

Environmental Effects on the Protonation States of Active Site Residues in Bacteriorhodopsin

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ABSTRACT Finite difference solutions of the Poisson-Boltzmann equation are used to calculate the pK_a values of the functionally important ionizable groups in bacteriorhodopsin. There are strong charge-charge interactions between the residues in the binding site leading to the possibility of complex titration behavior. Structured water molecules, if they exist in the binding site, can have significant effects on the calculated pK_a by strongly stabilizing ionized species. The ionization states of the Schiff base and Asp-85 are found to be strongly coupled. Small environmental changes, which might occur as a consequence of *trans-cis* isomerization, are capable of causing large shifts in the relative pK_a values of these two groups. This provides an explanation for the protonation of Asp-85 and the deprotonation of the Schiff base in the M state of bacteriorhodopsin. The different behavior of Asp-85 and Asp-212 is discussed in this regard.

INTRODUCTION

Elucidating the proton pumping mechanism of the membrane protein bacteriorhodopsin (bR) has been a problem of considerable interest for some time. The recent determination of the three-dimensional structure of bR has presented the possibility of correlating spectroscopically detected phenomena with specific events described at the molecular level. bR functions as a proton pump, using light energy to drive a series of steps marked by protein conformational changes and concurrent proton transfers between ionizable amino acids. It has become clear, primarily from a combination of genetic and FTIR experiments, that Arg-82, Asp-85, Asp-96, Asp-115, Asp-212, and Lys-216 are among the functionally significant residues involved in the proton pumping mechanism (see Mathies et al., 1991; Lanyi, 1992; Ebrey, 1993 for recent reviews). Understanding the source of the proton affinity changes that these groups undergo during the course of the bR photocycle is central to a clearer knowledge of the molecular basis of proton pumping. In this paper, we discuss the factors that determine the pK_a values of these groups in several structural models for bR. These are based on the structure of bR determined in the seminal electron diffraction study of Henderson and co-workers (Henderson et al., 1990).

The factors governing the pK_a values of amino acids are primarily electrostatic (Warshel, 1981; Bashford and Karplus, 1990; Sharp and Honig, 1990). When isolated in solution, the charged and neutral forms of a titrating group are stabilized to differing extents by interactions with the solvent. In the protein, these interactions are fully or partially replaced by those with protein dipolar groups, polarizable electrons and other titratable residues. Due to the electro-

static effects the protein environment exerts on each residue, individual pK_a values of amino acids can be either increased or decreased relative to those isolated in solution. In addition, protein conformational changes during the photocycle can alter the electrostatic environment of titratable groups and result in further pK_a shifts. Directed changes in individual pK_a values can drive sequential proton movement from groups with low pK_a to groups exhibiting a higher proton affinity.

Despite a qualitative understanding of these factors, the accurate calculation of pK_a values is quite difficult, primarily because the final value is the difference between relatively large numbers involving interactions of charged groups with the solvent and with each other. Nevertheless, there have been considerable advances in recent years in calculating pK_a values, due in large part to progress in the ability to treat theoretically the electrostatic properties of proteins (Sharp and Honig, 1990; Nicholls and Honig, 1991; Warshel and Aqvist, 1991). The approach adopted in this work is to use the Finite Difference Poisson-Boltzmann (FDPB) method, which has been applied successfully in accounting for a variety of electrostatic properties of globular proteins (see Harvey, 1989; Sharp and Honig, 1990 for reviews). Recently, a number of applications of the method to the calculation of pK_a values in proteins have been reported (Bashford and Karplus, 1990; Beroza et al., 1991; Bashford and Gerwert, 1992; Gilson, 1993; Yang et al., 1993).

The residues of interest in bR are buried primarily within the lipid bilayer. Asp-85 and Asp-212 lie below the protonated Schiff base linkage near the external side of the protein. They are located between the retinal chromophore and Lys-216. Asp-115 is situated near the β -ionone ring, and Asp-96 is close to the cytoplasmic surface. The Arg-82 side chain has been placed extending in the direction of the extracellular surface, but an alternate conformation where it faces the protein interior near Asp-85 and Asp-212 is also consistent with the diffraction data (Henderson et al., 1990). Asp-96 and Asp-115 appear to be neutral in the resting state of the protein

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while the remaining groups are charged (Engelhard et al., 1985; Eisenstein et al., 1987; Braiman et al., 1988; Gerwert et al., 1989).

