfers during transport: aspartic and glutamic acids (37), arginines (49), tyrosines (36), and serines and threonines (30) (the involvement of lysine residues was previously addressed by alkylation studies [1]). In addition, membrane-embedded residues that might be structurally important, such as prolines (35) and tryptophans (34) were mutated. Amino acid residues predicted to constitute the retinal binding pocket (19) have also been substituted (14, 52) to address protein-chromophore interactions. Finally, cysteines have been introduced at about 40 positions throughout the protein for derivatization with structural probes (3, 12, 13, 15).

IDENTIFYING AMINO ACIDS REQUIRED FOR PUMPING

We have classified amino acid residues in bR into three groups according to how their substitution affects proton translocation. Substitutions in the first group (shown in yellow in Fig. 1) result in wild-type or nearly wild-type proton transport activity. Most mutants in this group (100 of 120) have activity identical to that of wild-type bR. The activity of the remaining 20 mutants is greater than ~50% wild-type activity.

The second group (shown in blue in Fig. 1) includes positions where mutations result in 10 to 40% wild-type activity. Several findings suggest that substitutions at these positions perturb the structure of the protein-chromophore complex. (i) Their effect is often greatest when nonconservative replacements are made. For example, the proton pumping activities of P186L (17) and P186V (35) are 20 and 12%, respectively, of wild-type bR activity. In contrast, the activities of mutants containing flexible side chains, P186A and P186G, are 65 and 85%, respectively (35). Similarly, changing a bulky hydrophobic residue to alanine, as in M118A and M145A, results in activities of 20 and 10%, whereas the corresponding glutamic acid mutants have wild-type activity (14). (ii) Many of the substitutions result in shifted absorption spectra and slower retinal regeneration rates. Such mutants form retinal isomers other than 13-*cis* upon illumination, suggesting that the retinal environment is altered. (iii) The substituted residues are part of 21 amino acids facing the protein interior predicted to form the retinal binding pocket (19). With the exception of Trp-137 and Met-145, these residues are conserved among six retinal proteins related to bR (54). It is therefore likely that they are important for maintaining the structure of halobacterial retinylidene proteins.

The third group (shown in red in Fig. 1) includes amino acid residues at which substitutions reduce proton translocation to less than 10% of the wild-type activity. This group includes three aspartic acids located within the transmembrane portion of bR. The severe effects of isosteric substitutions at these positions suggest that these residues are essential for proton transport. Accordingly, this group of mutants has been studied in detail.

KEY STEPS IN PUMPING INVOLVE ASPARTIC ACIDS

The Schiff base proton is released to Asp-85 during the L→M transition. The release phase of the photocycle includes the intermediates K, L, and M (Fig. 2). Resonance Raman spectroscopy established that in all of these intermediates, the chromophore is in a 13-*cis* conformation, with slight differences in the rotation around other bonds in the polyene chain (32). Significantly, these studies indicated that the PSB loses its proton during formation of the M intermediate. Where does this proton go? FTIR studies had implicated aspartic acids as likely acceptors, since several of these residues undergo protonation changes during formation of the M intermediate (44).

Site-specific substitution of Asp-85 indicated that it acts as the proton acceptor. (i) Neutral substitutions of Asp-85 abolished proton translocation activity (37, 42). (ii) D85A and D85N had defective photocycles in which effectively no M intermediate was formed (42). (iii) Mutations of this residue affected the absorption spectrum and the pK_a of the PSB, consistent with the proximity of Asp-85 and the PSB (42, 53). Indeed, Asp-85 is placed ~4 Å (~0.4 nm) from the PSB in the structural model (19). (iv) The mutants were used to assign resonance peaks in FTIR studies (7), and the kinetics of Schiff base deprotonation were found to match the kinetics of Asp-85 protonation (44). The simplest explanation of these results is that Asp-85 is positioned to directly receive a proton from the Schiff base during the L→M transition.

