Water Is Required for Proton Transfer from Aspartate-96 to the Bacteriorhodopsin Schiff Base†

Yi Cao,‡ György Váró,§ Man Chang,‖ Baofu Ni,‖ Richard Needleman,‖ and Janos K. Lanyi*,‡

Department of Physiology and Biophysics, University of California, Irvine, California 92717, Biological Research Center of the Hungarian Academy of Sciences, H-6701 Szeged, Hungary, and Department of Biochemistry, Wayne State University School of Medicine, Detroit, Michigan 48201

Received June 18, 1991; Revised Manuscript Received August 23, 1991

ABSTRACT: During the M ↔ N → BR reaction sequence in the bacteriorhodopsin photocycle, proton is exchanged between D96 and the Schiff base, and D96 is reprotonated from the cytoplasmic surface. We probed these and the other photocycle reactions with osmotically active solutes and perturbants and found that the M ↔ N reaction is specifically inhibited by withdrawing water from the protein. The N → BR reaction in the wild-type protein and the direct reprotonation of the Schiff base from the cytoplasmic surface in the site-specific mutant D96N are much less affected. Thus, it appears that water is required inside the protein for reactions where a proton is separated from a buried electronegative group, but not for those where the rate-limiting step is the capture of a proton at the protein surface. In the wild type, the largest part of the barrier to Schiff base reprotonation is the enthalpy of separating the proton from D96, which amounts to about 40 kJ/mol. We suggest that in spite of this D96 confers an overall kinetic advantage because when this residue becomes anionic in the N state its electric field near the cytoplasmic surface lowers the free energy barrier of the capture of a proton in the next step. In the D96N protein, the barrier to the M → BR reaction is 20 kJ/mol higher than what would be expected from the rates of the M → N and N → BR partial reactions in the wild type, presumably because this mechanism is not available.

The transient isomerization of retinal in bacteriorhodopsin (BR),[1] a light-activated pump, drives the active transport of protons across the cytoplasmic membrane of halobacteria. A number of recent reports (Váró & Lanyi, 1990a, 1991a,b; Ames & Mathies, 1990; Gerwert et al., 1990; Milder et al., 1991) have described the reaction cycle after the absorption of the photon as a single linear sequence with several reversible reactions: BR $\xrightarrow{h\nu}$ K ↔ L ↔ M ↔ N ↔ O → BR. The M intermediate was resolved into two sequential states, M_1 and M_2, connected by an irreversible reaction (Váró & Lanyi, 1990a, 1991a). The latter step was suggested to function as the switch between the extracellular and cytoplasmic sides (Váró & Lanyi, 1991b); it also provides for a decrease of ≥13 kJ/mol in ΔG (Váró & Lanyi, 1991a), part of which is conserved as proton potential difference across the membrane. Three of the photocycle reactions consist of proton transfers. It is generally agreed that the L → M reaction represents proton transfer from the Schiff base to D85 (Braiman et al., 1988; Gerwert et al., 1989, 1990; Butt et al., 1989; Stern et al., 1989), a residue which communicates with the extracellular side of the membrane (Henderson et al., 1990). Likewise, a large amount of evidence indicates that the M → N reaction represents proton transfer from D96 to the Schiff base (Gerwert et al., 1989, 1990; Butt et al., 1989; Stern et al., 1989; Holz et al., 1989; Tittor et al., 1989; Otto et al., 1989, 1990). D96 communicates with the cytoplasmic side, and during the N → O step which follows the proton is replaced on D96 from the aqueous medium, completing the proton circuit across the membrane.

Little is known about the mechanism of the proton transfer reactions. The two-step reprotonation of the Schiff base from the cytoplasmic surface is particularly problematical because according to the most recent structure of the protein (Henderson et al., 1990) the proton moves over large distances: D96 and the Schiff base are separated by 10–12 Å and the surface and D96 by 6–8 Å. The two sequential proton transfers define a path from the Schiff base to the cytoplasmic surface, which was termed by Henderson et al. (1990) the *cytoplasmic proton channel*. The inner span contains only one residue, T89, which could participate in a hydrogen-bonded proton transfer chain (Nagle & Morowitz, 1978; Nagle & Tristram-Nagle, 1983). Further, replacement of residues in either span which might play proton transfer roles, i.e., T46, T89, T205, and S226, does not greatly affect the Schiff base reprotonation, although in some of these cases a slow M decay component did appear (Marti et al., 1991). On the other hand, a string of a fixed or mobile water molecules extending from the Schiff base toward the cytoplasmic surface would eliminate the need for protein residues [cf. Nagle (1987), Deamer (1987), and Deamer and Nichols (1989) for a discussion of such proton conduction in proteins and lipid bilayers]; what is known about the structure of the protein allows placing water molecules into this region (Henderson et al., 1990). Neutron diffraction localized about four tightly bound water molecules in the general neighborhood of the Schiff base (Papadopoulos et al., 1990).

In spite of these questions concerning the nature of proton conduction, it is clear that the strategically placed residue D96 plays the key role in the path of the proton from the surface to the Schiff base. Replacing D96 with asparagine, glycine, or alanine produced characteristic changes in M decay which shed some light on the mechanism of this proton transfer. In such mutated proteins, the usually bi-exponential M decay is

† Partly supported by grants from the National Institutes of Health (NIH GM 29498 to J.K.L.), the National Aeronautics and Space Administration (NAGW-212 to J.K.L.), and the National Science Foundation (DMB 9007365 to R.N.)

* To whom correspondence should be addressed.

‡ University of California.

§ Biological Research Center of the Hungarian Academy of Sciences.

‖ Wayne State University School of Medicine.

[1] Abbreviations: AMPSO, 3-[(1,1-dimethyl-2-hydroxyethyl)-amino]-2-hydroxypropanesulfonic acid; BR, bacteriorhodopsin.

0006-2960/91/0430-10972$02.50/0

monophasic and dependent on pH, indicating that protons in the aqueous phase are direct participants in the reprotonation (Otto et al., 1989; Tittor et al., 1989; Miller & Oesterhelt, 1990). The activation parameters of M decay in wild type as well as D96N and D96G proteins were calculated from the temperature dependencies of the relaxations (Tittor et al., 1989; Miller & Oesterhelt, 1990). This led to the suggestion that the importance of D96 is that it overcomes an otherwise unfavorably large negative entropy of activation for proton conduction. Thus, D96 will provide a kinetic advantage in spite of the much higher thermal barrier observed in the wild type than in the mutants. HN_3 and other weak conjugate acids act as external proton donors to the Schiff base of the related retinal protein halorhodopsin (Hegemann et al., 1985; Lanyi, 1986), which lacks a protonable residue analogous to D96. Azide was found to functionally replace D96 in D96N and D96G bacteriorhodopsins also: it restored the rate of M decay (and proton transport) to that in wild type and raised the activation enthalpy (Tittor et al., 1989), and made the M decay pH-independent (Miller & Oesterhelt, 1990). Azide was therefore suggested to bind in the proton channel of D96N where it reversibly protonates in the same manner as D96 in the wild type (Miller & Oesterhelt, 1990). However, it was reported elsewhere (Otto et al., 1989) that the M decay in D96N remains pH-dependent even in the presence of azide and at high concentrations of azide its rate far exceeds the rate in the wild type.

