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Minireview

Proton Transfer and Energy Coupling in the Bacteriorhodopsin Photocycle

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A description of the rate constants and the energetics of the elementary reaction steps of the photocycle of bacteriorhodopsin has been helpful in understanding the mechanism of proton transport in this light-driven pump. The evidence suggests a single unbranched reaction sequence, BR-*hv* → K ↔ L ↔ M_1 → M_2 ↔ N ↔ O → BR, where coupling to the proton-motive force is at the energetically and mechanistically important M_1 → M_2 step. The consequences of site-specific mutations expressed homologously in *Halobacterium halobium* have revealed characteristics of the Schiff base deprotonation in the L → M_1 reaction, the reorientation of the Schiff base from the extracellular to the cytoplasmic side in the M_1 → M_2 reaction, and the reprotonation of the Schiff base in the M_2 → N reaction.

KEY WORDS:

INTRODUCTION

Recent advances in the description of the structure of bacteriorhodopsin, low-temperature and time-resolved vibrational spectroscopy, and the consequences of single-residue replacements have produced the elements of the mechanism for proton translocation in this protein. The reaction cycle is initiated by light-induced isomerization of the *all-trans* retinal chromophore to 13-*cis*. The retinal Schiff base, D85, and D96 participate in the ensuing proton transfer. Vectorial proton movement is accomplished when the Schiff base transfers its proton to D85 with access to the extracellular side and regains it from D96 with access to the cytoplasmic side (cf. also Tittor *et al.*, 1992; Rothschild, 1992). The concept of ordered proton transfers between the Schiff base and the two strategic aspartate residues is the key to the transport mechanism. However, until recently there was neither a satisfactory description of the reaction sequence in the photochemical cycle nor an understanding how the excess free energy gained by the chromophore is coupled to the proton potential. This short review summarizes recent attempts to contribute information toward these questions, and the way the thermodynamic approach to the system has affected mechanistically oriented studies. Other work is cited when relevant or necessary to put the results into perspective; there is no intent to give a comprehensive review of the large bacteriorhodopsin literature.

CHROMOPHORE AND PHOTOCYCLE

The chromophore, which comprises the retinal and neighboring protein residues which influence it, has a single absorption band of distinct shape in the visible (e.g., Lozier *et al.*, 1975; Lozier and Niederberger, 1977; Shichida *et al.*, 1983) and many characteristic vibrational frequencies (reviewed by Smith *et al.*, 1985). The maximum in the visible is strongly red-shifted as compared to what is expected from model compounds. This "opsin shift," caused by interaction with protein residues in the retinal binding pocket, is explained for the most part by the external point-charge model (Kakitani *et al.*, 1985; Nakanishi *et al.*, 1980; Warshel, 1978). Because the distance between

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the protonated Schiff base and its counterion supplied by the protein is several angstroms greater than it would be in free solution the positive charge at the Schiff base is increased. This causes partial withdrawal of π-electrons from the polyene chain and their further delocalization, resulting in a substantial red shift. The existence of such a counterion effect on the absorption maximum is convincingly demonstrated by the observation that replacement of either of the two negatively charged residues near the Schiff base, D85 (Mogi *et al.*, 1988; Subramaniam *et al.*, 1990; Otto *et al.*, 1990) or D212 (Needleman *et al.*, 1991), with non-ionizable residues caused additional redshifts of significant magnitudes.[2] A second contribution to the opsin shift is the stabilization of the 6-*s-trans* retinal configuration in the retinal binding pocket so as to cause the ring to assume a more planar position relative to the π system than in free retinal (Harbison *et al.*, 1985). A third possible contribution is from a protein dipole near the β-ionone ring (Spudich *et al.*, 1986). Thus, the protein affects the retinal considerably, and electronic and vibrational spectra will be quite sensitive to changes in retinal–protein interaction. Indeed, time-resolved spectral changes at ambient temperature and spectra frozen in at low temperatures have detected at least six distinct transient states in the reaction sequence ("photocycle"). They include the following distinguishable species (with their order-of-magnitude lifetimes in parentheses): J_{610} (ps), K_{590} (ns), L_{550} (μs), M_{412} (ms), N_{560} (ms), and O_{640} (ms). There is general agreement that the same intermediate states are detected by either electronic or vibrational spectroscopy. The kinetic coupling between chromophore and opsin appears to be thus good: The time dependences of changes in the chromophore reaction sequence have counterparts in the FTIR spectra which originate from the protein (Gerwert *et al.*, 1990b).

A considerable amount is known about the molecular events which produce these intermediates states. The red-shifted K state(s) contain isomerized 13-*cis* retinal, but the Raman and FTIR spectra have strong HOOP amplitudes indicating a strained chain configuration (Pande *et al.*, 1981; Rothschild *et al.*, 1984; Braiman and Mathies, 1982). In the L state, which is blue-shifted relative to K (and BR), the strain will have relaxed. The Schiff base proton is transferred in the ensuing L $\rightarrow$ M reaction to D85, producing the M state which absorbs far to the blue, consistent with loss of charge from the Schiff base. Reprotonation of the Schiff base by D96 gives rise to the N state in which the retinal is still 13-*cis*, and which absorbs near the maximum of BR. While L and N both contain protonated Schiff base and 13-*cis* retinal, they are distinguished by numerous retinal and protein bond vibrations (Fodor *et al.*, 1988; Pfefferlé *et al.*, 1991). Reisomerization to a distorted *all-trans* configuration (Smith *et al.*, 1983), reprotonation of D96, and apparently the continued protonated state of D85 (Rothschild, 1992) produce the strongly red-shifted O state, which then decays to the initial BR. The evidence for these retinal transformations and internal proton transfers between the Schiff base and D85 and D96 was recently reviewed (Mathies *et al.*, 1991).