Asp-96 participates in reprotonation of the Schiff base during the M→N transition. In the uptake phase of the photocycle, the structural alterations that accumulated in the first portion of the photocycle are reversed and the protein is returned to its preillumination state. This phase includes the N and O intermediates. The first step that occurs in this phase is reprotonation of the Schiff base (32), and mutagenesis studies revealed that Asp-96 is required for this process. The mutants D96A and D96N reduce pumping activity to 2 and 3% of wild-type values, respectively (37, 41). Such mutants slowed the rate of M decay and proton uptake by more than 10-fold at neutral pH (20, 29, 41, 48). However, these effects were reversed by increasing the proton concentration or by providing a diffusable proton donor such as azide (41), suggesting that Asp-96 acts as an internal proton donor. The mutants were used to assign FTIR resonance peaks (7), establishing that Asp-96 is deprotonated during the M→N transition (44). These results indicate that Asp-96 mediates Schiff base reprotonation.

Asp-212 is required for bR structure. The function of Asp-212 in proton pumping has been more difficult to interpret. Neutral substitutions of Asp-212 severely reduce activity (37). These mutants also bleach during illumination or fail to form chromophore altogether (31, 37, 53), implying that Asp-212 is required for stabilizing the retinal linkage during the photocycle. Unlike Asp-85 and Asp-96, Asp-212 is invariant in six known halobacterial retinal-containing proteins (54), suggesting an important function for Asp-212, perhaps in the formation and stability of the PSB.

ADDITIONAL STEPS IN PROTON PUMPING

Arg-82 participates in proton release. During M formation, a proton is released from the extracellular face of bR, as monitored with a pH-sensitive dye (16). The fastest release times, determined by tethering such a dye to the extracellular surface of the protein (18), follow the same time course as

