PROGRESS REPORT/Thomas G. Ebrey

My laboratory has been actively involved in research on the purple membrane and bacteriorhodopsin for many years. In this section I will confine myself to our studies in the past 4 years. The numbers refer to the list of papers at the end of this section.

The Blue Membrane and Cation Binding to Bacteriorhodopsin

Our goal was to explore the ways in which cations affected the spectral and photochemical properties of the blue membrane. In the 1991 PNAS paper (#6), we showed that under the proper conditions the blue to purple transition is controlled by the binding of a unique divalent cation. This strongly argues for a direct role for cations in color regulation of bR.

The Photocycle Transitions and Their Correlation with Proton Movement

We developed a fast flash photolysis apparatus which enables us to accurately record either absorbance or photodocument signals from oriented purple membranes with a time resolution of tens of ns (9). Focusing on the step thought to be involved with proton release, our lab showed that the kinetics of both the absorbance and the photocurrent signals for the L to M transition were identical and had the same pH dependence. In addition the rate constants were found to be independent of pH, but the amplitudes of the components varied with pH. We showed that the photocurrent signal was associated with proton release from the membrane and so proton release took place, within experimental error, as fast as M was formed (#1,2,7).

Photochemistry of Bacteriorhodopsin

In the first paper in the series (#4), we reinvestigated the quantum efficiency of the photocycle and found that it is 0.64. We also reported that at very high flash energies a new stable photoprodut of bR is formed with a very low quantum efficiency. All these experiments were at room temperature. We then showed that the quantum efficiency was unchanged either upon lowering the temperature to 77 K or changing the pH from 5 to 10 (#11). From the amount of K in the photosteady state, which we could infer from the low temperature experiments, we could then accurately calculate the absorption spectrum (abs. max at 603 nm) and extinction coefficient (0.83 that of bH) of K, which had long been a source of uncertainty. Finally, we reinvestigated the question of the heterogeneity in the absorption spectrum of K at liquid nitrogen temperatures and confirmed Balashov’s earlier work done in Moscow that there were multiple forms of K at 90 K, with slightly different absorption spectra (#12, 18).

Photochemistry of Modified Bacteriorhodopsins

We studied the photochemistry of bacteriorhodopsins in which either the protein (fluorotryptamine substituted for about 40% of the tyrosines during the growth of the bacteria, #13), or the chromophore (fluoresubstituted, paper #3; ring oxidized, paper #10) was chemically modified. We found that if the Schiff base nitrogen were methylated so that no proton could be released from the Schiff base, then no M type intermediate could be formed and no proton pumping was detected. This shows that the deprotonation of the Schiff base and the formation of M are required for proton pumping.

The fluororetinal based pigments are interesting because some of them have very red shifted absorption spectra (abs. max=680 nm) while others have the pK of their Schiff bases lowered by 2 pH units or so (#3). The ring oxidized retinals are expected to have an altered interaction with charges/dipoles near the ring part of the chromophore of bR; these groups are expected to help modulate the color of the chromophore. However, all the pigments could undergo a photocycle that leads to the pumping of protons (#10).

Most recently we have studied the photochemistry of the Y57N mutant. We have confirmed that this pigment is normally photochemically dead, but have found that after raising the pH, some deprotonated Schiff base (M type) intermediate can be formed by light (#14). Most recently we have shown that by manipulating the pK of the protonated Schiff base we can restore light-induced deprotonation even at neutral pH (#22). The implications of this are pursued in the proposal.

We have also begun to study the mutants of the arginine at position 82 (R82A) (#15; #18), as expressed in the native membrane of H. halobium. We have found that the pK
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of the purple to blue transition changes from ca. 2.8 to 7.5. We have also found that the pattern of dark adaptation is quite unusual, with a very different pH dependence than is seen for the native pigment. We have also found that the rate of proton release from this mutant is 100 times slower than in the wild type, leading to quite altered pH-sensitive dye signals. An explanation for these and other observations is provided in terms of simple electrostatic calculations.

Measurements of the Surface Charge Density of the Purple Membrane
We have critically evaluated many of the measurements of the surface charge density of the purple membrane (#5) and concluded that it must be significantly lower than that predicted by the simple model of the membrane. We suggest that several divalent cations must be bound to both sides of the purple membrane, lowering the surface charge density from that predicted by the structural model without cation binding. The review also contained a number of original calculations on the effect of the surface charge density on a) on the number of cations associated with the purple membrane and b) the number bound to the membrane as a function of bulk pH and bulk divalent cation concentration.

pH Dependent Changes in the Absorption Spectrum of Bacteriorhodopsin
In this study (#8) we showed that in the alkaline and neutral pH range there are two spectrally quite close but distinct forms of bacteriorhodopsin. The alkaline form, bRh, absorbs at about 1.5 nm longer wavelength than the neutral form and the intrinsic pK of the transition between them is 8.3. We found that the pK of the red shift correlated with the appearance of the fast rise time component in the L to M transition and with an absorption increase at 238 nm associated with the formation of a tyrosinate. We suggest that the formation of the tyrosinate is responsible for the red shift and for the formation of the fast rising form of the M intermediate, although other residues such as arginine cannot be excluded. We then showed that the photocurrent signal associated with proton release disappears as the pH is raised with the same intrinsic pK as the spectral shift and that the change in photochemistry. This suggests that the light absorbed by bRh can lead to the deprotonation of the Schiff base but not to the release of a proton from the pigment (#19).

