We have no objection from a patent standpoint to the publication or dissemination of this material.

Mr. D. J. Donnell *Mar 10, 2000*
Office of Intellectual Property Counsel

DOE Field Office, Chicago

Final Report

DOE Grant 95ER20171

Molecular Mechanisms Controlling Proton Pumping by Bacteriorhodopsin

Rosalie K. Crouch, PI; Thomas G. Ebrey, Co-PI.

The focus of our studies was on the key mechanisms of light-induced proton transfer during light energy transduction in bR. Site directed mutagenesis, spectroscopic methods and methods of chemical modification were developed and applied. The most important accomplishments are:

- 1) discovery of the complex titration of the primary proton acceptor, Asp85;
- 2) development of the concept of coupling of the primary proton acceptor Asp85 with the proton release group;
- 3) discovery of the role of transient protonation of Asp85 in catalysis of thermal isomerization of the chromophore;
- 4) development of methodology to routinely map the technically challenging bR (being a hydrophobic integral membrane protein) by mass spectroscopy.

These findings comprise a new approach for investigation and interpretation of the properties of the pigment in the initial (ground state) and the photolyzed intermediate states. These properties are particularly important for the understanding of early and late light-induced proton release in bR and its mutants. The results are described in 7 papers, 1 book chapter, 2 manuscripts in preparation and 11 abstracts (not listed). Below is a short review of the results. The insights gained from these results have received considerable attention (e.g. Lanyi, 1996; Karlin, 1997) and are fundamental breakthroughs in the understanding of proton transport through membrane proteins and hence, the conversion of light into energy.

I. Construction of new mutants. A number of new mutants of bR were constructed and expressed in *Halobacterium salinarum*. They are: R82K, R82H, K129H, R134K, R134H, E204N, E204A, E204Q (expressed also earlier by Brown et al., 1995), E194C, M145C and some other mutants. Investigation of the properties of these mutants were crucial for a number of important findings.

II. Discovery of the complex titration of Asp85 in the purple to blue transition. Development of the concept of interacting (coupled) residues in the proton release channel in bR [1].

It was shown earlier that protonation of Asp85 is associated with a large red shift of the chromophore absorption band and formation of the so called blue membrane. Absorption at wavelengths longer than 680 nm is almost entirely due to the blue membrane. By measuring absorption changes at 680 nm as a function of pH we determined the pH dependence of the fraction of the blue membrane, f_{bm} , in R82K and thus the fraction of protonated Asp85 in a broad pH range. An important finding was that the titration curve for Asp85 has two pK_a 's [1].

The pK_a of the purple-to-blue transition at low pH (which reflects the pK_a of Asp85) is 3.6 for R82K. At high pH a second inflection in the purple-to-blue transition with $pK_a=8.0$ is found. The complex titration behavior of Asp85 indicates that the pK_a of Asp85 depends on the protonation state of another amino acid residue, X', which has a $pK_a=8.0$ in R82K.

Earlier we suggested a model of two interacting residues with coupled protonation states which could account for the complex titration of Asp85 (Balashov et al., 1993). The titration of Asp85 in R82K was the first experimental evidence for coupling of the pK_a of Asp85 with another titratable group, which presumably acts as the proton release group (PRG). The fit of the experimental data to the model of two interacting residues shows that deprotonation of X' at high pH causes a shift in the pK_a of Asp85 from 3.7 to 6.0. In turn, protonation of Asp85 decreases the pK_a of X' by 2.3 pH units. This suggests that at neutral pH, X' can release a proton upon formation of the M intermediate

RECEIVED
APR 04 2000
OSTI

DISCLAIMER

This report was prepared as an account of work sponsored by an agency of the United States Government. Neither the United States Government nor any agency thereof, nor any of their employees, make any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by trade name, trademark, manufacturer, or otherwise does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government or any agency thereof. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States Government or any agency thereof.

DISCLAIMER

Portions of this document may be illegible in electronic image products. Images are produced from the best available original document.

and the concomitant protonation of Asp85 in the photocycle.

The concept of coupling of protonation states of the primary proton acceptor and counterion to the Schiff base, Asp85, and the PRG has several implications for the mechanism of proton release, light-induced Schiff base deprotonation and interpretation of the phenotypes of several mutants with elevated fractions of blue membrane [1].