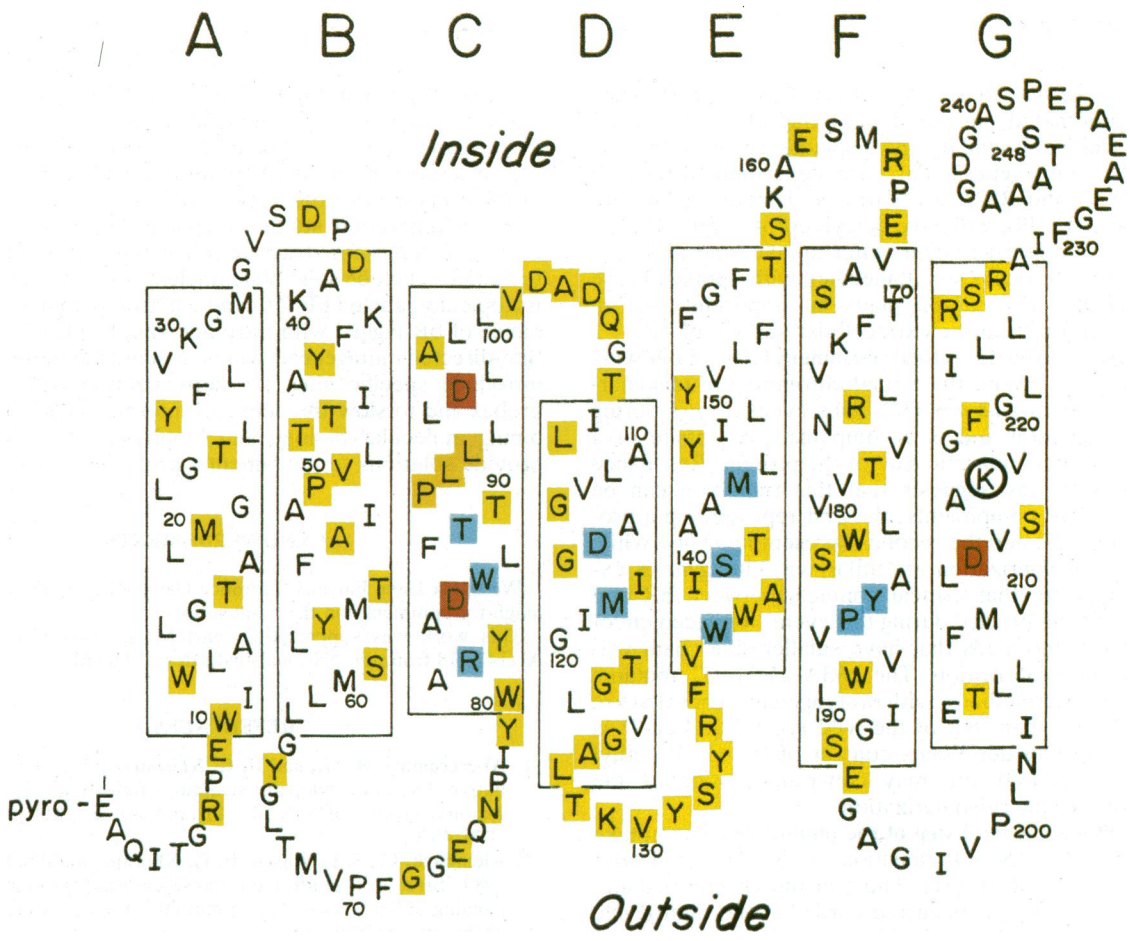

FIG. 1. Secondary structure model of bR. Boxed regions correspond to the seven transmembrane α-helices derived from the bR structural model (19). Amino acids facing the interior of the α-helical bundle run up the middle of each box. The lysine which binds retinal is indicated by a circle. The amino acids changed are shown in color, according to the effect of substitutions on the initial rate of proton translocation. Red, no activity; blue, low activity; yellow, wild-type activity. Amino acids were substituted one at a time as follows: Thr→Val and then Ser→Ala, except at position 169 (30); Tyr→Phe (36); Trp→Phe (34); Asp→Asn (37); Glu→Gln, except at position 9 (17, 33, 49a) Arg→Gln (49); Pro→Ala or Gly (35); and Met→Ala or Glu (14). In addition, the following substitutions were made: Asp (at positions 85, 96, 115, and 212)→Ala or Glu (20, 37, 41, 42); Glu (at positions 9 and 74)→Asp (33); Val-49→Leu or ala (14); Ala-53→Gly (14); Arg-82→Ala (42); Thr-89→Ala or Asp (30); Leu-93→Ala, Thr, or Val (52); Asp-85→His (51); Lys-129→Gln (33); Pro-186→Leu or Val (17, 35); Asp-212→Gln or Val (31); Phe-219→Ala (14). Finally, cysteines were introduced at positions 72, 90, 92, 169 (12), 74, 76, 79, 82, 85, 96, 98, 101, 103, 105, 109, 113, 116, 117, 122 (13, 15), and 125 to 142 (3).

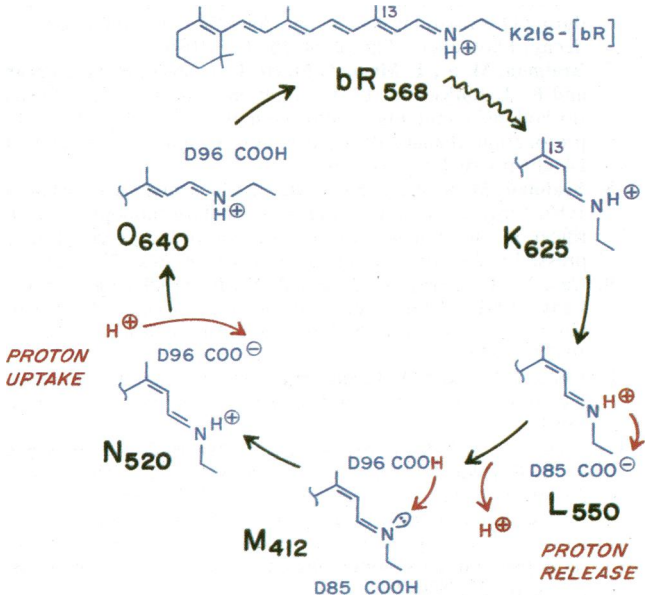

FIG. 2. bR photocycle and the mechanism of proton translocation. The ground state and photocycle intermediates are shown in black. Subscripts denote the absorption maxima (in nanometers). Arrows indicate the direction of the photocycle. The wavy arrow indicates the only light-dependent step. The retinal chromophore and key aspartic acids are shown in blue. The protonated Schiff base linkage of all-*trans* retinal to the protein is indicated for the ground state (bR_{568}). The protein moiety and the left hand portion of the retinal have been omitted in the intermediates for clarity. Proton transfer steps are shown in red.