We report here experiments designed to understand the mechanism of proton transfers on the cytoplasmic side of bacteriorhodopsin, their energetics, and particularly any participation of water. The results are generally consistent with water-mediated proton conduction, but where large amounts of water are required in the protein, they are to allow the proton exchange between D96 and the Schiff base in the M ↔ N equilibrium. It appears that extensive internal hydration of the protein is needed to stabilize transition states in which separation of a proton from its buried electronegative partner created an ion pair. The presence or absence of an ionizable residue at position 96 has an effect primarily on capture of the proton at the cytoplasmic surface. Thus, charge separations and their shielding at the surface and inside the protein have important influences on the late photocycle steps.

Materials and Methods

The bacteriorhodopsins were prepared as purple membrane sheets from *Halobacterium halobium* (Oesterhelt & Stoeckenius, 1974). The wild-type protein was from strain S9; the clone containing the D96N-mutated protein was constructed from strain L-33 by transformation with a shuttle vector containing the modified *bop* gene, described elsewhere (Ni et al., 1990; Needleman et al., 1991). All samples contained 100 mM NaCl, AMPSO,[1] and/or phosphate at 50 mM total concentration, plus solute at the indicated percent (w/w) concentration. Where the temperature was varied, phosphate was used as buffer because its pK_a is virtually temperature-independent. Unless otherwise mentioned, the measurements were at 22 °C. When M decay was slow, the samples were encased in a polyacrylamide gel as described before (Mowery et al., 1979) so as to avoid diffusional exchange of material between the illuminated and nonilluminated areas of the cuvette. The gel itself does not change any of the photocycle reactions. In no case did the measuring light cause the development of a photostationary state sufficient to affect the measured decay of the M intermediate. The osmolalities of sucrose, glucose, glycerol, and propylene glycol solutions at any given weight percent concentrations are from the CRC Handbook of Chemistry and Physics; where necessary, binomial extrapolation was used to calculate a few values somewhat outside the range in the tables. The water activities were calculated from the formula $a_w = 55.5/(55.5 + X)$, where X is the osmolality of the solute. The change in the chemical potential of water is $\Delta\mu_w = RT \ln a_w$. Viscosities were also from the CRC Handbook of Chemistry and Physics, but they were additionally determined with a flow viscometer directly on the solutions used.

Poly(vinylpyrrolidone) (average molecular weight 360000) was from Sigma Chemical Co. (St. Louis, MO).

The measurement of time-resolved spectra with a gated optical multichannel analyzer and the determination of single-wavelength kinetics were as previously described (Zimányi et al., 1989; Váró & Lanyi, 1990b). All lines drawn were calculated from least-squares fits.

Results

Influence of Osmotically Active Solutes on the Photocycle Reactions. The effects of withdrawing solute-inaccessible water from bacteriorhodopsin were explored by comparing the photocycle reactions at pH 9.0 in the presence and absence of 58% sucrose. Figure 1 shows spectra of the mixtures of intermediates at various delay times after flash excitation, obtained by adding appropriately scaled bacteriorhodopsin spectra (Váró & Lanyi, 1990a, 1991a) to the measured time-resolved difference spectra. Increased absorption is observed near 600 nm early in the photocycle (intermediate K), followed in sequence by the appearance of absorption near 530 nm (intermediate L), near 410 nm (intermediate M), and near 560 nm (intermediate N). The absorption changes overlap each other in time. The results in the absence of sucrose (Figure 1A) are similar to what we had reported earlier for pH 7 (Váró & Lanyi, 1991a), except that at the high pH the intermediate O does not accumulate significantly. This known fact (Lozier et al., 1978; Li et al., 1984; Váró et al., 1990) allowed the convenient omission of O from the subsequent analysis. Figure 1B shows that the absorption changes in the second half of the photocycle are noticeably changed in the presence of sucrose: the ratio of amplitudes for the maxima attributed to M and N is changed, the decay of M is slowed, and the maximum attributed to M overlaps less with absorption increases at higher wavelengths.

In previous reports (Váró & Lanyi, 1990c, 1991a), we described how the photocycle kinetics can be calculated from time-resolved difference spectra. First, we derive a set of estimated spectra for the photointermediates from several of the measured difference spectra. Figure 2A shows such spectra for K, L, M, and N from the experiment in the absence of sucrose; they are nearly identical to spectra at pH 7 (Váró & Lanyi, 1991a). These were then used to calculate the time-dependent concentrations of the intermediates from the difference spectra in the two experiments (points in Figure 2A,B). The lines in these graphs are the fits of the abbreviated model $K \leftrightarrow L \leftrightarrow M_1 \rightarrow M_2 \leftrightarrow N \rightarrow BR$. The kinetics confirm the visual impression of the effects of sucrose in Figure 1. The largest change is that k_{M_2N} and k_{NM_2} are decreased; this increased the accumulation of M at the expense of N, and delayed the formation of N, so that with sucrose present M was virtually the only photointermediate detected at 0.6 ms.

Earlier reports on the effects of high concentrations of solutes, such as glycerol, on the bacteriorhodopsin photocycle focused on viscosity rather than on osmotic pressure (Beece et al., 1981; Marque & Eisenstein, 1984). We examined the kinetics of M formation and decay in four solutions of equal and rather high viscosity (45 cSt): 60% sucrose, 35% glucose,