III. Elucidation of the key role of protonation of Asp85 in the catalysis of thermal isomerization in bR [1].

Our initial observation that the thermal isomerization, as a function of pH, is proportional to the fraction of blue membrane, was made on R82A (Balashov et al., 1993) where the titration of Asp85 is described by a simple monoprotic titration (Henderson-Hasselbalch equation). In R82K, we demonstrated that the rate constant of dark adaptation, k_{da} , is proportional to the fraction of blue membrane in a broad pH range (between pH 2 and 10), and most importantly, the pH dependence of blue membrane (and protonated Asp85) is described not by a simple monocomponent titration but by a more complicated, two component function with $pK_{a1}=3.9$ and $pK_{a2}=8.0$. This finding was important for two reasons:

- i) It demonstrated that the proportionality between the fraction of the blue membrane (f_{bm}) and the rate constant of thermal isomerization (k_{da}) is not just a peculiarity of R82A but a more general phenomenon. The results on R82K were encouraging so we next established this relationship for WT (Balashov et al., 1996) and K129H [3]. Based on these findings we suggested that the mechanism of thermal isomerization of the chromophore includes transient protonation of Asp85 which substantially reduces the barrier for isomerization and increases the rate by at least four orders of magnitude.
- ii) The proportionality between k_{da} and f_{bm} enabled us to use the *pH dependence of dark adaptation as a tool for investigating of effect of different mutations on coupling of Asp85 with the PRG* (measurements of pH dependence of dark adaptation in many cases is a much easier task than a spectral titration to determine very small amounts of protonated Asp85 at high pH). This approach was utilized recently by Lanyi and coworkers (Richter et al., 1996) to characterize Glu204 mutants and for identification of Glu204 as the PRG.

IV. Establishment of a link between the ground state properties of bR, which can be determined from the titration and dark adaptation experiments, with the proton release pattern (fraction of early and late proton release) [1,2,3].

From titration and dark adaptation experiments, the pK_a 's of Asp85 and the residue X', controlling the pK_a of Asp85, was determined (see Table 1 in [2]). Our hypothesis was that X' is in fact the PRG X. One of the predictions of this hypothesis was that the pK_a of the PRG in M should be close to the pK_a of X' in the state where Asp85 is protonated (because in M Asp85 is also protonated). Another prediction was that when coupling between Asp85 and X' is absent (because of removal of the PRG, or dramatic decrease of its pK_a in the ground state or by somehow reducing the interaction between the two groups), there should be no fast proton release. Both predictions were confirmed in the experiments. Three typical cases were observed: a) WT, strong coupling; b) R82K, weakened coupling; c) E194C and E204N, very small coupling or absence of coupling. In the last case there was no fast proton release in agreement with the prediction. In the first two cases the PRG pK_a in M determined from the fraction of the fast proton release, as suggested by Zimanyi et al. (1992) was systematically about 1 unit < the pK_a of X in the blue membrane. This difference we attributed to a different state of the Schiff base (protonated in the blue membrane/deprotonated in M). These experiments provided strong evidence that X' and the PRG is the same group. Further evidence in support of this conclusion was obtained in the lab of J. K. Lanyi (Richter et al., 1996). Using the same approach they found that Glu204 shows all the features of the PRG X and the residue controlling pK_a of Asp85, X'.

V. Establishment of a correlation between the rate of light-induced Schiff base deprotonation, and proton affinity of Asp85 [1-3,6,7,9].

In R82A, R82Q and R82K the formation of M is very fast 1.5 μ s [1, 2], and pH independent. We explain this by the high proton affinity (pK_a) of Asp85 in these mutants [1]. The pK_a of Asp85 for the first two mutants is 7.2, and 4.8 for R82K. In R82H, the pK_a of Asp85 is unusually low, 1, and the rate of M formation is slow (ca. 100 μ s) [9]. This correlation is observed not only in Arg82 mutants but in other mutants too: K129H [3], E194C [6], R134K [7], E204A and E204N [8]. The most likely explanation is that the initial high pK_a of Asp85 indicates more hydrophobic environment that favors the protonated state of Asp85 [1].