the absorbance changes associated with M formation. This result presents a puzzle: since the Schiff base proton is donated to Asp-85 and since Asp-85 remains protonated in M (and in O, as we discuss below), what is the source of the proton that is released into the medium?

One possibility is that a proton on Arg-82 is displaced during the events in the L→M transition. Arg-82 is likely to form a salt bridge with Asp-85 (49) and may lose a proton in response to changes in the protonation state of this residue. An alternative explanation is that in response to the neutral-

ization of negative charge in the protein interior when Asp-85 is protonated, the guanidino group of Arg-82 moves to the extracellular boundary and displaces a proton from the surface. The mutagenesis data are consistent with both models. R82Q and R82A are partially defective in proton translocation (31, 49), and have delayed proton release (42). In R82A, proton release occurs after uptake, and in R82Q, release appears to occur simultaneously with uptake. These results indicate that Arg-82 plays an important role in releasing a proton from the extracellular surface of bR.

Leu-93 is involved in retinal reisomerization. Following Schiff base protonation, the retinal chromophore reisomerizes from 13-*cis* to all-*trans* (32). This event occurs during the N→O transition and is accompanied by uptake of a proton from the cytoplasmic side of the protein. Resonance Raman spectroscopy suggests that this transition can be resolved into two components, the first representing a protonated form of N, and the second representing O (4). While the details of isomerization are unknown, mutagenesis experiments indicate that critical contacts between bR and retinal must be maintained during this event. Replacement of Leu-93 with amino acids that have smaller side chain volumes slows reisomerization. The L93A and L93T mutants have N and O intermediates with prolonged lifetimes that are 160 times longer than that of the wild type (52). Leu-93 is situated within van der Waals contact of the C-13 methyl group of retinal (19) and may constrain motion of the chromophore during reisomerization.

Tyr-185 affects the final step of the photocycle. The uptake of a proton in the N→O transition (4) is correlated with reprotonation of Asp-96 (41). Thus, in the O intermediate, most of the events that occurred earlier in the photocycle have been reversed. The retinal is in an all-*trans* configuration, the Schiff base is reprotonated, and Asp-96 has regained a proton. Nevertheless, the altered absorption (50), FTIR (44), and resonance Raman (32) spectra of the O intermediate indicate that further rearrangements to reach the bR_{568} form must occur. Mutant Y185F affects the O→bR transition (45). When illuminated, Y185F produces a long-lived species with spectral features characteristic of O, which exists in a pH-dependent equilibrium with bR (45). At high pH, the photocycle proceeds normally, but the decay of the O intermediate is greatly slowed. The slowed decay permits characterization of the protonation states of aspartic acids in this intermediate. FTIR studies of this O-like species in Y185F confirm that Asp-96 is reprotonated in the N→O transition and that Asp-85 remains protonated (6). The defective photocycle in Y185F is likely to result from a perturbed interaction with Asp-212. Tyr-185 has been suggested to form a hydrogen bond with Asp-212 (44) that would not exist in the phenylalanine substitution.

SUMMARY

Site-specific mutagenesis has identified amino acids involved in bR proton transport. Biophysical studies of the mutants have elucidated the roles of two membrane-embedded residues: Asp-85 serves as the acceptor for the proton from the isomerized retinylidene Schiff base, and Asp-96 participates in reprotonation of this group. The functions of Arg-82, Leu-93, Asp-212, Tyr-185, and other residues that affect bR properties when substituted are not as well understood. Structural characterization of the mutant proteins will clarify the effects of substitutions at these positions.