It has been known for some time that the photocycle intermediates follow one another in a roughly linear sequence (Lozier *et al.*, 1975). Nevertheless, until recently a kinetic scheme which would satisfactorily account for the observed multiple relaxations in the rise and decay of M and other intermediates has remained out of reach. A single linear sequence with unidirectional reactions was ruled out (Nagle *et al.*, 1982); most authors had suggested multiple bacteriorhodopsin species with different photocyles (Hanamoto *et al.*, 1984; Dancsházy *et al.*, 1988; Birge, 1990; Balashov *et al.*, 1991), photocycles containing an early branch and parallel reaction sequences (Sherman *et al.*, 1976, 1979; Lozier *et al.*, 1978; Beach and Fager, 1985), or a multiphoton photocycle (Kouyama *et al.*, 1988).

Finding a kinetic model which describes the reaction cycle is a considerable problem because the

[2] It appears that the properties of some mutated bacteriorhodopsins depend on whether they are expressed in *Escherichia coli* or *Halobacterium halobium*, the original host for the protein. Thus, the maximum of D212N from the *E. coli* system is 9–13 nm blue-shifted from wild type (Mogi *et al.*, 1988; Subramaniam *et al.*, 1990; Stern *et al.*, 1989) but 16 nm red-shifted when expressed in the *H. halobium* system (Needleman *et al.*, 1991). The maximum of D115N is 17–28 nm blue-shifted in the *E. coli* system (Mogi *et al.*, 1988; Subramaniam *et al.*, 1990) but unshifted relative to wild type in the *H. halobium* system (Váró *et al.*, 1992). The maximum of D85N is red-shifted from wild type by 22–35 nm in the *E coli.* system (Mogi *et al.*, 1988; Subramaniam *et al.*, 1990; Otto *et al.*, 1990; Miercke *et al.*, 1991) but by 46 nm in the *H. halobium* system (Cao and Lanyi, unpublished results). Many of the proteins from *E. coli* are light-unstable and show abnormal wavelength shifts upon illumination, reflecting changed *all-trans*/13-*cis* retinal isomeric ratios (Duñach *et al.*, 1990), but those from *H. halobium* so far examined appear like wild type in this regard. Most of these differences originate from the fact that in *H. halobium* the protein is assembled into purple membrane patches and this apparently does not occur readily after reconstituting the proteins from *E. coli.*