Although carboxylates in solution ionize around pH 4, burial in the low dielectric interior of the protein is expected to raise their pK_a values. This is a consequence of desolvation, the exclusion of water from the vicinity of the titrating group. Thus, the existence of Asp-96 and Asp-115 with pK_a values elevated to about 9 is not surprising. On the other hand, the fact that Asp-85 and Asp-212, which are both buried, are located only about 5.0 Å apart and are both charged is more difficult to understand. A structure-based explanation of the observed ionization states of these groups is crucial to understanding how light driven events can lead to pK_a changes during the course of the photocycle. The results obtained in this work describe the determinants of proton transfer in bR and provide constraints on possible models for the proton pumping mechanism.

This work is similar to a previous study in which the pK_a values of ionizable groups in bR also were calculated (Bashford and Gerwert, 1992). The major new features reported here are the inclusion in the calculations of individual water molecules located in a buried cavity near the Schiff base and the treatment of pK_a changes that occur as a result of *trans-cis* isomerization of the chromophore. Water molecules that are buried within internal cavities in an ordered state are found to have a significant effect on the predicted pK_a values. They are found to be capable of stabilizing ionized states to the extent that electroneutrality is not a requirement to maintain a binding site where Asp-85, Asp-212, and the Schiff base all exist in ionized forms. A second finding is that small changes in the pK_a of the Schiff base are sufficient to induce major changes in the protonation states of other ionizable groups. These observations have major implications for the proton transfer mechanism initiated by the light driven *trans-cis* isomerization.

MATERIALS AND METHODS

The fractional degree of protonation, f_i , of an ionizable group, i , in a protein is given by

$$\ln[f_i/(1 - f_i)] = 2.3(pK_{ai} - pH), \quad (1)$$

where pK_{ai} is the pK_a of the group and is given by

$$pK_{ai} = pK_i^{int} + \Delta pK_i^{tr} = pK_i^o - \gamma(i)\Delta\Delta G_i^{env}/2.3 kT + \Delta pK_i^{tr}. \quad (2)$$

$\gamma(i)$ equals -1 or 1 for an acidic or basic group, respectively. pK_i^{int} is the intrinsic pK of the group in the protein. It is pH-independent and defined as the hypothetical pK an ionizable group would have if all other ionizable groups in the protein are in their neutral form (Yang et al., 1993). The term ΔpK_i^{tr} is pH-dependent and is the pK shift due to the charge effects from all the other titratable groups in the protein. The intrinsic pK consists of two terms, a reference pK called pK^o and an environment term, $\Delta\Delta G_i^{env}$. pK^o is typically the known pK_a of the isolated amino acid in water, and $\Delta\Delta G_i^{env}$ is the change in electrostatic energy of dissociating the group in the protein environment relative to its reference state. The latter term can be divided into two contributions.

$$\Delta\Delta G_i^{env} = \Delta\Delta G_i^{rf} + \Delta\Delta G_i^{dip}. \quad (3)$$

$\Delta\Delta G_i^{rf}$ is the contribution of those parts of the system treated as a dielectric continuum and is the difference in reaction field energy of a group in the protein and in the reference state. It can be considered a desolvation penalty, which is the cost of moving a group from solvent into the protein environment. $\Delta\Delta G_i^{dip}$ is the contribution of the permanent dipoles in the protein relative to the reference state. In FDPB calculations, the reaction field of a group in the aqueous phase yields the solvation energy, whereas in the protein the effects from the polarizable electrons are included in addition to solvent interactions, which of course are weaker than in the bulk phase.

The average protonation state of each group is determined from a statistical mechanical average of all relevant states of the system (see Bashford and Karplus, 1990; Beroza et al., 1991; Yang et al., 1993 for a detailed discussion). If there are N ionizable residues, there are 2^N possible states because each ionizable group can be either charged or neutral. The average charge, $\langle\rho_i\rangle$, of each group at a specific pH is determined from the statistical mechanical average

$$\langle\rho_i\rangle = \sum_{i=1}^{2^N} \delta_n(i)\gamma(i)\exp(-\Delta G^n/kT)/Z \quad (4)$$

$\delta_n(i)$ is 1 when group i is charged in the n th state and is zero when it is neutral. Z is the total partition function of the system of 2^N states. ΔG^n is the free energy of the n th state and is given by

$$\Delta G^n = \sum_{i=1}^N (\delta_n(i)\gamma(i)[2.3kT(pH - pK_i^{int})] + \delta_n(i) \sum_{1 \leq j < i} \delta_n(j)\Delta G^{ij}) \quad (5)$$

where ΔG^{ij} is the electrostatic interaction energy between groups i and j . Equations 4 and 5 make it possible to calculate the fractional protonation of a group as a function of pH. The pH at which $\langle\rho_i\rangle$ is 0.5 provides a definition of the pK_a of that group.