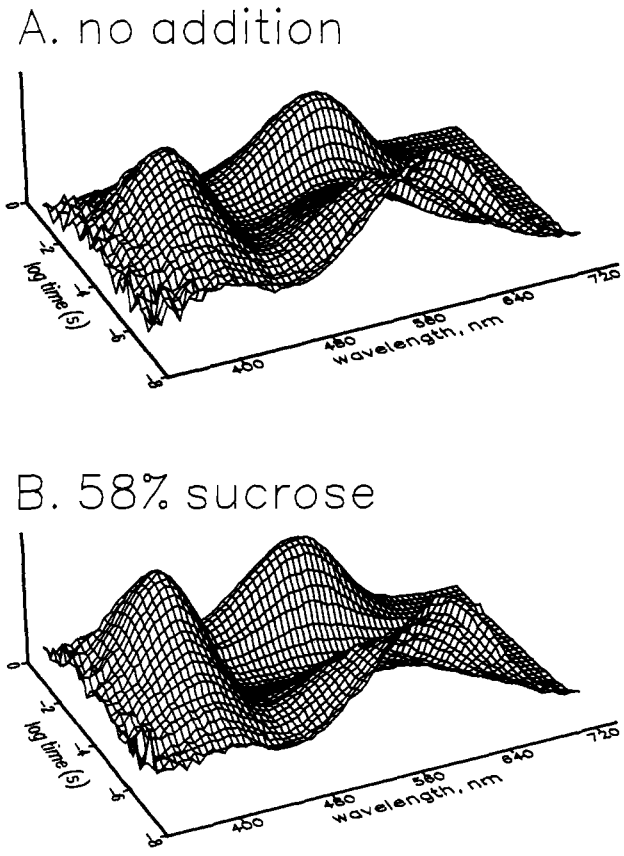

FIGURE 1: Time-resolved absorption spectra which describe the photointermediates of bacteriorhodopsin between 100 ns and 100 ms. The samples contained 20 nmol/mL BR, 100 mM NaCl, 50 mM AMPSO, pH 9.0, and either no added solute (A) or 58% (w/w) sucrose (B). The spectra represent the mixtures of intermediates at the indicated times after photoexcitation. They were calculated from each measured difference spectrum by adding an appropriately scaled BR spectrum. The latter was constant until 1 ms in (A) and 10 ms in (B) and declined after this time as the initial BR state was repopulated.

80% glycerol, and 4.1% poly(vinylpyrrolidone). Figure 3 shows absorption changes at 410 nm under these conditions, and in a BR sample without added solute. Sucrose, glucose, and glycerol at these concentrations affected primarily M decay (as 58% sucrose in Figure 2), but to considerably different extents. Further, poly(vinylpyrrolidone) had no detectable influence. Bulk viscosity is therefore not correlated with M kinetics. A possibility of lower "local" viscosity in the poly(vinylpyrrolidone) solution is discounted because this polymer behaves in solution as a hydrated random coil and affects essentially all of the volume. On the other hand, since the molar concentrations of the solutions in Figure 3 are different, they have considerably different osmotic pressures. The quantitative examination of M decay (cf. below) indicates that osmotic pressure, i.e., water activity, is the valid basis for analyzing the effects of these solutes.

Quantitation of the effect of solutes made use of kinetic analysis of the measured M relaxations according to the $M_2 \leftrightarrow N \rightarrow BR$ reaction sequence, similar to that used by Otto et al. (1989). The rate constants k_1, k_{-1}, and k_2, which describe the reactions $M_2 \rightarrow N$, $N \rightarrow M_2$, and $N \rightarrow BR$, respectively, were calculated from the equations:

$$[M]_t = \frac{p - q - k_1}{2p} \exp(q + p)t + \frac{p + q + k_1}{2p} \exp(q - p)t \quad (1)$$

$$p = (1/2)[(k_1 + k_{-1} + k_2)^2 - 4k_1k_2]^{1/2} \quad (2)$$

$$q = -(1/2)(k_1 + k_{-1} + k_2) \quad (3)$$

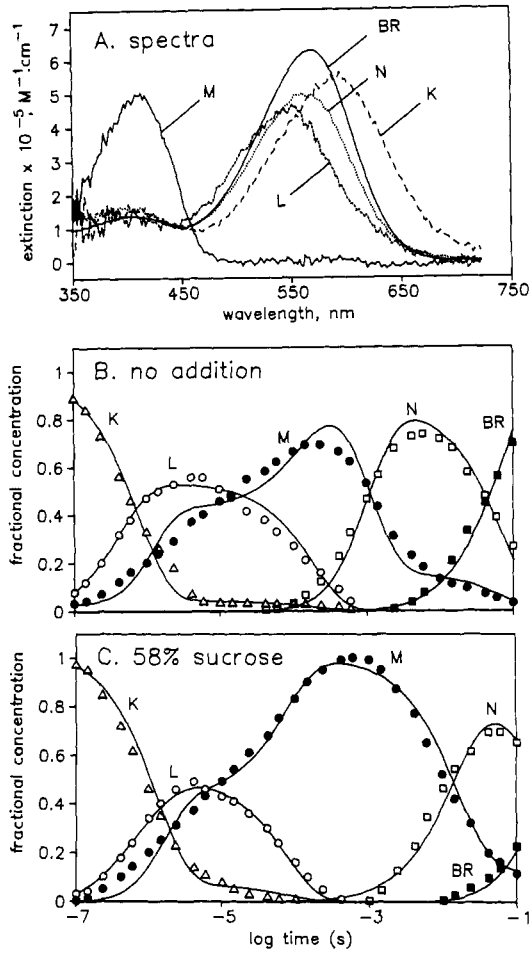

FIGURE 2: Component spectra and kinetics in the presence and absence of 58% sucrose. (A) Calculated component spectra for K, L, M (i.e., the sum of M_1 and M_2), and N from the experiment in Figure 1A). These were used to fit the measured spectra to generate the time-resolved concentrations in the absence of sucrose [points in (B)] and in the presence of sucrose [points in (C)]. Symbols: (△) K; (○) L; (●) $M_1 + M_2$; (□) N; (■) BR. The lines are the best fits of the model $K \leftrightarrow L \leftrightarrow M_1 \rightarrow M_2 \leftrightarrow N \rightarrow BR$. The rate constants in s^{-1} (in parentheses the values with sucrose): $k_{KL} = 1.25 \times 10^6$ (7.14×10^5); $k_{LK} = 1.0 \times 10^5$ (1.0×10^5); $k_{LM_1} = 1.0 \times 10^6$ (1.11×10^6); $k_{M_1L} = 1.25 \times 10^6$ (1.25×10^6); $k_{M_1M_2} = 1.25 \times 10^4$ (3.0×10^4); $k_{M_2N} = 833$ (56); $k_{NM_2} = 154$ (10); $k_{NBR} = 17$ (3.3).