VI. Increase in the rate of M formation at high pH can be attributed to the deprotonation of the PRG in the ground state [1-3, 6, 7, 9].

The well known dramatic increase in the rate of the L to M transition (light-induced deprotonation of the Schiff base) at high pH in the WT has always been related to the deprotonation of some residue at high pH (Balashov et al., 1991 and earlier work from several labs). Based on the ability of the PRG upon deprotonation to increase the proton affinity of Asp85 (pK_a) [1], we suggested that deprotonation of this group in the ground state is responsible for transition of the pigment into an alkaline form with slightly red shifted absorption spectrum and an increased rate of the L $\rightarrow$ M transition in the photocycle [1]. The properties of E204Q (Richter et al., 1996; Govindjee et al., 1996), E204A and E204N [8] which lack the PRG, and other mutants with an altered pK_a of the PRG in the ground state K129H [3], R134K [7], E194C [6], R82H [9], are consistent with this hypothesis.

VII. Evidence was obtained that partial transformation of the pigment into a blue shifted species absorbing at around 480 nm (the P480 species) may reflect deprotonation of the PRG. A small red shift of the chromophore and the tryptophan absorption band most likely are also indicative of deprotonation of the PRG [1].

We have noticed that besides the main transition of the purple pigment into P480 with $pK_a > 11$, there is partial transformation of a small fraction of pigment into P480 at lower pH, with a pK_a close to the pK_a of deprotonation of the PRG (as revealed from both titration and dark adaptation experiments). This observation led us to propose that this partial transformation into P480 is related to the deprotonation of the PRG [3]. Though this relationship has to be investigated further, the data obtained on a number of mutants are consistent with this idea. The pK_a 's of the small red shifts of the chromophore and tryptophan absorption bands also correlate with the pK_a of deprotonation of the PRG [1] in several studied cases (R82K, R82H). The shifts are absent in the mutants lacking the PRG or having weak coupling of this group with Asp85 (R82Q, E204Q, E204N, E194C).

VIII. Establishing the role of Arg82 in the control of the pK_a the PRG [1,2, 9].

In order to further characterize the role of Arg82 in the light-induced proton pumping in bR, we replaced Arg82 with either nonionizable alkaline or glutamine (R82A, R82Q), or ionizable lysine or histidine (R82K, R82H) and studied the ground state and photochemical properties of these mutants. Earlier studies by Subramaniam et al. 1990; Otto et al., 1990 with mutants expressed in *E.coli* clearly showed that substitution of Arg82 with neutral residues drastically increases the pK_a of Asp85. Our experiments with analogous mutants but expressed in native *H. salinarium* confirmed this conclusion (Balashov et al., 1993).

The next question was what is the role of Arg82 in the proton release. Since fast (early) proton release is inhibited in R82A and occurs after uptake, it was obvious that Arg82 is involved in this process directly or indirectly (Balashov et al., 1993). Replacement of Arg82 with lysine, which is also positively charged at neutral pH but has an intrinsic pK_a of about 1.7 pH units lower than Arg, causes similar 1.7 pH unit shift in pK_a of the PRG compared to WT. This supported the hypothesis that X' is

Arg82 in WT and Lys82 in R82K or at least that these groups are the principal part of a cluster of residues which constitute X'. Subsequent experiments in the laboratories of Lanyi and our group showed that the second possibility is correct. Evidence was provided that Glu204 is the PRG (Brown et al., 1995a; Richter et al., 1996; Govindjee et al., 1996) while *Arg82 is a group that strongly affects the pK_a of the PRG and is likely to serve as an important factor for the coupling between Asp85 and Glu204* (3).