Each of the terms in Eqs. 3 and 5 involves an electrostatic free energy. These were calculated with the DelPhi program (Nicholls and Honig, 1991). Atomic charges and radii were taken from the DISCOVER force field (Hagler et al., 1973) except for the retinal chromophore where charges calculated by Tavan et al. (1985) were used for the protonated and unprotonated states. The solvent accessible surface is determined by rolling a probe sphere of 1.4 Å radius over the van der Waals surface of the protein (Lee and Richards, 1971). The protein, as defined by the solvent accessible surface, is assigned a dielectric constant of 4. In addition to the regions outside this surface, all interior cavities and clefts are assigned a dielectric constant of 80. The effect of the low dielectric region of the lipid bilayer was not considered explicitly, because the protein was treated as being surrounded by the aqueous phase. The effects of this assumption will be considered below.

Separate structures were constructed for the charged and neutral configurations for each bR model investigated. Hydrogen atoms were added to polar groups in each structure using the program Proteus, which places protons on specified hydrogen bonding donors by choosing the acceptor that best satisfies experimental hydrogen bonding distance and angle criteria (Gunner, 1993). The program also checks for other donors that may be closer to the chosen acceptor. Cavities in bR structures were identified using the program GRASP (A. Nicholls and B. Honig, Department of Biochemistry and Molecular Biophysics, Columbia University, NY). They are defined as internal regions of the protein larger than the surfacing probe, yet inaccessible to the outer surface.

This study uses five bR models constructed on the basis of the structure solved by Henderson and co-workers (Henderson et al., 1990). They will be referred to as $bR_{out}(\epsilon)$, $bR_{in}(\epsilon)$, $bR_{out}(wat)$, $bR_{in/cis}(\epsilon)$, and $bR_{out/cis}(wat)$. The atomic coordinates of $bR_{out}(\epsilon)$ correspond to the original electron diffraction structure (Fig. 1). The side chain of Arg-82 points towards the extracellular surface of the protein, hence the subscript out. Several internal cavities are present in this structure, the largest lying below Asp-85 and Asp-212 and above the extended Arg-82 side chain (Fig. 2). All internal cavities in $bR_{out}(\epsilon)$ are assumed to be hydrated and are treated as regions with dielectric constant 80. The appearance of " ϵ " within the brackets indicates that rather than representing the water as individual molecules, it is treated macroscopically by assigning a dielectric constant to the cavity regions.

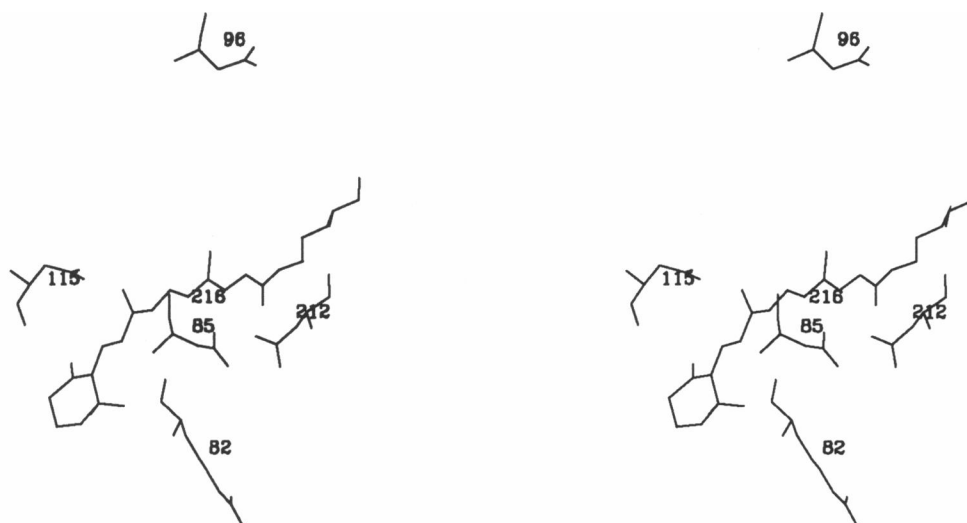


FIGURE 1 Stereo view of ionizable residues in the $bR_{out}(\epsilon)$ structure.