Photoshemical and Proton pumping Properties of the Archaerhodopsins
Working with samples of the Clarab Membrane provided by Mukohata, we found in aR-1 the order of proton release and uptake is opposite from that of bR even though all the amino acids in the binding site are identical (paper #21). The role of surface surface amino acids, especially lys 129, is currently being tested.

PUBLICATIONS


ABSTRACT

The aim of this proposal is to understand the molecular mechanisms controlling the proton pump, bacteriorhodopsin (bR). Thus, its focus is on the binding site of bacteriorhodopsin, from bR's initial state through the transformations that it undergoes after absorbing a photon and as it returns to the initial state. In particular, the following five questions will be probed: 1) Can we determine by specific chemical labeling what amino acids are near particular portions of the chromophore? 2) What is the role of bound water in controlling the pK_a's of the Schiff base and of D85, the initial proton acceptor and part of the counter ion to the Schiff base? 3) What changes occur in bacteriorhodopsin to allow the Schiff base to deprotonate and initiate the proton pumping process? What controls the rate of Schiff base deprotonation? and 4) What properties of the binding site are responsible for the temperature dependent photochemistry of bR? How do retinal modifications and amino acid mutations change this temperature independence?

To carry out these studies, we will manipulate not only the protein part of the pigment but also the light-absorbing chromophore using chemically modified retinals. The experiments proposed here will be done on mutants of bR expressed in the H. salinarium in which the pigment is folded into and surrounded by the native membrane.

The program outlined above offers the exciting possibility of beginning to understand the molecular mechanisms which underlie the light driven movement of protons through bacteriorhodopsin and to use this understanding to control proton movement.

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BACKGROUND AND RATIONALE

Under special conditions of oxygen and light the archaebacterium *Halobacterium salinarium* (formerly *halobium*) synthesizes a very unusual membrane, the purple membrane. This membrane contains just a single type of protein, bacteriorhodopsin (bR). Light energy absorbed by the chromophore of bacteriorhodopsin is used to transport a proton across the cell membrane. The resulting proton gradient is used by the cell to make ATP and for other transport purposes (recently reviewed in Oesterhelt and Tittor, 1989; Mathies et al., 1991; El-Sayed, 1992; Lanyi, 1992, 1993; Rothchild, 1992; Eberly, 1993).

There is strong interest in bacteriorhodopsin as an example of a proton pump that can be deeply understood. At the detailed molecular level we believe there is a good possibility of understanding the workings of the proton pump with respect to: structure of the protein (Fig. 1 and 2), photochemical transformations and energetics of the photocycle (Fig. 3), and movement of the proton (Fig. 3). Of course these three aspects of the proton pump are connected, and an understanding of all three will ultimately be required.

Present Status of Knowledge

There are at least five sequential steps in the proton pumping process (Fig. 3), inferred from a wide variety of photochemical, proton pumping, and structural studies: Light absorbed by the retinal chromophore of bacteriorhodopsin isomerizes the chromophore and stores part of the photon's energy in a high free energy primary photoprotected, K. Next, the energy stored in K causes the pigment to undergo some sort of change allowing the proton on the Schiff base to leave the Schiff base and go to a proton acceptor, Asp 85. Third, as a consequence of this movement an unknown group deprotonates with the proton appearing in solution on the extracellular side of the membrane. Next, Asp 96 donates its proton to the Schiff base, reprotonating it. Finally, a proton is taken up from solution on the cytoplasmic side of the membrane to reprotonate Asp 96, completing the transport of a proton across the cell membrane, and then the chromophore is thermally isomerized, resetting the pigment back to its initial state.

Bacteriorhodopsin

Bacteriorhodopsin exists in a number of pH dependent forms. At low pH the membrane is blue, due to the protonation of one of the carboxyls involved as counter ions, Asp 85 (see Subramaniam et al., 1990; Jonas and Eberly, 1991; Metz et al., 1992). For the normal purple color, the counter ions to the positively charged Schiff base are usually maintained in their
unprotonated state, even in the presence of a fairly high local proton concentration. At higher pH (intrinsic pK=8.3) Balashov et al., (1991) have recently shown that br converts to another pH dependent form, called br_{alkaline}.

A recent major event has been the publication of a moderate resolution structure for bacteriorhodopsin (Henderson et al., 1990). Fig. 1 and 2 are based on this structure. FTIR results have strongly suggested that for purple membrane the "buried" Asps, 85 and 212, are deprotonated (ionized) while 96 and 115 are protonated (Braiman et al., 1988; Gerwert et al., 1989).

**Light Driven Changes in Bacteriorhodopsin (Fig. 3)**

The Initial Photochemical Step: Light isomerizes the chromophore of br from the all-trans to the 13-cis conformation leading to the high free energy form of the pigment, K (discussed in Honig et al., 1979). We recently have determined the value of the quantum yield of the photocycle to be 0.64 (Govindjee et al., 1990; a similar value was obtained by Tittor and Oesterhelt, 1990; and Schneider et al., 1989). There probably are no major changes in the protein, although this point is not settled definitely.