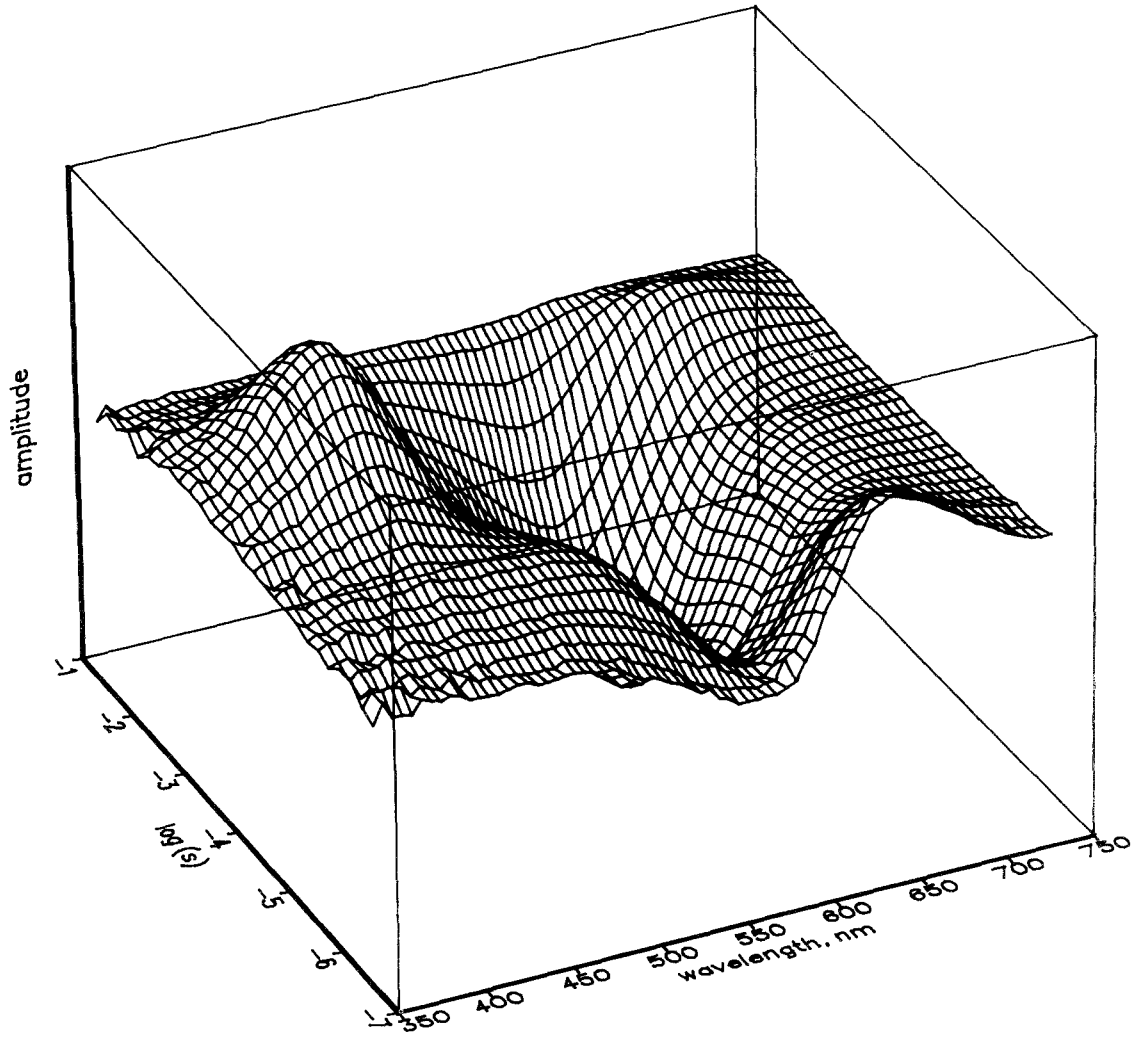

Fig. 1. Absorption changes during the bacteriorhodopsin photocycle. The axes are: wavelength, time as log of seconds, and absorption change. This example of a three-dimensional representation of time-resolved difference spectra is for data at pH 7, 100 mM NaCl, 30°C (Váró and Lanyi, 1991a).

absorption spectra of most of the intermediates overlap one another and the bacteriorhodopsin spectrum, and their exact amplitudes are not known. Global analyses of absorption changes at several wavelengths required five to seven relaxation time constants to describe the kinetics (Xie *et al.*, 1987; Maurer *et al.*, 1987; Gerwert *et al.*, 1990b; Milder *et al.*, 1991; Hofrichter *et al.*, 1989). However, as generally recognized, these phenomenological time constants have limited value because they are model-dependent functions of the desired rate constants of the elementary reactions in the cycle.

The earlier single-wavelength kinetic measurements are now supplanted by spectroscopy at multiple wavelengths, e.g., with gated optical multichannel analyzers which yield high-resolution difference spectra at various times delayed after flash excitation (Zimányi *et al.*, 1989; Hofrichter *et al.*, 1989; Milder *et al.*, 1991). Figure 1 shows an example of such difference spectra. From data of this kind the method of "singular value decomposition" calculates basis difference spectra, with a set of relaxation time constants (e.g., Hofrichter *et al.*, 1989; Milder *et al.*, 1991). Each basis spectrum consists of a model-dependent combination of the spectra of the intermediates. Deconvolution of the spectra into these components depends on (1) obtaining the analytical solution of the rate equations for each model considered, and (2) having kinetics which are non-degenerate with respect to relaxation times.

Unfortunately, in the complex bacteriorhodopsin photocycle both of these have turned out to be serious difficulties.

We obtained a compromise solution, based on the commonly held assumption that in the nanosecond to millisecond time domain under normal conditions there are only the five spectrally distinguishable intermediates listed above. A multiparameter search generated estimates for the spectra of these intermediates, which obeyed the following criteria: (1) the spectra could not contain negative absorptions, and (2) the spectra had to resemble known rhodopsin spectra, since the properties of polyene excited states predicted single maxima and half-widths within certain limits. When the spectra of each of the five intermediates were required to obey these criteria *simultaneously*, the positions of the absorption maxima become fixed to within a few nanometers and the range of possible amplitudes are narrowed to give virtually a single set of spectra (Váró and Lanyi, 1991a, b). Recent work using an unbiased grid search (Zimányi and Lanyi, manuscript in preparation) has indicated that the solutions obtained in this way converge to a strong global minimum. The spectra so obtained were essentially identical to spectra determined in earlier low-temperature measurements. An independent criterion for judging them was provided by the linear relationship of the visible absorption maxima of retinal chromophores to their ethylenic stretch frequency (Aton *et al.*, 1977); the positions of the maxima agreed well with those predicted. A set of spectra for K, L, M, N, and O from such calculations is given in Fig. 2A. Since the absorption maxima of the L and N states are close to each other, their kinetic separation is somewhat ambiguous. The K → L → M reaction segment could be investigated in spite of this problem (Váró and Lanyi, 1991a), by using D96N bacteriorhodopsin under conditions where the M → N reaction is so slow that N does not accumulate appreciably.

Once adequate estimates of the spectra of the intermediates were determined from the data, they provided a set of component difference spectra whose appropriately weighted sums reproduced the measured difference spectra at six temperatures between 5 and 30°C and yielded the fractional concentrations of the intermediates at each delay time (Váró and Lanyi, 1991a, d). Producing such fits is, by itself, an important criterion of self-consistency. Figure 2B (points) shows an example of the calculated transient concentrations of K, L, M, N, O, and BR.