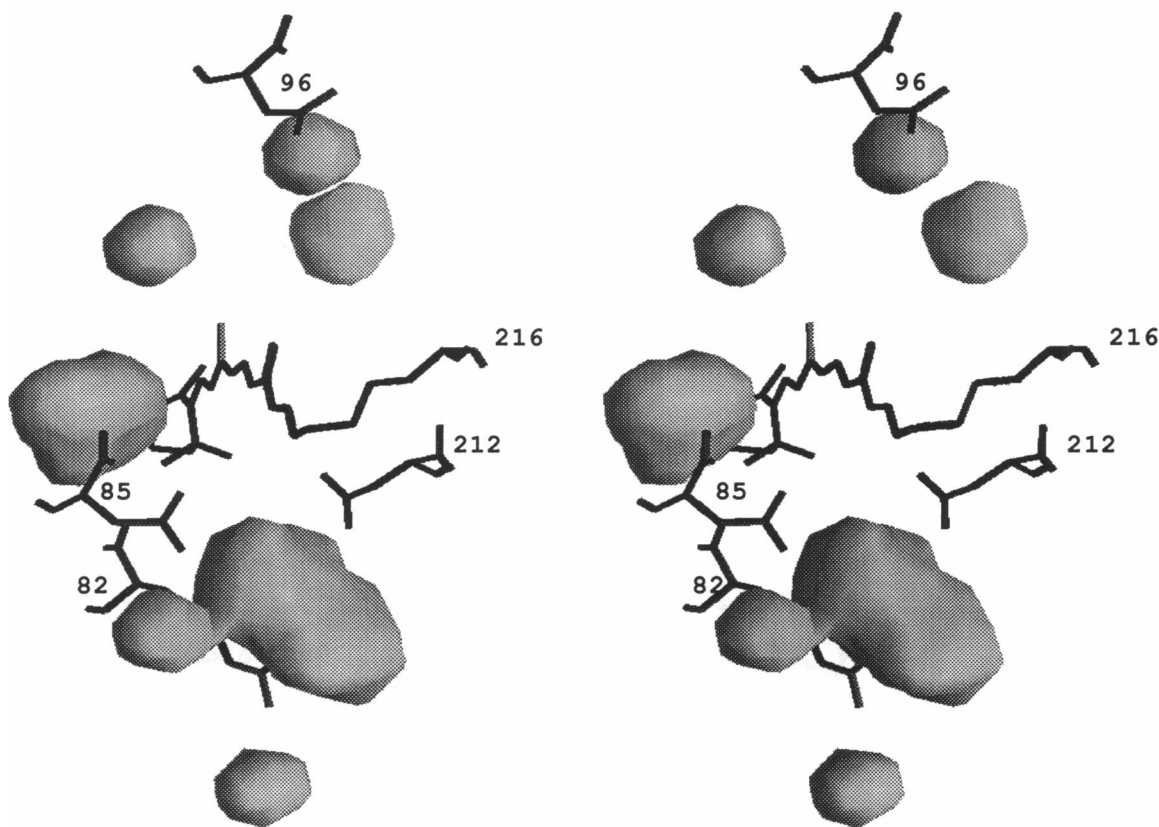


FIGURE 2 Stereo view of cavities in the $bR_{out}(\epsilon)$ structure. A probe radius of 1.2 Å was used to construct the cavities using the program GRASP.

The structure designated by $bR_{in}(\epsilon)$ contains a modified position for the Arg-82 side chain. It is positioned in the large internal cavity described above for $bR_{out}(\epsilon)$, but here the guanidinium group faces the protein interior and forms a salt bridge with Asp-85 (Fig. 3). This was accomplished by rotation about the χ_1 torsion angle and subsequent energy minimization of the protein side chains using the DISCOVER program (Biosym Technologies, 1992, San Diego, CA). The hydrogen-oxygen distance between the Arg-82 and Asp-85 side chains is 1.6 Å, as opposed to 10 Å in the $bR_{out}(\epsilon)$ structure.