We determined the (normalized) amplitudes and exponents of the biexponential M decays with nonlinear least-squares fitting of single-wavelength kinetic measurements as in Figure 3, and calculated the three rate constants from the values. This was possible because at pH 9 both exponential terms in eq 1 are large; at this pH, the decay of N is slow enough to cause substantial accumulation of N, and the $N \rightarrow M_2$ back-reaction has a large effect on the overall M kinetics. The results at various concentrations of the osmotically active solutes sucrose, glucose, glycerol, and the protein perturbant propylene glycol are plotted in Figure 4 as $-RT \ln (k_i/k_i^0)$ vs the change in the chemical potential of water, $\Delta\mu_w$, where k_i and k_i^0 are the rate constants in the presence and absence of solute, respectively. The calculated parameter is the additional free energy of activation imposed on the reactions by the added solutes. Figure 4 shows that decreasing the water activity inhibits the $M_2 \rightarrow N$ and $N \rightarrow M_2$ reactions, while the $N \rightarrow BR$ reaction is much less affected. The molecular sizes of the solutes have a significant influence also; the order of effectiveness is sucrose > glucose > glycerol (with molecular weights of 342, 180, and 92, respectively). Dextran is often used in such studies, but it would have produced much less decrease in water activity

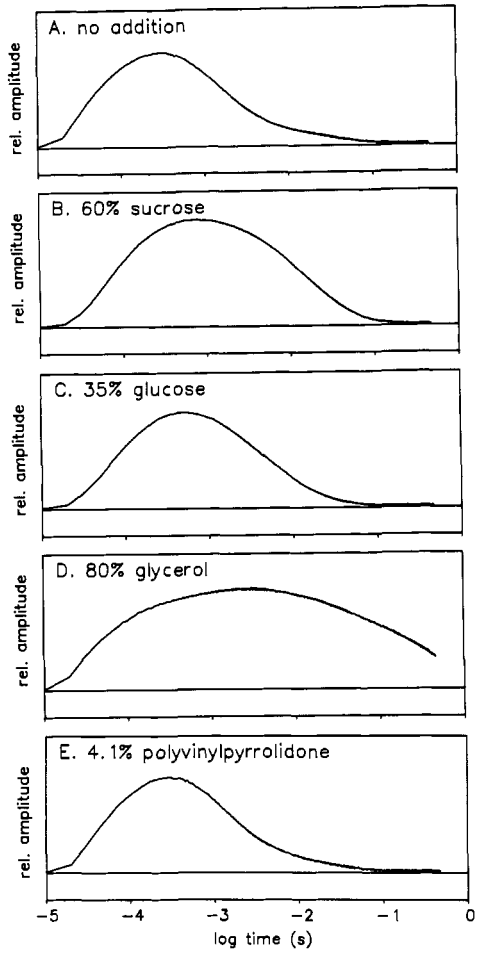

FIGURE 3: Kinetics of M rise and decay, measured at pH 9.0 as absorption changes at 410 nm, in the absence of solute (A) and with 60% sucrose (B), 36% glucose (C), 80% glycerol (D), and 4.1% poly(vinylpyrrolidone) (E). Time constant of the measuring system, 30 μs. The concentrations of solutes in (B)–(E) were chosen so as to make the viscosities the same (45 cSt, as determined in a kinematic viscometer). Otherwise, conditions as in Figure 1.

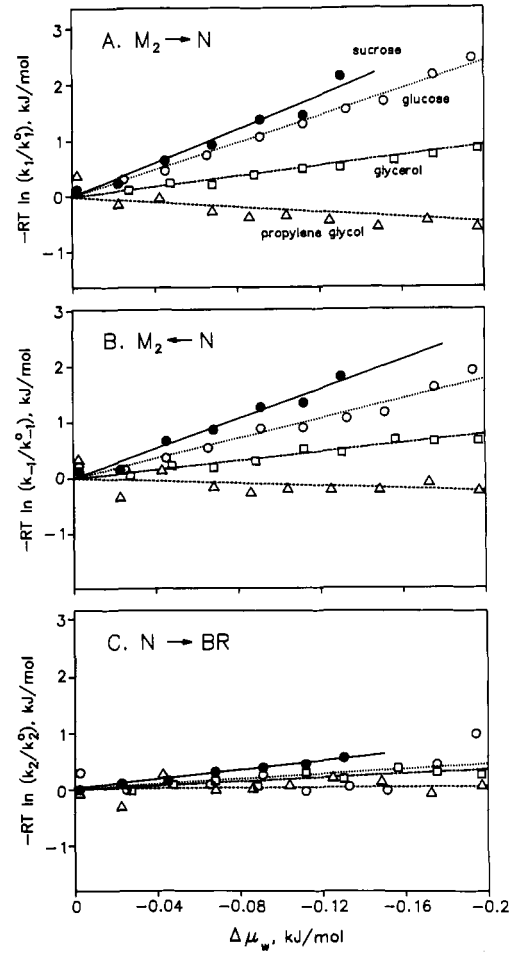

FIGURE 4: Effects of solutes on the $M_2 \rightarrow N$, $N \rightarrow M_2$, and $N \rightarrow BR$ reactions in wild-type bacteriorhodopsin (rate constants k_1, k_{-1}, and k_2, respectively). The additional free energy barrier to these reactions is plotted vs the change in the chemical potential of water in the presence of sucrose (●), glucose (○), glycerol (□), or propylene glycol (△). Conditions as in Figure 1.

than needed for observable inhibition. Propylene glycol, which is smaller than glycerol (its molecular weight is 76), slightly lowered the proton transfer barriers. The inhibitory effects of the solutes are thus readily interpreted as withdrawal of water required for the $M_2 \leftrightarrow N$ reaction inside the protein: the additional free energy barriers in Figure 4 reflect the consequences of shifting the water binding equilibrium. The size dependence of the slopes suggests that the smaller of the solutes have some degree of access to the water in question inside the protein. This is either because of their smaller size or because propylene glycol, and perhaps also glycerol, perturbs the protein sufficiently to abolish osmotic differences between the inside and outside of the structure. This possibility is supported by the fact that propylene glycol at higher concentrations than in Figure 4, and the still smaller ethylene glycol even at low concentrations, cause a blue shift and bleaching of the chromophore (not shown).

The consequences of withdrawing bound water by osmotically active solutes have been described for lipid bilayers (Parsegian et al., 1979; Rand, 1981), an ion channel (Zimmerberg & Parsegian, 1986), and a membrane protein (Kornblatt & Hoa, 1990). If the quantities determined had been free energies of water binding equilibria rather than free energies of activation, the slopes of the curves in Figure 4 would have given directly the moles of water in the protein. However, the changes in the activation energies for the proton transfer reactions do not reveal the desired free energies of the initial hydration equilibrium. Neither is a model available for how partial water withdrawal would affect proton transfer. Thus, the details of the hydration equilibria which underlie the results in Figure 4 could not be calculated. The only indication that the extent of hydration is already sufficient for protonation of the Schiff base is that the activation entropy of the $M_2 \rightarrow N$ reaction is small and positive (Váró & Lanyi, 1991b) rather than large and negative, as would be expected if binding of water were part of the free energy barrier to proton exchange. On the other hand, consideration of how various assumptions would affect calculation of the amount of water (i.e., using high binding energies for water and partial protonation rates after partial water withdrawal) indicates that the slopes in Figure 4 are *minimal* values. Thus, according to Figure 4, the $M_2 \rightarrow N$ and $N \rightarrow M_2$ reactions require at least 13–15 mol of sucrose-inaccessible water.