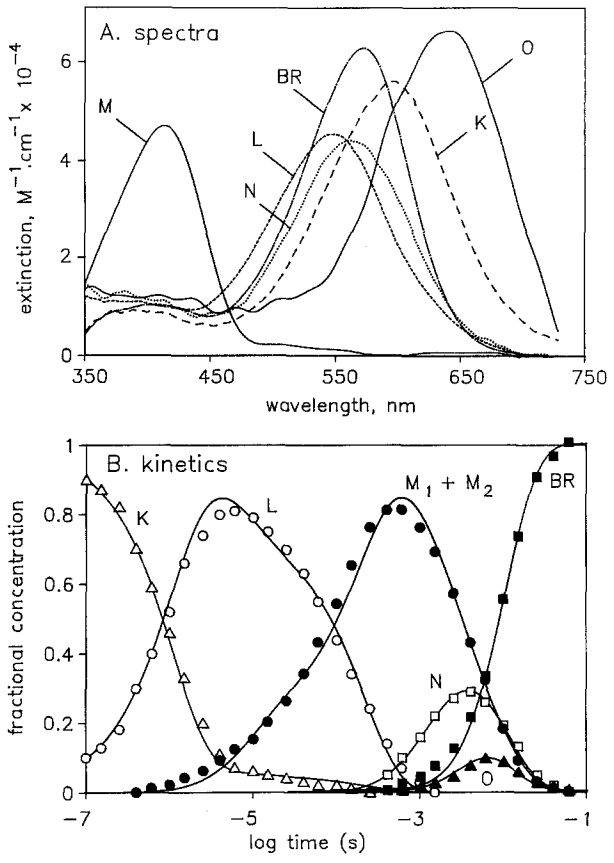

Fig. 2. Derived spectra for the intermediates (A) and the kinetics (B) of the photocycle. Data from Váró and Lanyi (1991d).

High-quality time-resolved resonance Raman spectra are more difficult to obtain but easier to interpret since intermediates with widely separated absorption maxima can be probed individually with probe beams of appropriate wavelengths. Essentially the same kinetic model, except for the existence of M substates (cf. below), was derived from the Raman spectra (Ames and Mathies, 1990) as from the spectra in the visible. Time-resolved FTIR (Gerwert *et al.*, 1990b) also suggested the same model.

The kinetic schemes which fit the time-resolved spectral data consist of single unbranched reaction sequences containing equilibration reactions between K and L, L and M, M and N, and N and O (Váró and Lanyi, 1990c, 1991a, b, d; Gerwert *et al.*, 1990b; Ames and Mathies, 1990; Milder *et al.*, 1991). The reversible reactions which connect K, L, and M together account for the observed three relaxation times in the rise of M. Likewise, they connect M, N, and O kinetically and account for the multiphasic M decay. Thus, all of the measured relaxations are functions of more than

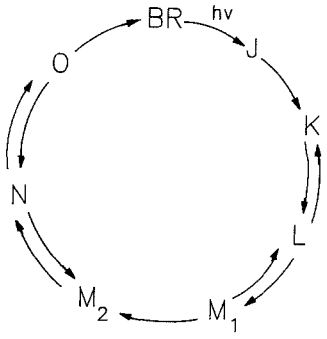

Fig. 3. Suggested photocycle model for bacteriorhodopsin (Váró and Lanyi 1990c, 1991b, d). The J intermediate is detected only on the picosecond time scale. Although not shown here, the K intermediate appears to consist of two substates (Shichida *et al.*, 1983). The L ↔ M_1 and M_2 ↔ N equilibria represent internal proton transfers from the Schiff base to D85 and from D96 to the Schiff base, respectively. A shunt reaction between N and BR, not shown here, is required additionally to fit the data.

one elementary rate constant in the cycle. However, the kinetics of k and L do not contain the time constants of N and O as predicted from a K ↔ L ↔ M ↔ N ↔ O sequence. The problem is most noticeable with L (Váró and Lanyi, 1990c, 1991a). If equilibration between L and M is what accounts for the biphasic L decay, the data indicate that the L ↔ M equilibrium is not far from unity. The observed disappearance of L as M reaches maximal concentration thus presents a paradox. One possible solution is to postulate two spectroscopically indistinguishable sequential M states connected by a unidirectional reaction: $M_1 \rightarrow M_2$ (Fig. 3). In such a scheme the K *plus* L mixture will reach equilibrium with M_1 but not with M_2, and K, L, and M_1 will decay together to zero with the time constant of the $M_1 \rightarrow M_2$ reaction. This model (and not others containing parallel pathways) explains the results, as well as the observation that the slower relaxations of the decay of K and L have the same time constant, which is the slowest of the three time constants for the rise of M (Váró and Lanyi, 1991a). The lines in Fig. 2B are calculated from such a model. Direct evidence for the existence of the postulated substates of M will be given below.