In WT, light-induced proton release occurs before uptake at neutral pH. In contrast, in mutants where Arg82 is replaced by a neutral residue (as in R82A and R82Q), only a small fraction of the protons is released before proton uptake at neutral pH; the major fraction is released after uptake. In R82Q we find that the relative amounts of the two types of proton release, "early" (preceding proton uptake) and "late" (following proton uptake), are pH dependent. The main conclusions are: (1) Arg82 is not the light-driven PRG; early proton release can be observed in R82Q at higher pHs, suggesting that the PRG has not been eliminated. (2) Arg82 affects the pK_a of the PRG, Glu204 (Brown et al., 1995), both in the unphotolyzed state of the pigment and during the photocycle. In the WT (in 150 mM salt) the pK_a of this group decreases from ca. 9.5 in the unphotolyzed pigment to ca. 5.8 in the M intermediate, leading to early proton release at neutral pH. In the Arg82 mutants, the respective pK_a 's of the PRG in the unphotolyzed pigment and in M are ca. 8 and 7.5 in R82Q (in 1 M salt), and ca. 8 and 6.5 in R82K (in 150 mM KCl). Thus in R82Q, the pK_a of the PRG does not decrease enough in the photocycle to allow early proton release from this group at neutral pH. (3) In R82Q, at neutral pH, proton uptake from the medium occurs during the formation of O. The proton is released during the O $\rightarrow$ bR transition, probably from Asp85 since the normal PRG cannot deprotonate at this pH. (4) The time constant of early proton release is increased from 85 μ s in the WT to 1 ms in R82Q (in 150 mM salt). This can be directly attributed to the increase in the pK_a of the PRG and also explains the uncoupling of proton release from M formation. (5) In E204Q only late proton release is observed both at neutral and alkaline pH, consistent with Glu204 being the PRG.

In R82K [1], a flash-induced transient signal of the pH-sensitive dye pyranine is similar to that in the WT (proton release precedes uptake) but the amplitude is small (about 15% of that found in the WT) indicating that only a small fraction of protons are released fast in R82K. This supports the suggestion that Arg82 is associated with the proton release pathway and that Lys cannot efficiently substitute for Arg in this process. The pH dependent light adaptation in R82K results in a pH-dependent yield of the M intermediate ($pK_a=8.0$). Two other reversible transitions with similar pK_a s are observed: a 2 nm red shift of the chromophore absorption band and a red shift of the absorption band of a Trp residue.

In R82H [9] interaction between Asp85 and His82 is stronger than interaction of Asp85 with Arg82 in WT as indicated by a lower pK_a of Asp85 in the ground state ($pK_a = 1$). At the same time coupling between Asp85 and the PRG (presumably Glu204) is weakened. This results in a small fraction of fast proton release at neutral pH. At pH 6.6, most of the proton release (ca. 80%) occurs with the time constant 40 ms, after the proton uptake (21 ms). At higher pH, the fraction of fast proton release increases with pK_a 7.2 (versus 5.8 in the WT).

IX. Identification of photocurrents that are related to proton release [2].

Early proton release in R82Q can be detected as a photocurrent signal which is kinetically distinct from those photocurrents due to proton movements from the Schiff base to Asp85 during M formation and from Asp96 to the Schiff base during the M $\rightarrow$ N transition. This opens the possibility of studying proton release using photoelectric techniques.

X. Glu194 is a part of a proton release complex, or is a group that strongly affects the pK_a of the PRG [6].

Substitution of Glu194, a residue on the extracellular surface of bR, with a cysteine inhibits the fast

light-induced proton release that normally is coupled with the deprotonation of the Schiff base during the L to M transition. Proton release in E194C occurs at the very end of the photocycle and coincides with deprotonation of the primary proton acceptor, Asp85, during the O to bR transition. The E194C mutation also results in a slowing down of the photocycle by about an order of magnitude as compared to WT, and produces a strong effect on the pH dependence of dark adaptation that is interpreted as a drastic reduction or elimination of the coupling between the primary proton acceptor Asp85 and the PRG [6]. The pH dependence of M formation is abolished in E194C. The lack of an increase in the rate constant of M rise in E194C at high pH is in agreement with the conclusion that the coupling between Asp85 and the PRG is absent or dramatically decreased in E194C and/or that the pK_a of the PRG is greatly decreased in the ground state.

These data indicate that Glu194 is a critical component of the proton release complex in bR and that in eliminating Glu194, we have removed a crucial portion of the normal proton release machinery, since light-induced fast proton release is no longer observed. Glu194 in native bR may serve as a part of the PRG, or as a structural constraint that keeps the pK_a of the PRG (presumably E204) high in the ground state.