Since in D96N bacteriorhodopsin the N state does not accumulate under these conditions (Váró & Lanyi, 1991a), a single-exponential M decay with the rate constant k' will describe the $M \rightarrow BR$ reaction pathway. Figure 5 shows the effects of sucrose and propylene glycol on k' in the presence and absence of azide. As in the wild-type protein, propylene glycol has little effect. Sucrose does not inhibit the rate of Schiff base protonation as in wild type, but remarkably somewhat increases it: the slope is about the same as for the $N \rightarrow BR$ reaction in the wild type (Figure 4C, sucrose curve),

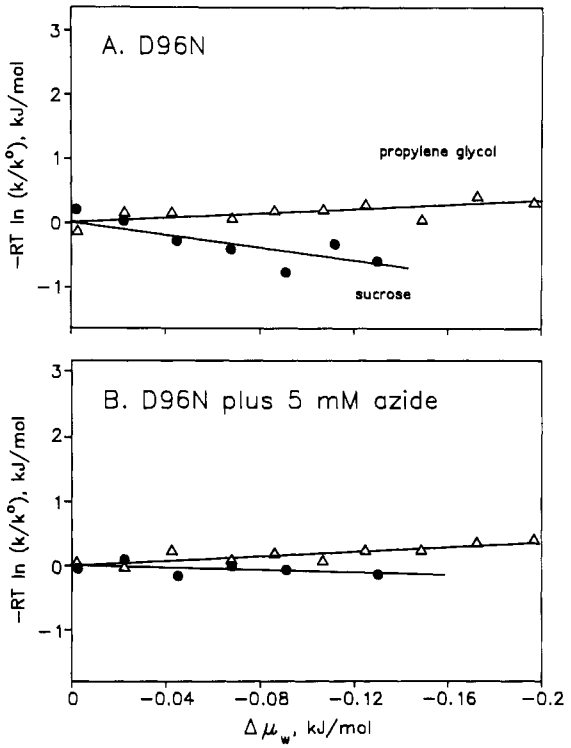

FIGURE 5: Effects of solutes on the rate constant of the $M_2 \rightarrow$ BR reaction in D96N bacteriorhodopsin in the absence of azide (A) and with 5 mM NaN_3 (B). The additional free energy barrier to the reaction is plotted vs the change in the chemical potential of water in the presence of sucrose (●) or propylene glycol (△). Conditions as in Figure 1, but the bacteriorhodopsin concentration was 32 nmol/mL.

but of the opposite sign. It would appear from this that hydration somewhat inhibits the reaction in D96N. On the other hand, the increased rate might be caused by enhanced hydration of the protein surface in the presence of sucrose, as described by Timasheff and Arakawa (1989) for some proteins. If so, the rate-limiting step for the Schiff base protonation in D96N must be at the surface, as suggested also by its pH dependency (cf. below). In the presence of 5 mM azide, k' is 60 times higher, and sucrose has virtually no effect (Figure 5B).

Effects of pH and Temperature on Schiff Base Reprotonation in the Wild-Type and D96N Proteins. Because the $M_2 \rightarrow N$ and $N \rightarrow M_2$ reactions are internal proton transfers, k_1 and k_{-1} should not depend on pH. On the other hand, according to transient absorption changes of a pH indicator dye which follow the kinetics of N decay (Váró & Lanyi, 1990b), the $N \rightarrow$ BR reaction utilizes protons in the aqueous phase. At pH >7 at least, log k_2 will be linearly dependent on bulk (or rather, surface) pH (Kouyama & Nasuda-Kouyama, 1989; Otto et al., 1989; Váró & Lanyi, 1990a; Ames & Mathies, 1990). In D96N, the $M \rightarrow$ BR rate, k', will be likewise dependent on pH since there is no internal proton donor (Tittor et al., 1989; Otto et al., 1989; Miller & Oesterhelt, 1990). In Figure 6A, pH dependencies are shown for k_1, k_{-1}, and k_2 in the wild type, and in Figure 6B,C for k' in D96N without and with 5 mM azide. The results confirm that k_1 and k_{-1} are not dependent on pH. More important, the slopes of pH dependencies are nearly, although not completely, the same for k_2 and for k' in the absence of azide. For k_2, the slope is 0.39 ± 0.01; for k', it is 0.47 ± 0.03 (Figure 6A,B). The latter had been reported as between 0.3 and 0.6 (Holz et al., 1989; Tittor et al., 1989; Miller & Oesterhelt, 1990). It is not 1 as expected from first-order kinetics, because the protons originate from the protein surface rather the bulk. The surface potential of purple membranes, due to fixed negative charges of the protein and the lipids, will lower the surface pH (Szundi & Stoeckenius, 1989; Miller & Oesterhelt, 1990). Calculations of the consequences of the pH-dependent ionization of surface groups indicate that the difference between surface and bulk pH will itself vary with pH, and the slope of dependency on bulk pH will be less than 1 (Miller & Oesterhelt, 1990). From the lesser slope for the wild-type protein relative to D96N (Figure 6A,B), it would appear that the negative charge of D96 during the N state has a measurable effect on the pH at the cytoplasmic surface. Nevertheless, the two proton uptake reactions clearly depend linearly, and in nearly the same way, on pH. This argues that in both the rate-limiting step is the capture of the proton by the opening of the cytoplasmic channel.

We find that azide increases the pH dependency of k' in D96N (Figure 6C). The slope of the line is 0.99 ± 0.03, suggesting that the proton donor in this case is HN_3 in the