DEPROTONATION OF THE SCHIFF BASE; PROTON RELEASE

D85 is located in a polar region of the protein which contains also the charged residues D212, R82, and the Schiff base, and constitutes a channel leading from the Schiff base to the extracellular side of the membrane (Henderson *et al.*, 1990). In the L to M reaction the Schiff base proton is transferred at 50–100 μs to the anionic D85. The evidence for this includes the following: (1) Time-resolved FTIR shows the appearance of a positive difference peak at about 1761 cm^{-1}, assigned to the protonation of D85, and this is coincident with the rise of M (Braiman *et al.*, 1988, 1991; Gerwert *et al.*, 1990b). (2) Replacement of D85 with asparagine or alanine produces a blue chromophore (see footnote 2) (the maximum of D85N is 615 nm) with a photocycle not containing a deprotonated Schiff base (Mogi *et al.*, 1988; Subramaniam *et al.*, 1990; Otto *et al.*, 1990; Stern *et al.*, 1989; Braiman *et al.*, 1991; Cao and Lanyi, unpublished results). Likewise, protonation of D85 in the wild-type protein at pH < 2 yields a blue protein (absorption maximum at 603 nm) with a similarly truncated photocycle (Fischer and Oesterhelt, 1979; Mowery *et al.*, 1979; Kimura *et al.*, 1984; Kobayashi *et al.*, 1983; Váró and Lanyi, 1989; Renthal *et al.*, 1990). (3) Replacement of D85 with glutamate raises the pK_a for this residue (Lanyi *et al.*, 1992); the protein appears to be in two conformations (a blue and a red) with pK_a's of about 5 and 9 (the difference may be in the spatial disposition of the nearby R82). An intermediate purple form was proposed (Duñach *et al.*, 1990), but action spectra for the photocycle intermediates do not reveal such a species (Lanyi *et al.*, 1992). As in wild type, in the blue form of the D85E protein the Schiff base does not deprotonate. Consistent with the observed higher pK_a for residue 85, in the red form the proton transfer from the Schiff base to E85 during the photocycle is about 100-fold accelerated.

D212 is also in the extracellular proton channel and much indirect evidence, as well as the recently derived structure for the protein (Henderson *et al.*, 1990), indicate that this residue and D85 and R82 are together in position to interact with the Schiff base. ^{15}N and ^{13}CNMR resonance of the Schiff base had shown (De Groot *et al.*, 1989, 1990) that the counterion supplied by the protein to the positive charge is unusually weak and diffuse, and led to the suggestion that the aspartate (and most likely also other) residues might form a three-dimensional network around a water molecule in which the net negative charge is delocalized. Proton transfer to such a complex would redistribute the proton occupancies of the various electronegative locations in the network. Consistent with this, replacement of D212 with asparagine (Needleman *et al.*, 1991; see also footnote 2) resulted

in a blue chromophore (maximum at 585 nm at pH > 7); the photocycle contained no M state, indicating that anionic residues at positions 212 and 85 have similar influences on the deprotonation of the Schiff base in L. The time-resolved difference spectra and the derived kinetics for D212N suggested the sequence $K \leftrightarrow L_1 \leftrightarrow L_2 \rightarrow BR$. The two L states might be analogous to the two M states, but with the Schiff base protonated. At lower pH, protonation of an unidentified residue caused a color change in the chromophore to purple, and the photocycle of this form included M. The proton transfers occurred with the correct vectoriality since this form transported protons. The M kinetics suggested, however, a widening of the pK_a difference between D85 and the Schiff base in the $L \leftrightarrow M$ reaction, relative to the wild type. Thus, D212 and D85, at least, seem to act in concert in the Schiff base deprotonation, D212 most likely by influencing the pK_a of either D85 or the Schiff base, or both. However, as mentioned above, it is D85 and not D212 which becomes protonated in M, and D212 is not essential for proton transport. D85 and D212 are unequal in other respects as well: (1) the red shift of the maximum in D85N relative to wild type is 46 nm but only 16 nm in D212N, and (2) only the D85 replaced mutants show lowered pK_a (from near 13 to about 7) for the Schiff base NH^+ in unilluminated bacteriorhodopsin (Otto *et al.*, 1990; Cao and Lanyi, unpublished results). These differences would seem to indicate that D85 interacts more directly with the Schiff base than D212 does, in both bacteriorhodopsin and the L state. Other models have been discussed where in L the Schiff base N–H bond moves away either from D212 and toward D85 (Mathies *et al.*, 1991), or from D85 and toward D96 (Pfefferlé *et al.*, 1991).

Transient absorption change of pH indicator dyes have detected proton release into the medium roughly concurrently with the $L \rightarrow M$ reaction. The time resolution of the dye response depends on how close the dye is located to the membrane surface. Highly negatively charged dyes, such as pyranine, show a several hundred μs time constant for proton release at 20°C unless buffer is present for rapid conduction of the protons into the bulk solution (Drachev *et al.*, 1984; Grzesiek and Dencher, 1986). The maximum rate detected is about 300 μs, while the less negatively charged phenol red detects proton release at about 100 μs (Váró and Lanyi, 1990b). Fluorescein covalently linked to the protein senses local pH change particularly rapidly; at 3°C it gave a 300-μs time constant (Heberle and Dencher, 1990). The fastest of these rates are in the same time range as the slower phase of the $L \rightarrow M_1$ reaction. In the model in Fig. 3 the first phase of the $L \rightarrow M_1$ reaction is the equilibration of the proton between the Schiff base and D85, while the second phase is the shifting of this equilibrium toward full deprotonation of the Schiff base by the $M_1 \rightarrow M_2$ reaction. Unfortunately, it is not clear whether a proton release component with a time constant the first phase of the $L \rightarrow M_1$ reaction would have been detectable with the dyes. Thus, the measurements so far do not decide whether proton release is triggered by the $L \rightarrow M_1$ or the $M_1 \rightarrow M_2$ reaction. The latter seems more likely since up to pH 9 the k_{KM1}/k_{M1L} ratio is pH independent (Váró and Lanyi, 1990c; Ames and Mathies, 1990), indicating that proton uptake from the medium cannot shift the proton from D85 back to the Schiff base. Proton release in the unidirectional $M_1 \rightarrow M_2$ switch reaction (cf. below) would be consistent with our suggestion that this is the step where the chromophore reaction is energetically coupled to the proton gradient.