$bR_{out}(wat)$ is identical to $bR_{out}(\epsilon)$ except that an internal cavity is no longer treated as a high dielectric region but, instead, is filled with five water

molecules. To guide the placement of these waters within the cavity, we studied the high resolution structure of intestinal fatty acid binding protein (2IFB) (Sacchetti et al., 1989; Brookhaven Protein Data Bank), which contains a number of internal cavities with clearly identifiable water molecules. The largest of these cavities contains seven water molecules, and is surrounded by an aspartic acid, glutamic acid, palmitate, and two lysines. This structure served as a model to gauge reasonable distances between charged groups and buried water molecules. In addition, packing information including the number of waters per cavity and average distances between waters was obtained. Using these values, water molecules were placed inside the largest cavity in $bR_{out}(\epsilon)$. The volume of the cavity is large enough

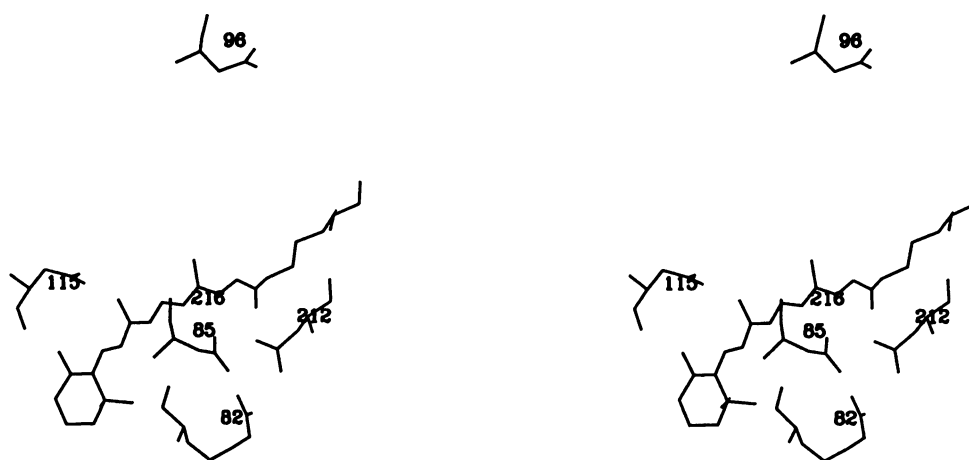


FIGURE 3 Stereo view of ionizable residues in the $bR_{in}(\epsilon)$ structure.

to accommodate five waters, consistent with experiment (Hildebrandt and Stockburger, 1984; Papadopoulos et al., 1990). Using DISCOVER, an energy minimization was carried out for the five water molecules keeping the protein atoms fixed. The minimization was performed separately for the charged and neutral forms of the protein (Yang et al., 1993). Water locations for the charged configuration are shown in Fig. 4. Note how the highly

directed electric field inside the protein causes the dipoles of all the water molecules to face in essentially the same direction.

It is worth noting that there is no possibility for a $bR_{in}(wat)$ structure without significant changes in the protein structure. When Arg-82 faces the active site, internal cavities that may accommodate water molecules are no longer present. Molecular dynamics simulations have produced conforma-