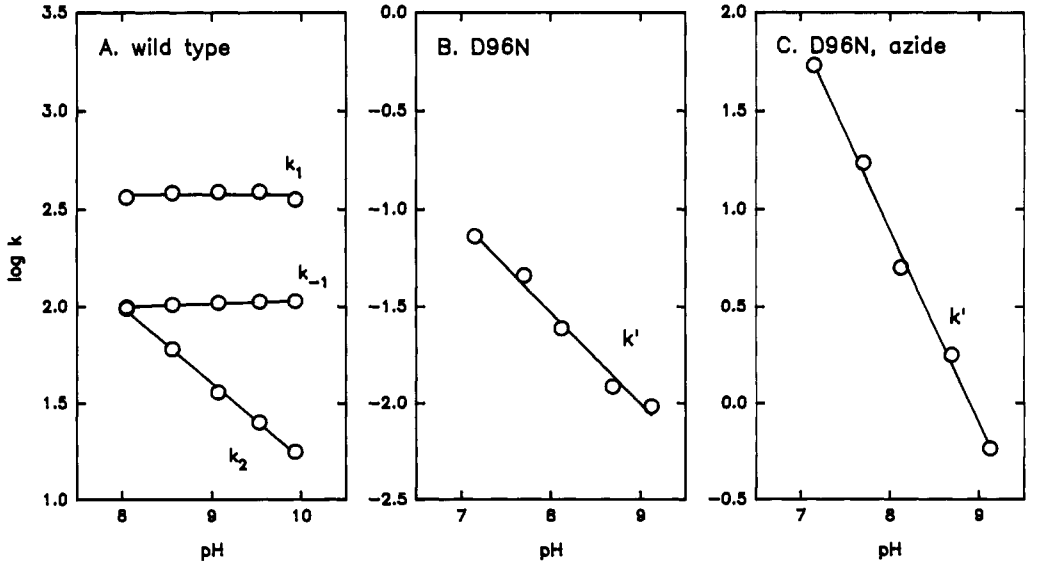

FIGURE 6: pH dependencies of proton transfer rate constants. Conditions as in Figure 1, but the buffer was 25 mM AMPSO plus 25 mM phosphate, at the indicated pH. (A) Wild-type bacteriorhodopsin; rate constants as in Figure 4; concentration 8 nmol/mL. (B) D96N bacteriorhodopsin; k' refers to the $M \rightarrow$ BR reaction. (C) as in (B), but in the presence of 5 mM NaN_3. In (B) and (C), the bacteriorhodopsin concentration was 32 nmol/mL.

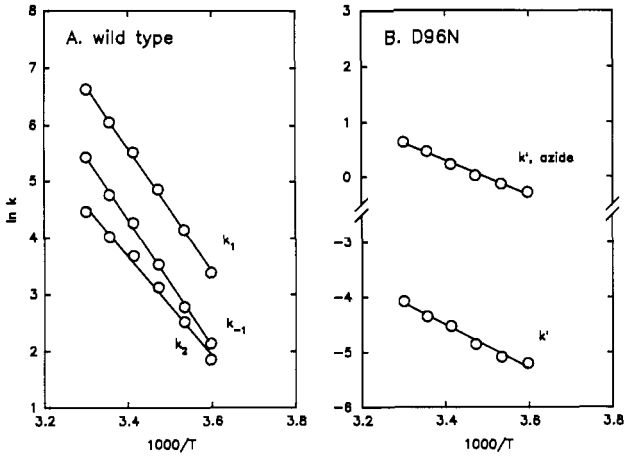

FIGURE 7: Temperature dependencies of proton transfer rate constants. Conditions as in Figure 1, but the buffer was 50 mM phosphate at pH 9.0. (A) Wild-type bacteriorhodopsin; concentration 8 nmol/mL; rate constants as in Figure 4. (B) D96N bacteriorhodopsin; concentration 32 nmol/mL, without and with 5 mM NaN_3; k' refers to the M → BR reaction.

bulk; the concentration of this conjugate acid will be strictly proportional to proton concentration whenever pH ≫ pK_a (the pK_a for azide is 4.7). Thus, when azide is present, the protons originate not from the protein surface but from the bulk, and as Otto et al. (1989) had suggested, the azide must function by shuttling protons between the proton channel and the bulk aqueous phase.

We have determined the activation barriers in wild-type and D96N bacteriorhodopsins from the temperature dependencies of the protonation reactions [for the equations used in the calculations, cf. Miller and Oesterhelt (1990) and Vârô and Lanyi (1991b)]. Figure 7 shows ln k_i as a function of $1/T$ for each of the measured reactions. In wild type, the three reactions described by k_1, k_{-1}, and k_2 have activation enthalpies 87 ± 2, 89 ± 2, and 69 ± 3 kJ/mol, respectively (Figure 7A). The activation enthalpy associated with k' in D96N is much smaller: 30 ± 2 kJ/mol; with 5 mM azide present, it decreases further to 24 ± 1 kJ/mol (Figure 7B). The latter is contrary to the increase in ΔH^* from 27 to 48 kJ/mol upon addition of azide to D96N reported by Tittor et al. (1989). These authors used 100 mM azide at pH 5.2, with 100 mM potassium phosphate buffer. Under these conditions, we obtain a ΔH^* of 50 ± 1 kJ/mol (data not shown), a comparable value. The discrepancy thus originates from the experimental conditions. At pH 5.2 and 100 mM azide, the concentration of HN_3 is 25 mM, or 10^5 times higher than what we employ in Figure 7C; when HN_3 is in a large excess (10^3 times) over bacteriorhodopsin, the mechanism of reprotonation is probably different.

Discussion

In this report, we attempt to describe how protons move in the cytoplasmic proton channel by comparing the wild-type protein with D96N. We make the following observation. Upon replacement of D96 with a nonprotonable residue, the two-step proton transfer described by the rate constants k_1 (D96 to the Schiff base) and k_2 (cytoplasmic surface to D96) merges into a single reaction with a rate constant k'. Thus, k' refers to proton transfer over the entire cytoplasmic proton channel. Because the transit times over the two adjoining segments of this trajectory are additive, i.e., $\tau' = \tau_1 + \tau_2$, and $\tau_i = 1/k_i$, the predicted value of k' is the harmonic mean of k_1 and k_2, i.e., $k' = k_1k_2/(k_1 + k_2)$. One should remember that the proton transfer described by k_1 is internal to the protein and independent of the external pH, while the reactions described by k_2 and k' depend linearly on the external proton concentration because here protons participate as reactants (Figure 6). By calculating a predicted k' value, we take this into account: k' contains k_2, determined at the same pH in the wild-type protein. This calculation gives an expected k' of 40 s^{-1} for the entire proton trajectory at pH 9 and 22 °C, which is considerably greater than the measured value of 0.013 s^{-1} in the D96N protein. The presence of D96 thus accelerates the proton transfer by about 3000-fold.

The data indicate that the free energy, enthalpy, and entropy of activation for the proton transfer are considerably changed when D96 is replaced with asparagine. From the difference in the rate constants, we calculate that the ΔG of activation for proton transfer over the entire trajectory is 20 kJ/mol greater in D96N than predicted on the basis of the two partial reactions in the wild type. The expected ΔH^* and ΔS^* for the entire trajectory were obtained by calculating k' from the measured k_1 and k_2 at each temperature in Figure 7A, and determining the activation parameters from the resulting curve. The expected ΔH^* is 72 ± 3 kJ/mol; the measured value in D96N is much lower, 30 ± 2 kJ/mol (Figure 7B). Similarly, there is a difference between the measured and expected ΔS^*; it amounts to −209 J/(mol·K). Thus, the presence of aspartate at position 96 results in an additional activation enthalpy of 42 kJ/mol, but a much more positive activation entropy. Direct comparisons of M relaxations in the wild-type to those in D96N also gave higher ΔH^* and more positive values for ΔS^* (Tittor et al., 1989; Miller & Oesterhelt, 1990).