According to time-resolved FTIR difference spectra, D85 remains protonated well into the millisecond time range (Braiman *et al.*, 1988, 1991; Gerwert *et al.*, 1990b; Pfefferlé *et al.*, 1991), indicating that the proton must be released from another group. The model of a diffuse counterion and proton acceptor complex would suggest that this group is R82, but this would be possible only if the pK_a of the guanidinium were unusually low.

THE SWITCH

The logical necessity for a switch which reorients the Schiff base from the extracellular to the cytoplasmic side has been voiced for some time. It was placed at M (Nagle and Mille, 1981; Schulten *et al.*, 1984; Gerwert and Siebert, 1986; Henderson *et al.*, 1990; Mathies *et al.*, 1991), N (Fodor *et al.*, 1988), or O (Milder, 1991). In a model with reversible $L \leftrightarrow M$ and $M \leftrightarrow N$ reactions (as in Fig. 3) the switch would be, by necessity, at M. Experimental evidence for two sequential M substates is now available. Although they were invoked originally to explain the anomalous kinetics of L decay (Váró and Lanyi, 1990c, 1991b), their existence was later confirmed by the observation of a 4-nm blue shift in the transient M spectrum of solubilized bacteriorhodopsin, which occurred at the time predicted for the postulated $M_1 \rightarrow M_2$ reaction

(Váró and Lanyi, 1991a). A blue shift for late M in detergent-solubilized bacteriorhodopsin is now reported by several other groups (Milder *et al.*, 1991; Subramaniam *et al.*, 1991). In the monomers therefore, although not in the purple membrane lattice, the two M states have slightly different spectra. More recently, we found that in the photocycle of monomeric D115N bacteriorhodopsin the two M maxima were split by as much as 15 nm (Váró *et al.*, 1992). This large shift amply confirms the existence of two distinct M states and justifies the earlier kinetic analysis, but raises questions about the origin of the spectral separation. The fact that it is observable only in the monomers suggests that motional freedom of the protein, and therefore the retinal, is required for the shift. The increased effect in the D115N mutant suggests that removal of the motional constraint might be near the β-ionone ring. This would exclude any mechanism for the switch which involves only the Schiff base region of the retinal.

The molecular mechanism of the switch is, in fact, still unknown. As argued in Váró and Lanyi (1991d), the problem is that the configuration of the retinal is 13-*cis* throughout most of the photocycle, i.e., well before and after the $M_1 \rightarrow M_2$ reaction. In one suggested mechanism the switch is a rotation around the C14–C15 single bond which would reorient the Schiff base (Gerwert and Siebert, 1986), in another the switch originates from protein conformational motions (Fodor *et al.*, 1988).

In purple membrane sheets, where protonmotive force cannot develop, the $M_1 \rightarrow M_2$ transition appears as an irreversible reaction. The magnitude of k_{M2M1} is clearly small; an estimation of the upper limit of the $M_2 \rightarrow M_1$ back-reaction was obtained from analyzing the quasi-equilibrium which develops before the greatly delayed M decay in the D96N mutant. In this system no significant change in the component concentrations occurs between 1 and 3 ms, and from the $[L]/[M_1 + M_2]$ equilibrium ratio during this time the rate of the back-reaction could be calculated (Váró and Lanyi, 1991a). This estimation gave $k_{M1M2}/k_{M2M1} > 200$, limited only by the signal/noise ratio in the time-resolved spectra.

REPROTONATION OF THE SCHIFF BASE; PROTON UPTAKE

D96 has an anomalously high pK_a for an aspartate residue (Otto *et al.*, 1990), most likely because of its location in a rather hydrophobic environment. Time-resolved FTIR measurements show the development of a peak at 1755 cm^{-1} as the Schiff base is reprotonated (i.e., in the $M_2 \leftrightarrow N$ reaction); this is attributed to deprotonation of D96 (Gerwert *et al.*, 1989, 1990b; Braiman *et al.*, 1991; Pfefferlé *et al.*, 1991). Thus, kinetically at least D96 is the immediate proton donor to the Schiff base. Replacing D96 with nonprotonable groups greatly slows the reprotonation of the Schiff base; protonation in this system appears to be directly from the aqueous phase (Tittor *et al.*, 1989; Miller and Oesterhelt, 1990; Otto *et al.*, 1989; Cao *et al.*, 1991). The path of the transferred proton is a problem. The distance between the Schiff base and D96 is 10–12 Å in unilluminated bacteriorhodopsin (Henderson *et al.*, 1990), and it is unlikely that this can change significantly in the M intermediate. Protonable groups between the Schiff base and D96 are absent, and there is only one residue, T89, which might participate in proton conduction. It is widely held therefore that water trapped in single file in the narrow proton channel on the cytoplasmic side of the protein is the most reasonable candidate for conducting protons.