Current efforts in the field remain directed at understanding how retinal isomerization is coupled to proton transport. In particular, there has been more emphasis on determining the structures of bR and its photointermediates. Since well-ordered crystals of bR have not been obtained, continued electron diffraction studies of purple membrane offer the best opportunity for structure refinement. Other informative techniques include solid-state nuclear magnetic resonance of isotopically labeled bR (56) and electron paramagnetic resonance of bR tagged with nitroxide spin labels (2, 3, 13, 15). Site-directed mutagenesis will be essential in these studies to introduce specific sites for derivatization with structural probes and to slow the decay of intermediates. Thus, combining molecular biology and biophysics will continue to provide solutions to fundamental problems in bR.

ACKNOWLEDGMENTS

We thank Dave Farrens, Florence Davidson, and Duncan Greenhalgh for comments on the manuscript.

This work was supported in part by grants GM28289-12 and AI11479-18 from the National Institutes of Health.

REFERENCES

1. **Abercrombie, D. M., and H. G. Khorana.** 1986. Regeneration of native bacteriorhodopsin structure following acetylation of ε-amino groups of Lys-30, -40, and -41. J. Biol. Chem. **261:** 4875–4880.
2. **Altenbach, C., S. L. Flitsch, H. G. Khorana, and W. L. Hubbell.** 1989. Structural studies on transmembrane proteins. 2. Spin labeling of bacteriorhodopsin mutants at unique cysteines. Biochemistry **28:**7806–7812.
3. **Altenbach, C., T. Marti, H. G. Khorana, and W. L. Hubbell.** 1990. Transmembrane protein structure: spin labeling of bacteriorhodopsin mutants. Science **248:**1088–1092.
4. **Ames, J. B., and R. A. Mathies.** 1990. The role of back-reactions and proton uptake during the N→O transition in bacteriorhodopsin's photocycle: a kinetic resonance Raman study. Biochemistry **29:**7181–7190.
5. **Antoniou, M., M. P. Krebs, R. M. Mollaaghababa, and M. P. Heyn.** Unpublished results.
6. **Bousché, O., S. Sonar, M. P. Krebs, H. G. Khorana, and K. J. Rothschild.** 1992. Time-resolved FTIR spectroscopy of the bacteriorhodopsin mutant Tyr-185 → Phe: Asp-96 reprotonates during O formation; Asp-85 and Asp-212 deprotonate during O decay. Photochem. Photobiol. **56:**1085–1096.
7. **Braiman, M. S., T. Mogi, T. Marti, L. J. Stern, H. G. Khorana, and K. J. Rothschild.** 1988. Vibrational spectroscopy of bacteriorhodopsin mutants: light-driven proton transport involves protonation changes of aspartic acid residues 85, 96 and 212. Biochemistry **27:**8516–8520.
8. **Braiman, M. S., L. J. Stern, B. H. Chao, and H. G. Khorana.** 1987. Structure-function studies on bacteriorhodopsin. IV. Purification and renaturation of bacterio-opsin polypeptide expressed in *Escherichia coli*. J. Biol. Chem. **262:**9271–9276.
9. **Cao, Y., G. Váró, M. Chang, B. Ni, R. Needleman, and J. K. Lanyi.** 1991. Water is required for proton transfer from aspartate-96 to the bacteriorhodopsin Schiff base. Biochemistry **30:** 10972–10979.
10. **Cline, S. W., and W. F. Doolittle.** 1987. Efficient transfection of the archaebacterium *Halobacterium halobium*. J. Bacteriol. **169:**1341–1344.
11. **Dunn, R., J. McCoy, M. Simsek, A. Majumdar, S. H. Chang, U. L. RajBhandary, and H. G. Khorana.** 1981. The bacteriorhodopsin gene. Proc. Natl. Acad. Sci. USA **78:**6744–6748.
12. **Flitsch, S. L., and H. G. Khorana.** 1989. Structural studies on transmembrane proteins. 1. Model study using bacteriorhodopsin mutants containing single cysteine residues. Biochemistry **28:**7800–7805.