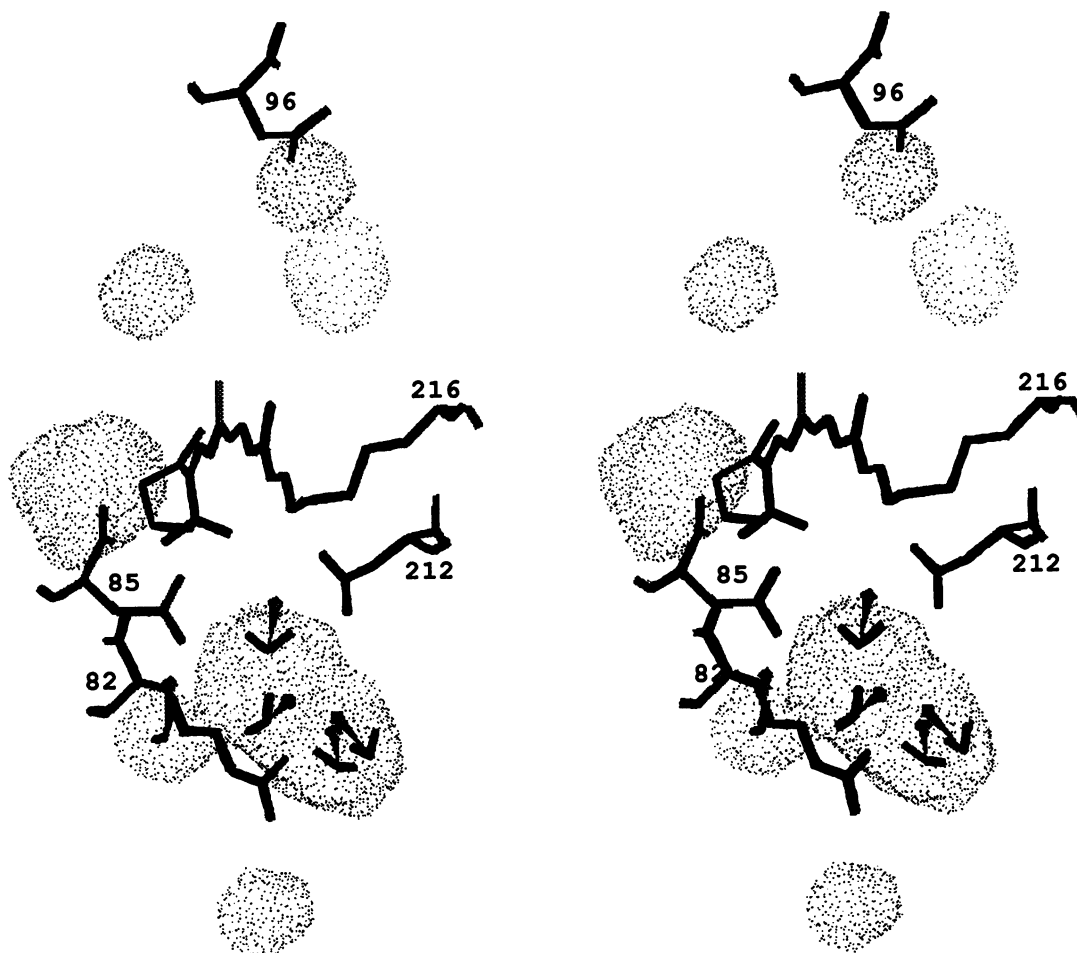


FIGURE 4 Stereo view of ionizable residues in the $bR_{out}(wat)$ structure. Field vectors have been calculated at water oxygen positions. Arrows have been generated using the GRASP program.