Osmotically active solutes, in fact, specifically inhibit the $M_2 \leftrightarrow N$ reaction in the photocycle; we estimate that proton exchange between D96 and the Schiff base requires about 15 molecules of water inside the protein (Cao *et al.*, 1991). This is much more than what is needed for a string of water along the proton trajectory. The results suggest that the detected water stabilizes the transition state in the proton transfer, which consists of the proton–aspartate dipole. Comparison of the rate of the $M_2 \leftrightarrow N$ reaction with the rate of water-mediated proton conduction in gramicidin revealed an additional 40 kJ/mol activation energy. This appears to be the enthalpic barrier of producing the transition state dipole because replacement of D96 with asparagine lowered the activation enthalpy by 42 kJ/mol. Thus, the rate of proton transfer from D96 to the Schiff base is accounted for by gramicidin-like water-mediated proton conduction plus the additional barrier consisting of the Born self-energy of the proton–aspartate pair. In contrast, the N decay in the wild-type protein at pH > 8, and the Schiff base reprotonation in the D96N protein directly from the surface at all pH values, are limited by capture of a proton on the cytoplasmic surface. These reactions are much less influenced by withdrawal of water from the protein, similarly to the azide-catalyzed protonation of the Schiff base in D96N

(Tittor *et al.*, 1989; Miller and Oesterhelt, 1990; Otto *et al.*, 1989) which is virtually unaffected by water (Cao *et al.*, 1991). In the latter case the proton transfer must be in a direct donor–acceptor pair between the Schiff base and HN_3, and the acceleration of the protonation is caused by the higher capture rate for HN_3 than H^+.

THE COUPLING OF CHROMOPHORE REACTIONS TO THE PROTON POTENTIAL

Calorimetric measurements had shown (Birge and Cooper, 1983; Ort and Parson, 1979b) that only a small portion of the energy of the photon is conserved in K. A recalculation of the excess enthalpy content of K, based on recently accepted quantum yields, gave 49 kJ/mol (Birge *et al.*, 1991). Assuming that most or all of the excess free energy of K is enthalpy, this is sufficient to drive only one proton to the observed 180-mV proton potential at realistic thermodynamic efficiencies.

Even at first glance, the existence of only two irreversible reactions ($M_1 \rightarrow M_2$, and $O \rightarrow BR$) in the photocycle of an open syste, such as the purple membrane sheets, indicates that the excess free energy is lost from the system at discrete steps rather than gradually throughout the entire reaction sequence. Which of these is the reaction which drives the proton transport? Since proton back-pressure in *H. halobium* cells causes accumulation of M (Dancsházy *et al.*, 1983; Groma *et al.*, 1984) rather than N or O, the immediate driving reaction must be the $M_1 \rightarrow M_2$ step. This proposition is consistent with two other findings: (1) The proton transport is essentially over once N is reached (Otto *et al.*, 1989; Kouyama and Nasuda-Kouyama, 1989; Váró *et al.*, 1990a; Subramaniam *et al.*, 1991). (2) The ΔG lost at the $M_1 \rightarrow M_2$ reaction in purple membrane sheets would be sufficient to produce protonmotive force of a reasonable size. A protonmotive force of 180 mV is equivalent to a free energy of -17 kJ/mol. The free energy change at the $M_1 \rightarrow M_2$ step will be $RT\ln(k_{M1M2}/k_{M2M1})$. As discussed above, k_{M2M1} was estimated to be no greater than $k_{M1M2}/200$, which provides a ΔG of at least 13 kJ/mol. Thus, the $M_1 \rightarrow M_2$ reaction may be sufficiently exergonic to generate a physiologically relevant protonmotive force. In fact, the actual ΔG loss at this step could be as much as 25–30 kJ/mol without dissipating an excessive fraction of the free energy on the proton transport and thus jeopardizing full recovery of bacteriorhodopsin at the final $O \rightarrow BR$ step. The free-energy diagram of the photocycle based on such considerations (Váró and Lanyi, 1991d) is given in Fig. 4.

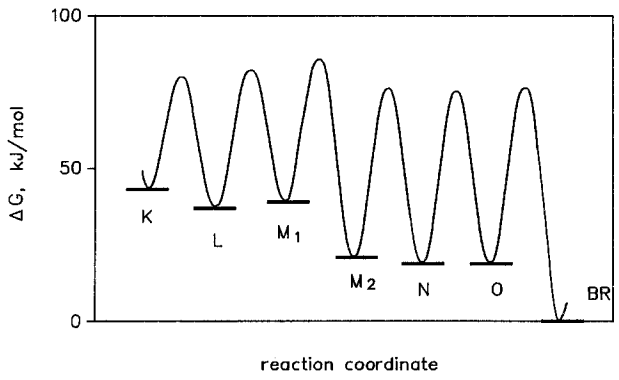

Fig. 4. Free-energy diagram of the bacteriorhodopsin photocycle, based on data and calculations in Váró and Lanyi (1991d). The shunt reaction between N and BR is not shown here.