The K to L Transition: Little is known about the transformation from the first photoproduct to the L intermediate. The lifetime of K at room temperature is a little over a μs (e.g. Liu and Ebrey, 1988), and an electrical current is observed in the direction that the proton will move during proton pumping. The L intermediate can be stabilized at low temperature. There probably is a conformational change in the protein upon the formation of L since the pigment cannot be photoreversed back to its initial state if recooled to 77 K (Hurley et al., 1978).

The L to M Transition: After the initial energy storage event, this transition can be seen as the next crucial event in proton pumping. The product of the transition, M is an unprotonated Schiff base so the Schiff base deprotonates during this transition. On a time scale that closely matches the L to M optical transition, a proton appears in solution (Drachev et al., 1984; Liu et al., 1990; Heberle and Dencher, 1992). Siebert et al. (1982) first showed that a carboxylic acid was protonated at the same time that the Schiff base lost its proton. Khorana, Heyn, Rothschild, and co-workers (Braiman et al., 1988; Otto et al., 1990) and Oesterhelt, Bamberg and co-workers (Butt et al., 1989) showed that this carboxylic acid was Asp 85. At room temperature and neutral pH, the L to M transition takes about 85 μs. However, Siebert et al. (1982) also showed that this protonated carboxyl group does not deprotonate on the time scale that the proton appears in solution, so the released proton must originate from a second type of group, which we have designated X in Fig. 3.

Balashov et al. (1991) have shown that the transition from the neutral to the alkaline form
of bR has the same pK as the transition from the slow to the fast kinetics for the L to M transition. Light-induced proton release from the membrane also falls off with the same intrinsic pK, 8.3 (Kono et al., 1993). These observations suggest two pH dependent forms of the pigment, each with its own photocycle, with the high pH form not releasing protons efficiently.

The M to N Transition: In 1986 both Ebrey's group (Dancshazy et al., 1986; 1987a, 1987b; Govindjee et al., 1988) and Skulachev and Drachev's group (Drachev et al., 1986, 1987) discovered a new photocycle intermediate, most prominent at high pH, that controlled the rate of return of the initial pigment from M during the decay of the photocycle. Drachev et al. showed that the decay of N coincided with the uptake of protons by the purple membrane, completing the proton translocation process. Experiments by Fodor et al. (1988) suggested that M did decay into N and thus was on the normal pathway of the photocycle. In important papers Otto et al. (1989), Varo and Lanyi (1990), Ames and Mathies (1990), and Brown et al. (1993) provided evidence that M and N were in equilibrium, providing a rationale for the biphasic decay of M seen at most pHs. Otto et al. (1990) and Butt et al. (1989) also suggested that Asp 96, which was initially protonated, donates its proton to the Schiff base, reprotonating it and forming N. Asp 96 is then reprotonated from the intracellular side, leading to proton uptake and the completion of proton translocation.

The N to O to bR Transition: Several groups (Chernavskii et al., 1989; Varo and Lanyi, 1990; Ames and Mathies, 1990; Cao et al., 1993) have recently provided evidence that N is in equilibrium with O and so M, N, and O usually are all in equilibrium with each other, helping to explain the complex but mostly biphasic decay of the M intermediate. The best available evidence (Drachev et al., 1987; Varo and Lanyi, 1990) is that the proton that completes the proton pumping cycle is picked up from the cytoplasmic side of the membrane during the N to O transition, leading to the reprotonation of Asp 96. The decay of O to bR, which may involve the deprotonation of Asp 85 and the reprotonation of the actual donor of a proton to the extracellular side of the membrane, leads to the restoration of the initial state of the pigment.

RESEARCH PLAN AND METHODOLOGY

Our goal is to understand the molecular mechanisms controlling the photochemical and proton pumping properties of bacteriorhodopsin. The following four questions will be probed: 1) Can we determine by specific chemical labeling what amino acids are near particular portions of the chromophore? 2) What is the role of water in controlling the pK_a's of the Schiff base and of D85, the initial proton acceptor and part of the counter ion to the Schiff base? 3). What changes occur in bacteriorhodopsin to allow the Schiff base to deprotonate and initiate the proton pumping process? What controls the rate of Schiff base deprotonation? and 4) What properties of the binding site are responsible for the temperature dependent photochemistry of bR? How do retinal modifications and amino acid mutations change this temperature independence? Experiments to elucidate these properties are given below.