13. **Greenhalgh, D. A., C. Altenbach, W. L. Hubbell, and H. G. Khorana.** 1991. Locations of Arg-82, Asp-85, and Asp-96 in helix C of bacteriorhodopsin relative to the aqueous boundaries. Proc. Natl. Acad. Sci. USA **88:**8626–8630.
14. **Greenhalgh, D. A., D. L. Farrens, S. Subramaniam, and H. G. Khorana.** Unpublished results.
15. **Greenhalgh, D. A., and H. G. Khorana.** Unpublished results.
16. **Grzesiek, S., and N. A. Dencher.** 1986. Time-course and stoichiometry of light-induced proton release and uptake during the photocycle of bacteriorhodopsin. FEBS Lett. **208:**337–342.
17. **Hackett, N. R., L. J. Stern, B. H. Chao, K. A. Kronis, and H. G. Khorana.** 1987. Structure-function studies on bacteriorhodopsin. V. Effects of amino acid substitutions in the putative helix F. J. Biol. Chem. **262:**9277–9284.
18. **Heberle, J., and N. A. Dencher.** 1992. Surface-bound optical probes monitor proton translocation and surface potential changes during the bacteriorhodopsin photocycle. Proc. Natl. Acad. Sci. USA **89:**5996–6000.
19. **Henderson, R., J. M. Baldwin, T. A. Ceska, F. Zemlin, E. Beckmann, and K. H. Downing.** 1990. Model for the structure of bacteriorhodopsin based on high-resolution electron cryomicroscopy. J. Mol. Biol. **213:**899–929.
20. **Holz, M., L. A. Drachev, T. Mogi, H. Otto, A. D. Kaulen, M. P. Heyn, V. P. Skulachev, and H. G. Khorana.** 1989. Replacement of aspartic acid-96 by asparagine in bacteriorhodopsin slows both the decay of the M intermediate and the associated proton movement. Proc. Natl. Acad. Sci. USA **86:**2167–2171.
21. **Huang, K.-S., H. Bayley, M.-J. Liao, E. London, and H. G. Khorana.** 1981. Refolding of an integral membrane protein. Denaturation, renaturation, and reconstitution of intact bacteriorhodopsin and two proteolytic fragments. J. Biol. Chem. **256:**3802–3809.
22. **Karnik, S. S., M. Nassal, T. Doi, E. Jay, V. Sgaramella, and H. G. Khorana.** 1987. Structure-function studies on bacteriorhodopsin. II. Improved expression of the bacterio-opsin gene in *Escherichia coli*. J. Biol. Chem. **262:**9255–9263.
23. **Khorana, H. G.** 1988. Bacteriorhodopsin, a membrane protein that uses light to translocate protons. J. Biol. Chem. **263:**7439–7442.
24. **Khorana, H. G., G. E. Gerber, W. C. Herlihy, C. P. Gray, R. J. Anderegg, K. Nihei, and K. Biemann.** 1979. Amino acid sequence of bacteriorhodopsin. Proc. Natl. Acad. Sci. USA **76:**5046–5050.
25. **Krebs, M. P., T. Hauss, M. P. Heyn, U. L. RajBhandary, and H. G. Khorana.** 1991. Expression of the bacterioopsin gene in *Halobacterium halobium* using a multicopy plasmid. Proc. Natl. Acad. Sci. USA **88:**859–863.
26. **Krebs, M. P., R. M. Mollaaghababa, and H. G. Khorana.** Gene replacement and expression of bacteriorhodopsin mutants in *Halobacterium halobium*. Proc. Natl. Acad. Sci. USA, in press.
27. **Lanyi, J. K.** 1992. Proton transfer and energy coupling in the bacteriorhodopsin photocycle. J. Bioenerg. Biomembr. **24:**169–179.
28. **Lozier, R. H., A. Xie, J. Hofrichter, and G. M. Clore.** 1992. Reversible steps in the bacteriorhodopsin photocycle. Proc. Natl. Acad. Sci. USA **89:**3610–3614.
29. **Marinetti, T., S. Subramaniam, T. Mogi, T. Marti, and H. G. Khorana.** 1989. Replacement of aspartic residues 85, 96, 115, or 212 affects the quantum yield and kinetics of proton release and uptake by bacteriorhodopsin. Proc. Natl. Acad. Sci. USA **86:**529–533.
30. **Marti, T., H. Otto, T. Mogi, S. J. Rösselet, M. P. Heyn, and H. G. Khorana.** 1991. Bacteriorhodopsin mutants containing single substitutions of serine or threonine residues are all active in proton translocation. J. Biol. Chem. **266:**6919–6927.
31. **Marti, T., S. J. Rösselet, and H. G. Khorana.** Unpublished data.
32. **Mathies, R. A., S. W. Lin, J. B. Ames, and W. T. Pollard.** 1991. From femtoseconds to biology: mechanism of bacteriorhodopsin's light-driven proton pump. Annu. Rev. Biophys. Biophys. Chem. **20:**491–518.