The enthalpy and entropy changes during the photocycle provide more detailed information about the flow of energy through the system. The temperature dependences of the observable microscopic rate constants in the photocycle define the activation enthalpies and entropies, and for many of the intermediates also their enthalpy and entropy levels relative to each other. Thus, the thermodynamic parameters of the K—L—M_1 and M_2—N—O reaction segments could be described in detail, both in purple membranes (Váró and Lanyi, 1991d) and in bacteriorhodopsin monomers (Váró and Lanyi, 1991c). The reactions in the first segment take place roughly isoenergetically and without significant changes in ΔH, ΔS, and ΔG. The reactions in the second segment are also isoenergetic, but entail the large-scale conversion of $T \cdot \Delta S$ to ΔH. This is consistent with the earlier finding (Ort and Parson, 1979a; Garty *et al.*, 1982) that there is a large enthalpy loss in the system at a step, undefined at the time, between proton release and uptake, resulting in negative ΔH relative to the initial state (about 80 kJ/mol). The enthalpy diagram of the photocycle identifies this step as the $M_1 \rightarrow M_2$ reaction (Váró and Lanyi, 1991d). Assuming that the free-energy decrease between K and M_2 is 17 kJ/mol, the total of 110 kJ/mol enthalpy decrease requires an entropy decrease of about 300 J/mol K (this value is not greatly changed if the free-energy decrease is in fact 25–30 kJ/mol). Thus, after the $M_1 \rightarrow M_2$ reaction the remaining free energy of the system will be in the

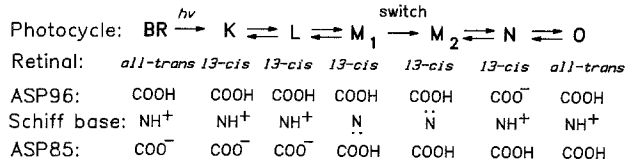

Fig. 5. Summary of the isomeric states of the retinal and the protonation states of D85 and D96 throughout the photocycle.

form of negative entropy; during the rest of the photocycle $T \cdot \Delta S$ is reconverted to ΔH. An entropy decrease of the required size can be produced only by a system of a large degree of internal freedom. This rules out the sole participation of the chromophore, and demands that a change in protein configurational/conformational states be invoked in the second half of the photocycle, beginning with M_2. Time-resolved FTIR (Gerwert *et al.*, 1990a, b; Ormos, 1991; Braiman *et al.*, 1991) and X-ray diffraction (Koch *et al.*, 1991) do, in fact, reveal protein conformational changes after M. In the latter case, at least, the changes are mainly in the N → BR reaction, where the largest entropy changes take place also (Váró and Lanyi, 1991d). The second half of the photocycle is thus driven by conformational recovery of the protein. Consistently with this, the thermodynamic parameters of the first half of the photocycle in monomeric bacteriorhodopsin are little changed from those in the purple membrane lattice, but greatly altered in the second half (Váró and Lanyi, 1991c). Importantly, the protein conformational change plays little or no role in the energetics of the proton translocation itself. Rather, its relaxation will reset the system after the chromophore had accomplished the essential steps in the proton transport.

CONCLUSIONS

The thermodynamic description gives an explicit model for the flow of energy during the bacteriorhodopsin photocycle. It is a good framework for organizing what is known or surmised at present about the chromophore and the protein into a self-consistent mechanism for the proton transport, as follows. Figure 5 summarizes the isomeric states of the retinal, and the protonation states of the Schiff base, D85, and D96 in each of the intermediates. Absorption of a photon gives rise to the sequential J and K intermediates. In these intermediates the excess free energy partly or more likely wholly resides in retinal-bond torsions and angular distortions. The strained 13-*cis* configuration in K is relaxed upon thermal decay to the L state. In L, free energy is conserved mainly as the lowered pK_a of the Schiff base; since this pK_a now roughly equals that of D85, reversible proton exchange with this residue is possible. Proton release into the extracellular medium appears to be an indirect consequence of the protonation of D85. In the first deprotonated M state (M_1) the proton is already physically separated from the Schiff base, and proton potential develops as a result of this separation and the ensuing proton release. Energy transfer to the transported proton takes place therefore as the deprotonated Schiff base moves away from its proton exchange partner to produce M_2. At this reaction most or all excess free energy is lost from the chromophore. Free energy other than what is transferred to the proton gradient is conserved in the protein as a large-scale conformational change. Relaxation of the low-entropy conformation will drive the completion of the photocycle and ensures that the initial state fully recovers. Besides fulfilling these energetic requirements, the $M_1 \rightarrow M_2$ reaction serves two mechanistic functions which are necessary for the directional proton transfer: (1) It changes access of the Schiff base from the extracellular to the cytoplasmic side, and (2) it restores the high pK_a of the Schiff base as the excess free energy leaves the chromophore at this time. Thus, in the next step the Schiff base can regain the proton from D96, the internal proton donor with high pK_a on the cytoplasmic side.

Importantly, the free-energy diagram of the cycle (Fig. 4) indicates that both $L \leftrightarrow M_1$ and $M_2 \leftrightarrow N$ proton transfers are isoenergetic, and thus have mechanistic rather than energetic functions. Reprotonation of D96, apparently directly from the cytoplasmic surface, is also near zero ΔG. By a mechanism so far not understood, the conformational relaxation at lower pH and the reprotonation of D96 at higher pH allow the fast reisomerization of the retinal to *all-trans*, which leads to the O state. The kinetics suggest that depending under conditions the initial bacteriorhodopsin will recover either from O or directly from N.

The finding that the orientational switch reaction also entails energy transfer to the ion gradient and enthalpy–entropy conversion was not anticipated. The latter, at least, is not inherent in the concept of the switch. The rationale for this complexity in the $M_1 \rightarrow M_2$ reaction is obviously that the thermodynamic and mechanistic requirements of the proton transport are inseparably interwoven in this system. It

remains to be seen if this design principle will be observed in other ion pumps as well.

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