XI. K129H mutation strongly affects the pK_a of the PRG and Asp85 [3].

We suggest that Lys129 may be involved in stabilizing the hydrogen bonding network that couples Glu204 and Asp85. Substitution of Lys129 with a histidine residue causes structural changes which alter this coupling and affect the pK_a s of Glu204 and Asp85. Lysine-129 is a residue located in the extracellular loop connecting transmembrane helices D and E of bR. Replacement of Lys129 with a histidine alters the pK_a s of two key residues in the proton transport pathway, Asp85 and the PRG (probably Glu204); the resulting pigment has properties which differ markedly from those of WT.

1) In the unphotolyzed state of K129H, the pK_a of Asp85 is 5.1 in 150 mM KCl (compared to ~2.6 in WT), while the unphotolyzed-state pK_a of Glu204 decreases to 8.1 (from ~9.5 in the wild type pigment). 2) The pK_a of Glu204 in the M state is 7.0 in K129H, compared to ~5.8 in the wild type pigment. 3) As a result of the change in the pK_a of Glu204 in M, the order of light-induced proton release and uptake exhibits a dependence on pH differing from the wild type: at neutral pH and moderate salt concentrations (150 mM KCl), light-induced proton uptake precedes proton release, while it follows proton release at higher pH. This pumping behavior is similar to that seen in a related bacterial rhodopsin, archaerhodopsin-1, which has a histidine in the position analogous to Lys129. 4) At alkaline pH, a substantial fraction of all-*trans*- K129H pigment (ca. 30%), undergoes a conversion into a shorter wavelength species, P480, with pK_a ~ 8.1, close to the pK_a of Glu204. 5) Guanidine hydrochloride lowers the pK_a 's of Asp85 and Glu204 in the ground state and the pK_a of Glu204 in the M intermediate, and restores the normal order of proton release before uptake at neutral pH. 6) The coupling between Asp85 and Glu204 is weaker in K129H than in WT. The change in the pK_a s of either residue in the unphotolyzed pigment is only 1.5 units compared to 4.9 units in WT. In the M state, the corresponding change of photolyzed K129H is 1 unit, compared to 3.7 units in WT.

XII. In several mutants, proton uptake coincides with the N->O transition [2,3,4,6,8].

In the absence of the putative PRG, Glu204 (e.g., E204Q), the second half of the photocycle is slowed down more than 10-fold compared to the wild type. Parallel measurements of light-induced pH changes and formation of the O intermediate in E204Q, and the effects of pH and D₂O on the M decay and O formation rates in E204Q suggest that *proton uptake occurs concurrently with the N <-> O transition*, possibly coupled with the thermal reisomerization of the retinal. Hence, one of the rate limiting steps in the slow E204Q photocycle is proton uptake from the outside medium, coincidentally with the decay of the slow component of M (the N <-> O transition). The close kinetic correspondence between proton uptake and the N->O transition was also observed in R82Q [2], E194C [6] and R82H at low pH [9].

XIII. In the absence of Glu204, the PRG (or the key element of the proton release complex), proton transfer from Asp85 to the surface becomes the rate limiting step; the rate of proton transfer can be

increased by addition of azide and weak acids (cyanate and formate). This effect of exogenous acids suggests that they can serve as proton acceptors in the extracellular channel of bR [4].

In the photocycle of E204Q, the long lifetime of decay of the O state, due to a high activation barrier for the deprotonation of Asp85 in the O $\rightarrow$ bR step, becomes the rate-limiting step. Addition of the weakly acidic anions azide, cyanate, or formate accelerates the decay of the O intermediate, and restores the total photocycling time to that observed in WT, by accelerating the deprotonation of Asp85. We also find that azide similarly accelerates the decay of O in the wild type under conditions in which Glu204 does not deprotonate during the photocycle (pH < 6). It has previously been shown that azide and other weak acids can influence proton transfers in the cytoplasmic half of the protein (Tittor et al., 1989); we suggest that these weak acids can affect proton transfers in the extracellular half of the protein as well.

XIV. The photocurrents associated with the photoconversion of 13-cis-bR were detected. Their dependence on D₂O points out that proton movement is involved. It is suggested that transient uptake of proton and protonation of Asp85 is involved in photoconversion of 13-cis-bR [5].