A. Chemical labeling of Specific Amino Acids near Particular Portions of the Bacteriorhodopsin Binding Site

Although Henderson's moderate resolution structure provided a set of amino acids believed to form the binding pocket of the chromophore, independent confirmation of these residues and the details of their interaction and distances from the chromophore are lacking. The importance of specific interactions was demonstrated in the study of Subramaniam et al. (1991), which indicated that an interaction between Leu93 and a specific region of the chromophore probably is crucial in controlling the N and O reactions of the photocycle. We propose to investigate the binding site using a unique approach combining site directed mutagenesis with chemically modified chromophores. Bacteriorhodopsin has the interesting property of not having any cysteines present in the native structure. This is especially convenient as cysteines can be easily introduced by the molecular genetics techniques discussed below into specific sites. Thiol chemistry has been extensively applied in protein chemistry for affinity labeling (e.g. Hartman, 1977). Selective
alkylation of the thiol functionality is possible due to its high nucleophilic reactivity relative to other amino acid side chains. Hubbell and Khorana have used this approach by specifically placing cysteines at various positions along the helix and then labeling these cysteines with an agent containing a spin label (Altenbach et al., 1990). We propose to extend this concept to exploring the interaction of these cysteine groups with the chromophore (suitably derivatized with good leaving groups) and using other agents to tag the protein via a thiol linkage. Through these experiments, we should be able to crosslink the chromophore within the protein binding site. For the chromophore crosslinking experiments, we have studied BR extensively by molecular modeling using Henderson’s model on the SYBYL molecular modeling program. Allowing the analogues to rotate about the C 6-7 bond, we identified 9 amino acids that are in the correct position to be the likely positions for crosslinking to synthetic retinals. See Figure 2.

**Overall Scheme:**

1. Bacteriorhodopsin mutants will be generated by site directed mutagenesis by reintroducing transformed bacterioopsin (bop) genes into bop- strains of H. salinarium. Mutants will be made in which a cysteine is located so that there is the potential of this nucleophilic group displacing a good leaving group positioned on the retinal chromophore (e.g., M118C, S141C, and T121C).
2. Retinal analogues will be synthesized that contain a specific derivatization that can serve as a good leaving group for electrophilic affinity labeling for forming crosslinks with the protein (e.g., 4-Bromo-retinal).
3. After removing the native chromophore, analogues of the native chromophore will be introduced into both the native and the site-mutated BRs. If the (now cysteine) residue proposed to be near the Bromo- group of the retinal is in the appropriate position, then the retinal will be covalently crosslinked to the protein. After crosslinking the retinal, the location of the chromophore within the binding pocket of both the native and the site-specific mutated BRs will be determined by peptide chemistry and mass spectrometric methods.

An outline of the proposed scheme for the project is presented below. In order to have all the appropriate controls, for each retinal derivative incorporated into a BR mutant, four pigments will be regenerated. As this is a new approach, it is essential that we be certain which changes in function and/or physical property are due to which alteration. Therefore, in Scheme 1, each of the six shaded pigments will be examined under identical conditions for spectral properties, stability to hydroxylamine and all-trans retinal, photocycle intermediates, and proton pumping function.
General Procedures:

Site Directed Mutagenesis of bR

Oligonucleotide-directed site specific mutagenesis will be performed essentially as described (Menick, 1991; Kunkel, 1985). The 2.7 Kb BamH1-HindIII bop gene insert will be restricted from the \textit{H. salinarium} shuttle vector pMC-1 (gift of R. Needleman) and ligated into M13mp18 or pUC 118. Mutagenetic primers will be made that are complementary to the \textit{bop} template with the exception of designed mismatches to make the appropriate mutation. Phage harboring the mutation will be initially screened by colony blot hybridization then plaque purified and the mutation verified by sequencing. The entire coding region of the \textit{bop} gene will be sequenced to insure that no additional mutations have occurred. The BamH1-HindIII \textit{bop} gene insert from each mutant will be restricted from the M13mp18 RF or from pUC118 and ligated into pXLI10a (gift of R. Needleman). The pXLI10a expression vector contains a pH19 replicon which allows expression without integration into the host chromosome and can be selected and maintained in either \textit{H. salinarium} or \textit{E. coli}. Each of the bR mutants and wild type controls will be transformed into the \textit{H. salinarium} bop* strain L33 which contains an ISH2 insert within the \textit{bop} gene. Transformation will be performed as described (Lam and Doolittle, 1989; Ni et al., 1990; Needleman et al., 1991). L33 cells will be grown to mid-log phase, pelleted and resuspended in spheroplasting buffer (2 M NaCl, 27 mM KCl, 50mM Tris-HCl, pH 8.75, 15% sucrose), which is then brought to 85 mM EDTA. When nearly all the cells have formed spheroplasts, 200 μl of spheroplasts will be gently transferred to a tube containing 1-10 μg of plasmid DNA. After 20 minutes of incubation, 220 μl of filter sterilized 60% PEG will be added and gently mixed. The mixture will be incubated 20 min, then 1 ml of regeneration salt buffer will be added. The spheroplasts will be pelleted by centrifugation, resuspended in growth media (plus 15% sucrose) and incubated at 37°C overnight. Aliquots will be plated in soft agar overlays (growth media 0.8% agar, 1.5% sucrose and 5 μg/ml Mevinolin) containing the same media but no Mevinolin. Several independent clones for each mutant will be picked (after 2 weeks growth) and struck out on growth media plates and single colony isolates will be grown up in liquid culture to characterize each bR mutant.