33. **Mogi, T., and H. G. Khorana.** Unpublished data.
34. **Mogi, T., T. Marti, and H. G. Khorana.** 1989. Structure-function studies on bacteriorhodopsin. IX. Substitutions of tryptophan residues affect protein-retinal interactions in bacteriorhodopsin. J. Biol. Chem. **264:**14197–14201.
35. **Mogi, T., L. J. Stern, B. H. Chao, and H. G. Khorana.** 1989. Structure-function studies on bacteriorhodopsin. VIII. Substitutions of the membrane-embedded prolines 50, 91, and 186: the effects are determined by the substituting amino acids. J. Biol. Chem. **264:**14192–14196.
36. **Mogi, T., L. J. Stern, N. R. Hackett, and H. G. Khorana.** 1987. Bacteriorhodopsin mutants containing single tyrosine to phenylalanine substitutions are all active in proton translocation. Proc. Natl. Acad. Sci. USA **84:**5595–5599.
37. **Mogi, T., L. J. Stern, T. Marti, B. H. Chao, and H. G. Khorana.** 1988. Aspartic acid substitutions affect proton translocation by bacteriorhodopsin. Proc. Natl. Acad. Sci. USA **85:**4148–4152.
38. **Nassal, M., T. Mogi, S. S. Karnik, and H. G. Khorana.** 1987. Structure-function studies on bacteriorhodopsin. III. Total synthesis of a gene for bacterio-opsin and its expression in *Escherichia coli*. J. Biol. Chem. **262:**9264–9270.
39. **Needleman, R., M. Chang, B. Ni, G. Váró, J. Fornés, S. H. White, and J. K. Lanyi.** 1991. Properties of $Asp^{212} \rightarrow Asn$ bacteriorhodopsin suggest that Asp^{212} and Asp^{85} both participate in a counterion and proton acceptor complex near the Schiff base. J. Biol. Chem. **266:**11478–11484.
40. **Ni, B., M. Chang, A. Duschl, J. Lanyi, and R. Needleman.** 1990. An efficient system for the synthesis of bacteriorhodopsin in *Halobacterium halobium*. Gene **90:**169–172.
41. **Otto, H., T. Marti, M. Holz, T. Mogi, M. Lindau, H. G. Khorana, and M. P. Heyn.** 1989. Aspartic acid-96 is the internal proton donor in the reprotonation of the Schiff base of bacteriorhodopsin. Proc. Natl. Acad. Sci. USA **86:**9228–9232.
42. **Otto, H., T. Marti, M. Holz, T. Mogi, L. J. Stern, F. Engel, H. G. Khorana, and M. P. Heyn.** 1990. Substitution of amino acids Asp-85, Asp-212, and Arg-82 in bacteriorhodopsin affects the proton release phase of the pump and the pK of the Schiff base. Proc. Natl. Acad. Sci. USA **87:**1018–1022.
43. **Ovchinnikov, Y. A., N. G. Abdulaev, M. Y. Feigina, A. V. Kiselev, and N. A. Lobanov.** 1979. The structural basis of the functioning of bacteriorhodopsin: an overview. FEBS Lett. **100:**219–224.
44. **Rothschild, K. J.** 1992. FTIR difference spectroscopy of bacteriorhodopsin: toward a molecular model. J. Bioenerg. Biomembr. **24:**147–167.
45. **Sonar, S., M. P. Krebs, H. G. Khorana, and K. J. Rothschild.** Static and time-resolved absorption spectroscopy of the bacteriorhodopsin mutant Tyr-185 → Phe: evidence for an equilibrium between bR_{570} and an O-like species. Biochemistry, in press.
46. **Soppa, J., and D. Oesterhelt.** 1989. Bacteriorhodopsin mutants of *Halobacterium* sp. GRB. 1. The 5-bromo-2′-deoxyuridine selection as a method to isolate point mutants in *Halobacteria*. J. Biol. Chem. **264:**13043–10348.
47. **Soppa, J., J. Otomo, J. Straub, J. Tittor, S. Meessen, and D. Oesterhelt.** 1989. Bacteriorhodopsin mutants of *Halobacterium* sp. GRB. II. Characterization of mutants. J. Biol. Chem. **264:**13049–13056.
48. **Stern, L. J., P. L. Ahl, T. Marti, T. Mogi, M. Duñach, S. Berkowitz, K. J. Rothschild, and H. G. Khorana.** 1989. Substitution of membrane-embedded aspartic acids in bacteriorhodopsin causes specific changes in different steps of the photochemical cycle. Biochemistry **28:**10035–10042.
49. **Stern, L. J., and H. G. Khorana.** 1989. Structure-function studies on bacteriorhodopsin. X. Individual substitutions of arginine residues by glutamine affect chromophore formation, photocycle, and proton translocation. J. Biol. Chem. **264:**14202–14208.