What determines then the rate of the two-step proton transfer between the Schiff base and the cytoplasmic surface? The most important thermal barrier for the reprotonation of the Schiff base must be the Born self-energy of the ion pair created as the proton leaves D96. In a water-filled narrow channel containing no charged groups, as in gramicidin, the rate of proton transfer across the membrane is 1 × 10^9 s^{-1} (Akeson & Deamer, 1991). In bacteriorhodopsin, the proton transfer over the 10-Å distance from D96 to the Schiff base is just 10^7 times slower than what is expected on this basis, which is equivalent to an additional barrier of 40 kJ/mol. That this is virtually entirely attributable to the ΔH of the charge separation at D96 is suggested by the fact that when D96 is replaced with asparagine the measured ΔH^* of the M_2 → BR reaction is decreased by 42 kJ/mol from that calculated from the two reaction segments in the wild type, i.e., by about the amount of the additional barrier attributed to the ionization of D96. Thus, in the wild-type protein, the rate of proton transfer from D96 to the Schiff base is accounted for by a water-mediated conduction as in gramicidin, but with an additional barrier from the high enthalpy of the aspartate–proton ion pair. The lower enthalpy barrier when HN_3 replaces D96 as the proton donor in D96N (by 48 kJ/mol) indicates that the rate-limiting step in this protonation is different. We suggest that here the proton interacts directly with both N_3^- and the Schiff base in the transition state and the proton transfer step itself is very fast because it is determined only by the chemical reactivities of the donor and acceptor groups.

The results indicate that of all photocycle reactions it is proton exchange between the Schiff base and D96 (M_2 ↔ N) which is most strongly influenced by removal of internal water in the protein. The subsequent protonation of D96 from the cytoplasmic aqueous phase (N → BR) is distinctly less dependent on water, and the rest of the photocycle steps are even less affected. Greatly slowed M decay rates at much lower water activities have been observed in bacteriorhodopsin films

(Korenstein & Hess, 1977; Váró & Keszthelyi, 1983; Braiman et al., 1987; Váró & Lanyi, 1990c; Pfefferlé et al., 1991). The pattern of inhibition by various solutes suggests that the water is in space distinctly less permeable to sucrose and glucose than to glycerol and propylene glycol. Although the amount of water in these effects cannot be calculated, it appears even without assumptions that at least 15 mol is required for maximal proton transfer to and from the Schiff base. This is a much larger amount than needed to form a single chain of water molecules between the Schiff base and D96. It probably represents diffuse hydration of the interhelical region on the cytoplasmic side of the protein in spite of the hydrophobic nature of the proton channel itself. In the D96N protein, Schiff base reprotonation is much less affected and in the opposite direction, and sucrose has virtually no effect when the protonation is facilitated by HN_3. The transition states for the various kinds of proton transfer reactions must therefore be different. Taken together with the calculated activation barriers (cf. above), the results suggest the following. (1) In long-range proton transfer reactions (over 10 Å) where the proton originates from a conjugate acid like D96, the transition state consists of a proton–anion pair, which is stabilized by an amount of water comparable to hydration in the bulk solution. (2) In the short-range proton transfer between HN_3 and the Schiff base, the transition state consists of the donor–acceptor pair, which is virtually indifferent to the presence of water in the protein. (3) Proton transfers from the protein surface are limited by the capture of the proton at the protein–water interface (cf. below) and are much less affected by removal of water than the reactions in (1).

It might be supposed that the much more rapid proton transfer in the wild type relative to D96N results from the presence of a proton on D96, i.e., the high effective proton concentration in the channel leading to the Schiff base. Indeed, fixing the reactant at a binding site near the active center is usually an important factor in accelerating the rate of a reaction by enzyme catalysis. However, by comparison of the measured rate with an expected rate which contains k_2, the contribution of proton concentration is eliminated. We suggest that the much slower protonation rate in D96N is not due to the absence of a dissociable proton on residue 96 in the channel but the absence of a negative charge at this residue at any time during the reaction. Importantly, since they are linearly dependent on the surface proton concentration (Figure 6A,B), the proton transfers to D96 in the wild type (k_2) and to the Schiff base in D96N (k') are both limited by the entry of protons at the cytoplasmic surface. We suggest that in D96N it is this step which is slower: the free energy activation barrier to the capture of the proton at the surface is higher in the absence of the electric field of the ionized D96 which extends to the cytoplasmic surface in the wild type during state N. The magnitudes of electric fields of ionized residues and how they are affected by the local structure inside proteins and the distance from the surface are of great interest in protein chemistry, and have been explored from theoretical [e.g., see Honig et al. (1986)] and experimental [e.g., see Yam et al. (1991)] points of view. That in bacteriorhodopsin the influence of an anionic residue at position 96 extends to the surface is indicated by the pH dependencies of the rate of proton capture in the wild-type and D96N proteins (compare the slopes for k_2 and k' in panels A and B of Figure 6).

Since the lowering of the proton capture barrier in the wild-type protein appears to be entirely by a more positive activation entropy term, we must consider entropy-related effects, i.e., how D96 might influence access of the protein to those conformations which allow entry of the proton into the channel. There are at least two specific possibilities: (1) A negatively charged residue at position 96 will form more effective proton exchange pairs with donor residues located near the cytoplasmic surface than other residues or bound water. Interaction of D96 with T46 and S226 has been suggested by Marti et al. (1991). When these residues were replaced with valine and alanine, respectively, a rather slow M decay component appeared which indeed suggests a slowed N → BR reaction. (2) The negative charge appearing at residue 96 causes reorganization of water in state N so as to maximize hydration of the carboxylate, and the rearranged hydration facilitates proton conduction also. In D96N such organization of water near the surface is not supported by the accumulated ΔH of the anionic D96 and requires therefore a considerable expenditure of $T\Delta S$. The observed slight lowering of the barrier by sucrose (Figure 5A) in D96N may be by a changed degree of hydration of the protein surface.

The results in this study suggest that azide overcomes the hindrance to proton conduction in D96N by shuttling between the immediate vicinity of the Schiff base and the bulk aqueous phase. It is not certain that the HN_3 passes through the proton channel as it approaches the Schiff base. Hydration has little effect because in this reaction the proton is passed directly and rapidly from donor to acceptor in a ligand transfer pair, and the rate-limiting step is the entry of HN_3 into the protein. Since HN_3 is not very polar, it should partition much more readily into the protein than H^+. Such a mechanism might explain why at high concentrations azide accelerates Schiff base protonation by as much as 2 orders of magnitude over the rate in wild type (Otto et al., 1989).

ACKNOWLEDGMENTS

We are very grateful to D. W. Deamer for providing a manuscript before its publication, to S. H. White, M. Gutman, and A. Parsegian for valuable discussions, and to L. Zimányi for analytical solutions of rate equations.

Registry No. Aspartic acid, 56-84-8.

REFERENCES

Akeson, M., & Deamer, D. W. (1991) *Biophys. J. 60*, 101–109.

Ames, J. B., & Mathies, R. A. (1990) *Biochemistry 29*, 7181–7190.