Stability of plasmid linked \textit{bop} gene - Preliminary work by the Crouch/Menick lab and others (Lam and Doolittle, 1989; Needleman et al., 1991; Krebs et al., 1991) suggest that the pMC-1 plasmid containing the wild type or mutant \textit{bop} gene can exist both as an autonomously replicating form and integrated into the \textit{H. salinarium} chromosome. Expression of the \textit{bop} gene does not appear to be greatly affected by integration (Ni et al., 1990; Needleman et al., 1991; Fan and Menick; preliminary data). The only events which could directly effect the expression and phenotype of mutant \textit{bop} genes are 1) The excision or replacement of the ISH2 element in the wild type chromosomal \textit{bop} gene. Although this event has never been reported (Ni et al., 1990) we will perform a Southern analysis of each of the transformants to insure this has not taken place. 2) Integration of the plasmid borne \textit{bop} gene which results in the deletion or base change in the coding region. In order to insure that the \textit{bop} gene is intact and retains only the base changes that we have engineered, the integrated \textit{bop} gene will be amplified by PCR, subcloned, and sequenced. The amplified mutant \textit{bop} gene is easily distinguished from the amplified chromosomal \textit{bop} gene with the ISH2 insert because it is 1.1 Kb smaller. This effective transformation-expression system allows the structure-function relationship study of bR in halophilic archaebacteria.

Synthesis of Retinal Analogues

Retinals will be synthesized by established methods of preparing these somewhat complex, unstable molecules. The Crouch laboratory has had extensive experience in this area, with over forty new retinals/retinols being synthesized over the past fifteen years. The specific compounds proposed in this study are presented under the specific experiments below. The compounds fall into two general classes: retinals in which there is derivatization at the 3 or 4 position on the ring and which can be prepared from retinal itself, and retinals which contain
photoaffinity labels which require a full synthesis. The two general schemes for preparing the compounds are presented below in Scheme 2a and 2b. All compounds will be fully characterized by standard methods employed in analysis of organic compounds (FTIR, NMR (400 MHz, C and H), mass spectrometric analysis, uv-visible absorption).

Scheme 2a

1. base
2. reduction
3. oxidation

\[ R = \text{acylated, spinlabeled, etc.} \]

Scheme 2b

Protein Crosslinking

Crosslinking reactions will be conducted in two sets of experiments: 1) the case where retinals containing a good leaving group are introduced into bR mutants containing a cysteine and 2) pigments containing a photoaffinity labeled retinal. In both cases, having a radiolabeled retinal will facilitate the procedure of identifying if crosslinking has occurred and the position of the crosslink. In the first case, it is anticipated that the reaction will occur in situ with the addition of ammonia. In the second case, specific radiation will be needed to activate the label. We have developed a flash apparatus suitable for these reactions (Beischel et al., 1992). For both sets of experiments the following general procedure will be used: a) pigments will be formed using a slight excess of the protein; b) unreacted chromophore will be removed by washing 3x with buffer; in experiments where gel electrophoresis is being used for purification of the protein, a 1% BSA wash will proceed the buffer washes; c) pigment will be divided into equal aliquots one being reserved as the unreacted control; d) the experimental half will be exposed to light or dilute base depending on the experiment; e) either, the pigments will receive exhaustive treatment of both samples with hydroxylamine to cleave the Schiff base and washing to remove hydrolyzed retinal and the samples counted. Alternatively, the pigments will be electrophoresed on 12.5% precasted polyacrylamide gels under denaturing conditions and the gel slices counted in Solvable according to manufacturer's instructions (DuPont) and/or the gel autoradiographed. We have successfully used both procedures in control experiments.

Localization of Labeled Amino Acids

Both the in situ displacements and the photoaffinity labeling experiments will require the identification of the labeled amino acid. We propose to accomplish this by use of a combination of protein chemistry methodology and mass spectrometric analysis. The labeled protein will be delipidated in formic acid on a Sephadex LH-20 column (Gerber et al., 1979). After the addition of tryptamine to protect the tryptophans, the protein is treated with CNBr (60 fold molar excess over
the methionine content). The CNBr digest goes to completion but some of the hydrophobic peptides are difficult to fractionate by HPLC. Therefore, tryptic digestion is carried out after the CNBr procedure without intermediate HPLC separation. HPLC separations are performed on reverse-phase C-4 and C-18 columns. Fractions are collected, 1 μl glycerol added, samples concentrated by speed-vac and stored in the matrix to be used (e.g., 5-10% acetic acid or p-nitrobenzoyl alcohol). The samples are analyzed with a JEOL HX110/HX110 high performance tandem mass spectrometer equipped with a high voltage (35kV) cesium gun (10KV) (Bieman and Scoble, 1987). Molecular weight measurements on larger molecules may be obtained by electrospray ionization on a Nermag R30-10 triple quadrupole tandem mass spectrometer (Papac et al., 1991) or by laser desorption time of flight mass spectrometry (Schey et al., 1992) on an instrument similar to that described by Beavis and Shait (1990). The mass spectral data will be reviewed with Dr. Daniel Knapp (see letter of collaboration, Appendix 4). In some cases preliminary data will be obtained by classical Edman degradation and amino acid analysis using the MUSC protein chemistry facility.

2. The Role of Water in Controlling the pK of the Schiff Base and of Asp 85, the Initial Proton Acceptor from the Schiff Base.