49a. **Stern, L. J., and H. G. Khorana.** Unpublished data.

50. **Stoeckenius, W., R. H. Lozier, and R. A. Bogomolni.** 1979. Bacteriorhodopsin and the purple membrane of halobacteria. Biochim. Biophys. Acta **505:**215–278.
51. **Subramaniam, S., D. A. Greenhalgh, and H. G. Khorana.** 1992. Aspartic acid 85 in bacteriorhodopsin functions both as proton

acceptor and negative counterion to the Schiff base. J. Biol. Chem. **267**:25730–25733.

52. **Subramaniam, S., D. A. Greenhalgh, P. Rath, K. J. Rothschild, and H. G. Khorana.** 1991. Replacement of leucine-93 by alanine or threonine slows down the decay of the N and O intermediates in the photocycle of bacteriorhodopsin: implications for proton uptake and 13-*cis* retinal → all-*trans*-retinal reisomerization. Proc. Natl. Acad. Sci. USA **88**:6873–6877.
53. **Subramaniam, S., T. Marti, and H. G. Khorana.** 1990. Protonation state of Asp(Glu)-85 regulates the purple-to-blue transition in bacteriorhodopsin mutants Arg-82 → Ala and Asp-85 → Glu: the blue form is inactive in proton translocation. Proc. Natl. Acad. Sci. USA **87**:1013–1017.
54. **Uegaki, K., Y. Sugiyama, and Y. Mukohata.** 1991. Archaerhodopsin-2, from *Halobacterium* sp. aus-2 further reveals essential amino acid residues for light-driven proton pumps. Arch. Biochem. Biophys. **286**:107–110.
55. **Váró, G., L. Zimányi, M. Chang, B. Ni, R. Needleman, and J. K. Lanyi.** 1992. A residue substitution near the β-ionone ring of the retinal affects the M substates of bacteriorhodopsin. Biophys. J. **61**:820–826.
56. **Zheng, L., and J. Herzfeld.** 1992. NMR studies of retinal proteins. J. Bioenerg. Biomembr. **24**:139–146.