Two photocurrent components were found in R82K, with 30 and 300 μ s lifetimes; they are due to the photocycle of the 13-cis rather than the all-trans form of the pigment. A large D₂O effect suggests that the charge motions producing these photocurrents are related to proton movement within the molecule. The current amplitudes depends on the protonation states of at least two residues, Asp85 and the PRG, presumably Glu204. In R82Q, a 10 μ s photocurrent is observed that also depends on the protonation state of Asp85 and is similar to the 30 μ s current in R82K. We attempt to explain these currents in terms of a model for interacting residues in the extracellular channel and catalysis of chromophore isomerization [1] suggesting that transient protonation of Asp85 may take place to allow thermal isomerization of the chromophore.

XV. In mutants with a nonfunctional PRG (E204N, E194C), the pH dependence of the fraction of O intermediate is similar to the WT. This indicates that though the pK_a of the PRG is a key factor in diverting the photocycle into two pathways (having fast or slow proton release), it is not the major factor controlling accumulation of the O intermediate [8].

The maximal transient concentration of the O intermediate in E204N and E194C decreases at high pH with pK_a 7.2-7.3 [8]. This is close to the value found earlier for WT (around ~7, Einfeld & Stockburger, 1993; Cao et al., 1993). This observation indicates that removal or inhibition of the PRG in these mutants does not affect the pH dependencies (pK_a) of the apparent yield and lifetime of O. This means that the transition between the low pH photocycle with a large amount of O intermediate and the high pH photocycle with a large amount of N intermediate is not completely determined by the pK_a of the PRG. Other groups, presumably Asp85 and Asp96, may control this process.

XVI. Analogue pigments formed with 9-desmethyl and 13-desmethyl retinal have shown that alteration of the chromophore structure in combination with protein mutation provides an innovative combination of methods for manipulating the photocycle [10]

The use of analogues of retinal lacking the 9 and/or 13 methyl in combination with the R82A mutation have allowed us to examine the photocycle and function of pigments in which the 13-cis to all-trans isomeric ratio is expected to be increased. The 9-methyl is critical to maintaining the absorption maximum of bR (Gartner et al, 1893) and the environment of interaction which causes this shift has not been altered in this mutant. The photocycles of the pigments are affected in that the R82A/9-dm shows an inhibition of M-formation and all the R82A/dm pigments show the 600 nm species. Proton pumping is eliminated in the R82A/13-dm pigment. These results support previous findings on the importance of the Arg82 residue and the retinal polyene methyl groups to the function of bR and illustrate the value of the combination of the two analogue approaches in

probing the complicated chromophore-protein interactions in the proton pumping process.

XVII. The technology to routinely sequence bR by mass spectrometry has been developed. This will allow for the crosslinking experiments described in the present proposal.

We have developed methodology to reliably map bR by mass spectrometry to confirm mutation and locate sites of chemical modifications and cross-links. Key to this methodology is the development of a chromatographic system with a mobile phase gradient of acetonitrile/isopropanol in aqueous TFA using a C-4 reversed phase column. For the cysteine mutants, we have found that it is essential to derivatize the protein before removal from the membrane. Analysis of the peptide fragments is done by online HPLC-MS and -MS/MS using an ion trap mass spectrometer.

Correspondence to the original plan of experiments:

Discovery of the complex titration of Asp85 [1] focused our research towards understanding the origin of this phenomenon and its role in the energy transduction process and particularly on light-induced Schiff base deprotonation and proton release. This became the most developed direction of the initial plan. The experiments on the determination of the sites of labeling have been slow, mostly due to the need to develop methodology for studying membrane proteins by mass spectroscopy. Finally, we have mapped bR by mass spectroscopy (aided by new instrumentation) and now results are being obtained. Progress was made on all of the proposed experiments although the direction did change with the discoveries discussed above.