The pK of the Schiff base of bacteriorhodopsin is extraordinarily high (ca. 13) compared to model Schiff bases (ca. 6) while that for the primary proton acceptor, D85, is somewhat low for an aspartic acid, 2. Proton pumping in bR probably involves light lowering the pK of the Schiff base by a very large amount and perhaps raising that of D85, so as to allow proton transfer between the two groups; the total pK change should be about 11 pH units. It is becoming clear, from the study of mutations of amino acids which are thought to be near Asp 85, that several amino acid residues control the pK of this amino acid. For example changing R82 to an Ala, raises the pK of D85 by about 5 pH units (see e.g. Subramaniam et al., 1990; Balashov et al., 1992; 1993). Likewise, the high pK of the Schiff base is controlled by a complex set of interacting amino acids and perhaps bound water. We plan to investigate the contribution of water to the control of the pK of the Schiff base and D85.

Several reports have suggested that water may play a special role in the active site of bR (Hildebrandt and Stockburger, 1984; deGroot et al., 1989; Cao et al., 1991; Maeda et al., 1992). Sheves and co-workers (Gat and Sheves, 1993) have proposed that the high pK of the Schiff base of bacteriorhodopsin is due in part to the particular hydrogen bonding geometry that the water molecules make with the Schiff base as the waters bridge nearby hydrogen bonding amino acid residues to the Schiff base. Although the Gat and Sheves paper was not specific on what residues might be involved, clearly they had in mind those that have been mentioned in the background that are part of the environment of the Schiff base such as D85, D212, R82, Y57, Y185, and others (discussed by Sheves, Schulten and co-workers in Humphrey et al., 1994).

There are few ways to investigate if water is helping control the pK of the Schiff base; an especially promising one is to remove/replace the proposed special bound waters and see what effect this has on either the pK or some other aspect of the Schiff base environment. Molecules like ethylene glycol or methanol would be good candidates to replace the water in that they are small and polar but would not be able to hydrogen bond in the same way that water does. At the same time, it is known that if small neutral molecules are added to water, they increase the osmolarity and hence can force water from binding sites in proteins into the bulk solution. That is, since osmotic pressure controls the activity of water in an aqueous compartment inaccessible to neutral solutes (osmolytes), osmotic stress induces the release of bound water from the macromolecules to bulk solvent (Robinson and Silgar, 1994). Many of these osmolytes can also denature the protein at high concentrations, but the osmotic effects usually occur at much lower concentrations of the osmolyte (for references see: Robinson and Silgar, 1994; Rand, 1992; Wolfenden, 1983). Cao et al. (1991) has used this approach to demonstrate that water is involved in controlling the rate of decay of the M intermediate. Initially we will study the effects of ethylene glycol and of methanol. Preliminary experiments by us have found that bR is stable in ethylene glycol/water or methanol/water mixtures at concentrations of less than 8 M for at least a day (see also Mitaku et al., 1988; Cao et al., 1991; Fukada and Kouyama, 1992). Thus these samples are sufficiently stable that we can do experiments on them. At these concentrations
osmotic pressures of up to 100 Atm can be achieved; in other systems these concentrations of osmolytes are sufficient to cause the displacement of bound water molecules although we have no guarantee that they will here. It is interesting that if water is displaced by these concentrations of ethylene glycol or methanol then this displacement does not greatly alter the absorption spectrum of bR. On the other hand it is known that osmolytes such as sucrose, glycerol, DMSO, and ethanol do change some properties of bR such as the extinction coefficient of bR (Oesterhelt et al., 1973), the circular dichroism spectrum of the purple membrane (Draheim et al., 1985, 1991), the 13-cis to all-trans ratio in light adapted bR (Balashov et al. 1988), and the photochemical cycle of bR and some of its mutants (Cao et al., 1991; Govindjee, Yan, Crouch, Menick, and Ebrey, unpublished observations).

Three methods will be used to see if the ethylene glycol/methanol is affecting the Schiff base and its environment. The straightforward procedure of just spectrally titrating the Schiff base of bR (as we have done earlier, see e.g. Balashov et al., 1991, 1983) in a water/ethylene glycol mixture to see if the pK has changed is very reasonable. A second method, which will probe the ability of ethylene glycol (methanol) to change the electrostatic and hydrogen bonding environment of the Schiff base but not give a direct reading of the Schiff base pK, would be to assay the light and dark adapted pigments in the ethylene glycol (methanol)/water mixture for the percent of all-trans and 13-cis isomers present, since it has been shown that this ratio is affected by dehydration (Kouyama et al., 1985; Balashov et al., 1988). Their equilibrium concentration will depend on the Schiff base environment. The amount of 13-cis and all-trans isomers present can be analyzed with a flash photolysis assay (described in Balashov et al., 1993). Finally one can assay for changes in the water hydrogen bonding during the photocycle by looking at the FTIR difference spectra of bR versus L (following Maeda et al., 1992, who showed that there are bands in the difference spectra that can be assigned to water in different environments in these two states of bR) and then repeating the experiment with ethylene glycol or methanol instead of water. We have previously been involved in FTIR experiments and have excellent equipment at Illinois (Bagley et al., 1985; 1989).