Beece, D., Bowne, S. F., Czege, J., Eisenstein, L., Frauenfelder, H., Good, D., Marden, M. C., Marque, J., Ormos, P., Reinisch, L., & Yue, K. T. (1981) *Photochem. Photobiol. 33*, 517–522.

Braiman, M. S., Ahl, P. L., & Rothchild, K. J. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 5221–5225.

Braiman, M. S., Mogi, T., Marti, T., Stern, L. J., Khorana, H. G., & Rothchild, K. J. (1988) *Biochemistry 27*, 8516–8520.

Butt, H.-J.; Fendler, K., Bamberg, E., Tittor, J., & Oesterhelt, D. (1989) *EMBO J. 8*, 1657–1663.

CRC Handbook of Chemistry and Physics (1980) (Weast, R. C., Ed.) CRC Press, Boca Raton, FL.

Deamer, D. W. (1987) *J. Bioenerg. Biomembr. 19*, 457–479.

Deamer, D. W., & Nichols, J. W. (1989) *J. Membr. Biol. 107*, 91–103.

Gerwert, K., Hess, B., Soppa, J., & Oesterhelt, D. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 4943–4947.

Gerwert, K., Souvignier, G., & Hess, B. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 9774–9778.

Hegemann, P., Oesterhelt, D., & Steiner, M. (1985) *EMBO J. 4*, 2347–2350.
Henderson, R., Baldwin, J. M., Ceska, T. A., Zemlin, F., Beckmann, E., & Downing, K. H. (1990) *J. Mol. Biol. 213*, 899–929.
Holz, M., Drachev, L. A., Mogi, T., Otto, H., Kaulen, A. D., Heyn, M. P., Skulachev, V. P., & Khorana, H. G. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 2167–2171.
Honig, B. H., Hubbell, W. L., & Flewelling, R. F. (1986) *Annu. Rev. Biophys. Biophys. Chem. 15*, 163–193.
Korenstein, R., & Hess, B. (1977) *Nature 270*, 184–186.
Kornblatt, J. A., & Hoa, G. H. B. (1990) *Biochemistry 29*, 9370–9376.
Kouyama, T., & Nasuda-Kouyama, A. (1989) *Biochemistry 28*, 5963–5970.
Lanyi, J. K. (1986) *Biochemistry 25*, 6706–6711.
Li, Q., Govindjee, R., & Ebrey, T. G. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 7079–7082.
Lozier, R. H., Niederberger, W., Ottolenghi, M., Sivorinovsky, G., & Stoeckenius, W. (1978) in *Energetics and Structure of Halophilic Microorganisms* (Caplan, S. R., & Ginzburg, M., Eds.) pp 123–139, Elsevier/North-Holland, Amsterdam.
Marque, J., & Eisenstein, L. (1984) *Biochemistry 23*, 5556–5563.
Marti, T., Otto, H., Mogi, T., Rösselet, S. J., Heyn, M. P., & Khorana, H. G. (1991) *J. Biol. Chem. 266*, 6919–6927.
Milder, S. J., Thorgeirsson, T. E., Miercke, L, J. W., Stroud, R. M., & Kliger, D. S. (1991) *Biochemistry 30*, 1751–1761.
Miller, A., & Oesterhelt, D. (1990) *Biochim. Biophys. Acta 1020*, 57–64.
Mowery, P. C., Lozier, R. H., Chae, Q., Tseng, Y. W., Taylor, M., & Stoeckenius, W. (1979) *Biochemistry 18*, 4100–4107.
Nagle, J. (1987) *J. Bioenerg. Biomembr. 19*, 413–426.
Nagle, J. F., & Morowitz, H. J. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 298–302.
Nagle, J. F., & Tristram-Nagle, S. (1983) *J. Membr. Biol. 74*, 1–14.
Needleman, R., Chang, M., Ni, B., Vârô, G., Fornes, J., White, S. H., & Lanyi, J. K. (1991) *J. Biol. Chem. 266*, 11478–11484.
Ni, B., Chang, M., Duschl, A., Lanyi, J. K., & Needleman, R. (1990) *Gene 90*, 169–172.
Oesterhelt, D., & Stoeckenius, W. (1974) *Methods Enzymol. 31*, 667–678.
Otto, H., Marti, T., Holz, M., Mogi, T., Lindau, M., Khorana, H. G., & Heyn, M. P. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 9228–9232.
Otto, H., Marti, T., Holz, M., Mogi, T., Stern, L. J., Engel, F., Khorana, H. G., & Heyn, M. P. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 1018–1022.
Papadopoulos, G., Dencher, N. A., Zaccai, G., & Büldt, G. (1990) *J. Mol. Biol. 214*, 15–19.
Parsegian, V. A., Fuller, N., & Rand, R. P. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 2750–2754.
Pfefferlé, J.-M., Maeda, A., Saski, J., & Yoshizawa, T. (1991) *Biochemistry 30*, 6548–6556.
Rand, R. P. (1981) *Annu. Rev. Biophys. Bioeng. 10*, 277–314.
Stern, L. J., Ahl, P. L., Marti, T., Mogi, T., Duñach, M., Berkovitz, S., Rothschild, K. J., & Khorana, H. G. (1989) *Biochemistry 28*, 10035–10042.
Szundi, I., & Stoeckenius, W. (1989) *Biophys. J. 56*, 369–383.
Timasheff, S. N., & Arakawa, T. (1989) in *Protein Structure: A Practical Approach* (Creighton, T. E., Ed.) pp 331–345, IRL Press/Oxford Press, Oxford.
Tittor, J., Soell, C., Oesterhelt, D., Butt, H.-J., & Bamberg, E. (1989) *EMBO J. 8*, 3477–3482.
Vârô, G., & Keszthelyi, L. (1989) *Biophys. J. 43*, 47–51.
Vârô, G., & Lanyi, J. K. (1990a) *Biochemistry 29*, 2241–2250.
Vârô, G., & Lanyi, J. K. (1990b) *Biochemistry 29*, 6858–6865.
Vârô, G., & Lanyi, J. K. (1990c) *Biophys. J. 59*, 313–322.
Vârô, G., & Lanyi, J. K. (1991a) *Biochemistry 30*, 5008–5015.
Vârô, G., & Lanyi, J. K. (1991b) *Biochemistry 30*, 5016–5022.
Vârô, G., Duschl, A., & Lanyi, J. K. (1990) *Biochemistry 29*, 3798–3804.
Yam, R., Nachliel, E., Kiryati, S., Gutman, M., & Huppert, D. (1991) *Biophys. J. 59*, 4–11.
Zimányi, L., Keszthelyi, L., & Lanyi, J. K. (1989) *Biochemistry 28*, 5165–5172.
Zimmerberg, J., & Parsegian, V. A. (1986) *Nature 323*, 36–39.