LIST OF PUBLICATIONS THAT ACKNOWLEDGE DOE FUNDING

1. Balashov, S. P., R. Govindjee, E. S. Imasheva, S. Misra, T. G. Ebrey, Y. Feng, R. K. Crouch, and D. R. Menick. 1995. The two pK_a 's of aspartate-85 and control of thermal isomerization and proton release in the arginine-82 to lysine mutant of bacteriorhodopsin. *Biochemistry* **34**: 8820-8834.
2. Govindjee, R., S. Misra, S. P. Balashov, T. G. Ebrey, R. Crouch, and D. R. Menick. 1996. Arginine-82 regulates the pK_a of the group responsible for the light-driven proton release in bacteriorhodopsin. *Biophys. J.* **71**: 1011-1023.
3. Govindjee R., E.S. Imasheva, S. Misra, S.P. Balashov, T.G. Ebrey, N. Chen, D.R. Menick, and R. K. Crouch. 1997. Mutation of a surface residue, lysine-129, reverses the order of proton release and uptake in bacteriorhodopsin; guanidine hydrochloride restores it. *Biophys. J.* **72**: 886-898.
4. Misra, S., R. Govindjee, T.G. Ebrey, N. Chen, J.-X. Ma, and R.K. Crouch. 1997. Proton uptake and release are rate-limiting steps in the photocycle of the bacteriorhodopsin mutant E204Q. *Biochemistry* **36**: 4875-4883.
5. Misra, S., T.G. Ebrey, R.K. Crouch, D.R. Menick. 1997. Charge movements in the 13-*cis* photocycles of the bacteriorhodopsin mutants R82K and R82Q. *Photochem. Photobiol.* **65**:1039-1044.
6. Balashov, S. P., E. S. Imasheva, T. G. Ebrey, N. Chen, D. R. Menick, and R. K. Crouch. 1997. Glutamate-194 to cysteine mutation inhibits fast light-induced proton release in bacteriorhodopsin. *Biochemistry* **36**: 8671-8676, 1997.
7. Misra, S., T.G. Ebrey, R.K. Crouch, and D.R. Menick. 1997. Mutation of arginine 134 to lysine alters the pK_a s of key groups involved in proton pumping by bacteriorhodopsin. *Photochem. Photobiol.* **66**: 774-783, 1997.
8. Balashov, S. P., M. Lu, E. S. Imasheva, B. Othersen, III, R. K. Crouch and D. R. Menick. The proton release group of bacteriorhodopsin controls the rate of the final step of its photocycle at low pH. *Biochemistry* **38**: 2026-2039, 1999.
9. Crouch, R.K., D.R. Menick, Y. Feng, R. Govindjee and T.G. Ebrey 1997. Use of Demethyl Retinal Analogues for Probing of the Structure/Function of the Arg82Ala Bacteriorhodopsin Mutant. Monograph for Koji Nakanishi. pp 343-362, 1999.
10. L.E. Ball, J.E. Oatis, K. Dharmasiri, M. Busman, J. Wang, S. Cowden, A. Galijatovic, N. Chen, and D.R. Knapp. Mass spectrometric analysis of integral membrane proteins. Application to complete mapping of bacteriorhodopsins and rhodopsin. *Protein Science*, **7**:758-764, 1998.

MOLECULAR MECHANISMS CONTROLLING PROTON PUMPING BY BACTERIORHODOPSIN

Summary

Bacteriorhodopsin (bR) is the simplest biological system for the transduction of light energy. Light energy is directly converted to transmembrane proton gradient by a single, small membrane protein. The extraordinary stability of bR makes it an outstanding subject for bioenergetic studies.

This project has focused on the role of interactions between key residues of the pigment involved in light-induced proton transfer. Methods to estimate the strength of these interactions and their correlation with the rate and efficiency of proton transfer have been developed. The concept of the coupling of the protonation states of key groups has been applied to individual steps of the proton transfer with the ultimate goal of understanding on the molecular level the driving forces for proton transport and the pathway of the transported proton in bR. The mechanism of light-induced proton release, uptake and the mechanism of recovery of initial state of bR has been examined.

The experiments were performed with genetically engineered, site-specific mutants of bR. This has enabled us to characterize the role of individual amino acid residues in bR. Time resolved and low temperature absorption spectroscopy and light-induced photocurrent measurements was used in order to study the photochemical cycle and proton transfer in mutant pigments. Chemical modification and crosslinking of both the specific amino acids to the chromophore or to other amino acids were used to elucidate the role of light-induced conformational changes in the photocycle and the structure of the protein in the ground state.

The results of this project provided new knowledge on the architecture of the proton transfer pathways inside the protein, on the mechanism of proton release in bR, and on the role of specific amino acid residues in the structure and function of bR.