If changes are seen in the pK, cis/trans isomer ratio or water hydrogen-bonding changes during the photocycle, it is necessary to check for other variables that are also changed with solvent that may be affecting these properties rather than direct water involvement at the Schiff base. We would do a concentration series, with several different osmolytes, to see if the changes observed correlate with osmolarity or with other properties such as viscosity, dielectric constant, size, or water molarity.

Since water at locations in bR other than near the Schiff base might be the origin of any osmotic effects we discover, we can localize the water effects specifically to the Schiff base region by using site directed mutants involving residues near the Schiff base. It is difficult to predict exactly what hydrogen bonding changes of the water to the Schiff base will occur if a specific residue is altered, but changing one of these residues should have a large effect on the strength and number of waters bound. We propose to study our osmotic pK (or other ) effect initially with six mutant pigments: R82A, R82K, Y57N, Y57F, D212N and D85E. If bound water is an important determinant of the Schiff base properties, then at least one of these should have drastic effects on water bound near the Schiff base and should show a quite altered osmolyte concentration dependence.

3. Origin and Effects of Steric Restrictions on Chromophore Conformational Change

One of the most amazing properties of bacteriorhodopsin is its ability to undergo temperature independent photoisomerization at temperatures down to that of liquid nitrogen (e.g. Hurley et al., 1977; Balashov et al., 1992) and even liquid helium (Iwasa et al., 1980). Since the initial step is a photoisomerization, it must be concluded that there is no large barrier, such as that which would exist if the chromophore had to move through either the solvent or an amino acid side chain, since this would impart temperature dependence to the event. So chromophore photoisomerization must take place in a "pseudo-vacuum" within the pigment. The narrowness of the limits in which this happens is demonstrated by the observations that rather small changes in the chromophore of retinal proteins can abolish the temperature independence. For example, we showed that, unlike rhodopsin, the primary photochemistry of 9-cis rhodopsin is not temperature independent.
(Hurley et al., 1977) and that for bR after adding a double bond to the ring of retinal, the synthetic bacteriorhodopsin's photochemistry is now temperature dependent (Iwasa et al., 1981). Most recently, Yan et al. (1993) reported that the primary photochemistry of sensory rhodopsin from *H. salinarum* showed temperature dependence.

First, we will make mutants of bR in which the amino acids, proposed by Henderson et al. to be in the retinal binding site of bacteriorhodopsin (Fig. 1), are changed to those present in sensory rhodopsin to see if temperature dependence of the quantum yield for the primary photoprodut, K, seen by Yan et al. in sensory rhodopsin can be introduced. The candidate amino acids changes are M20I, V49I M145H, and W138F; all the other amino acids in the binding site are identical.

Second, since several interesting mutants have already been made by us for other purpose, we will do the relatively easy job of screening these mutants to see if temperature dependent photochemistry has been introduced. This screening can be done by undergraduate honors students or first year graduate students doing research rotations. Those to be screened include R82A, R82K, Y57N, Y57F, Y57V, M118C, M121C, and M145C.

Third, since we have identified a retinal analogue, 3,4 dehydroretinal, that is expected to have very small steric differences from retinal, but which has temperature dependent primary photochemistry when incorporated to form a pigment, we will try to "heal" this temperature dependence by changing amino acids in the binding site to less bulky analogues. [need a sentence about which ones to try]

Finally, we are in a unique position to perform an extremely interesting experiment if the cross-linking experiment proposed in section 1 is successful (as noted, we have preliminary evidence that we can cross link the ring of the chromophore to the protein). This is to see what effect this restriction of chromophore motion set by the cross-linking event has on a) the primary photochemistry and its temperature dependence and b) on the ability of the chromophore's Schiff base to deprotonate as a result of light absorption and, if a) is normal, photoisomerization.

4. Light-induced Deprotonation of the Schiff base of Bacteriorhodopsin

The proton affinities of both the Schiff base and of the protonatable amino acid residues in the pigment determine the pH dependent properties of the initial pigment; their alteration as a consequence of the light-induced changes in the pigment probably constitute the key mechanism of the proton pump. We propose to study these changes at the L to M step in the photocycle, where deprotonation of the Schiff base takes place.

A. Function and properties of the L Intermediate: Evidence for a major conformational change

The L intermediate is the starting point for the intramolecular proton transfer from the Schiff base to Asp 85. Under normal conditions simultaneous transfer of the proton from the proton release group "X" to the bulk occurs. Our study of the Y57N mutant indicated that at pH 7, the L to M transition is blocked; the M intermediate is not formed while the lifetime of the L intermediate is increased from 85 us to 500 ms (Govindjee et al., 1992). This makes the Y57N intermediate an attractive object for studying the properties of the L intermediate. We plan to test the hypothesis that in the L state conformational changes in the protein occur that increase the accessibility of the Schiff base to the bulk solution. We have found that in the presence of hydroxylamine, Y57N bleaches several orders of magnitude faster than the wild type (unpublished observations). Subramaniam et al. (1991) have shown that hydroxylamine attacks the protonated Schiff base of retinal, consistent with it reacting with the L intermediate. We plan to measure the lifetime of the L intermediates of a set of mutants of bR that we have or will construct in which we believe the L lifetime will be altered from that of the wild type and correlate this with the rate of bleaching of the mutants by hydroxylamine in light. We expect the L lifetimes to vary by at least three order of magnitude and thus the rates of bleaching by hydroxylamine should vary by this same amount. Besides Y57N, which we know has a very long lifetime, it is known that R82A (Balashov et al., 1993) and Y57F have very short L lifetimes. We will also construct and study R82K and Y57T
and V mutants because we suspect that they will also have greatly altered L lifetimes compared with the wild type.

These experiments would not only provide strong evidence for an opening up of the bR molecule in the L state (so that hydroxylamine is now able to attack the Schiff base) but also, with proper controls, enable us to check if that hydroxylamine is attacking the chromophore during the N or O states.

B. Control of the rate of Schiff base deprotonation, the L to M transition, by cations.

The complex nature of the L and M transition has been recently reviewed (Ebrey, 1993; Lanyi, 1993). Because the proton leaves the Schiff base and goes to Asp 85 during the formation of M and a proton appears in solution almost simultaneously with this event, the L to M transition is a key step in the proton pump. Several mutants of bR show a very rapid rate of light-induced Schiff base deprotonation, the L to M transition compared to the wild type at pH 7. The case which we have studied most carefully is the R82A mutant, in which the L to M transition is changed from ca. 85 us in the wild type to ca. 1 us in the mutant (Balashov et al., 1993). Other examples which have increased rates for L to M compared to the wild type at pH 7 include Y57F (Govindjee et al., in prep.) and D85E (Lanyi et al., 1992). Simple calculations suggest that this rate effect in R82A could be explained in terms of changes in the electrostatic potential on D85 due to the change in the charge at position 82 (Balashov et al., 1993). Such reasoning also provides a simple explanation for the speeding up of the L to M rate as the pH is raised, for if the effect of raising the pH is to deprotonate a group near D85, like Y57 (Balashov et al., 1991; Govindjee et al., 1992) or R82 (Balashov et al., 1993), then the removal of the proton on Y57 (or R82) should allow the proton to move from the Schiff base to D85 more easily, manifesting itself in the increased rate constant. Assuming that electrostatic potential can control the L to M transition, then if a cation is part of the binding site, as has been hypothesized by ourselves and others (e.g. Jonas and Ebrey, 1991; Sweetman and El-Sayed, 1991), the rate of the L to M transition should be very sensitive to the valence of the cation bound. Previous we have shown either sodium (+1), calcium (+2), or lanthanum (+3) can be the sole cation bound to bR (Chang et al., 1995), so preparation of such cation specific bR should give quite different rates of the L to M transition if a cation is close to the Schiff base. By using mutants of specific amino acids near the Schiff base (R82A and K; D85A and E; Y57F and N; D212Q) we can confirm that the cation effect, if seen, is due to binding near the Schiff base.

C. Light-induced Charge Motions in bR: Detection of Spectroscopically Silent Transitions

After excitation of bR, the photocycle is accompanied by intramolecular charge displacements due to the movement of proton and protein charges. This charge movement can be detected by either voltage or current measurements from oriented purple membranes (Triissl, 1990). Our measurements have indicated that the $\mu$s phase of the photocurrent is due to proton release from the purple membrane (Liu, 1990; Liu et al., 1990).

Photocurrent measurements on mutant of bR are much less developed than absorption spectroscopic studies. Our preliminary data (Misra, Ebrey, Yan, Crouch and Menick) indicate that on a time scale of the B2 signal (associated with proton release) there are components of the photocurrent which are not seen in the optical signal for M formation. This leads us to the idea that some important transitions and charge motions in bR may not be easily detectable as absorbance changes but are easily observable with photocurrent measurements.

We plan to investigate systematically this possibility and make quantitative correlations of optical and photocurrent signals in mutants which have (or probably have) accelerated M formation (R82A, Y57F, R82K) and in mutants with inhibited or strongly delayed M formation (Y57N, especially at high pH, Govindjee et al., in prep.) We expect that this study will reveal the components directly associated with proton release and clarify the nature of the different M forms that have been proposed (see reviews of Ebrey, 1993; Lanyi, 1993)
What work will be done at the Medical University of South Carolina and what will be done at the University of Illinois at Urbana-Champaign.

This grant is being submitted as a competing renewal of DE-FG02-88ER13948 (to TGE). However, since crucial parts of the work will be done at MUSC, since this work represents a larger portion of the budget than that to be done at UIUC, and since it is easier to do a subcontract (without double overhead!) through MUSC, the grant is being submitted from that institution.

In general all of the mutants that are proposed that are not presently available (i.e. R82K, M118C, S141C, T121C, M145C). In addition, all the artificial chromophores specified in Project 1, starting with 4-bromo retinal, will be synthesized at MUSC. Initial pigment binding and spectroscopic characterization of the mutants and synthetic retinal/mutant pigments will be done at MUSC. Flash photolysis, pH dependent dye measurements, and photocurrent measurements will be done at UIUC.