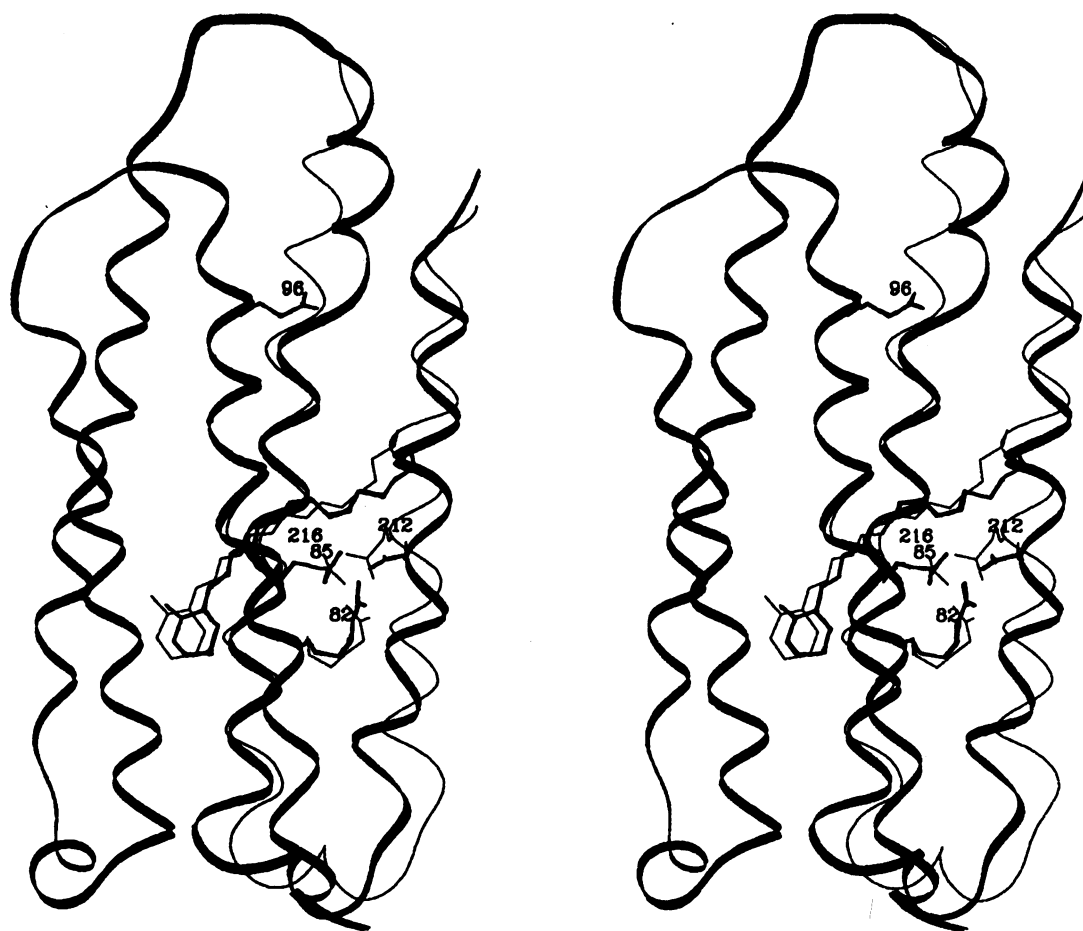


FIGURE 5 Comparison of $bR_{in}(\epsilon)$ (thin lines) and $bR_{in/cis}(\epsilon)$ (bold lines) structures. For clarity, helices A and B are not shown.

tions in which Arg-82 faces inward and that also contain buried waters (Zhou et al., 1993; Humphrey et al., 1994). However, our goal in this study was to obtain results with minimal modification of the experimentally determined coordinates. In future work, we plan to carry out similar calculations on structures that rely more heavily on theory-based model building.

The fourth structure, $bR_{in/cis}(\epsilon)$ is based on the $bR_{in}(\epsilon)$ structure, but the retinal has been rotated about the 13,14 double bond into the *cis* conformation. The chromophore conformation was taken from the crystal structure of 13-*cis* retinal (Simmons et al., 1981). A least-squares fit was used to superimpose all atoms from the β -ionone ring through C13 on the corresponding atoms of the all *trans* retinal in the binding site of the bR_{in} protein. The lysine-216 side chain was then extended and the nitrogen attached via a double bond to C15 of the *cis* retinal. Using DISCOVER, dynamics and minimization were performed to relax strained regions in the protein. The retinal isomerization requires the movement of Lys-216 and surrounding residues. Because the G-helix is free at one end and can move in response to the retinal isomerization with minimal disruption of the rest of the structure, it shifts slightly toward the extracellular side. Asp-212 and the Schiff base, located on G, also move in this direction and away from Asp-85, which is anchored on helix C. The flexible Arg-82 side chain moves to a position equidistant between the two aspartic acids (Fig. 5).

The final structure, $bR_{out/cis}(\text{wat})$, in addition to fixing retinal in the 13-*cis* conformation as described above, contains five water molecules in the internal cavity lying below the Schiff base, Asp-85, Asp-212, and above Arg-82. The arginine extends outward toward solution in this conformation to provide space for the explicit waters. Initial water positions were obtained from the $bR_{out}(\text{wat})$ structure. An energy minimization was then carried out in which the coordinates of the water molecules and protein side chains were allowed to vary. This was done for both the fully charged and fully neutral forms of the protein (Yang et al., 1993).

RESULTS

Table 1 contains experimental pK_a values of various ionizable groups studied in this work. Table 2 lists electrostatic interactions and pK_a values calculated for each of the structures studied. The first column in Table 2 is the reaction field term that describes the loss of solvation energy resulting from the complete or partial burial of the ionizable group within the protein. This term is quite large for all the groups and by itself would cause significant pK_a shifts favoring the neutral form of each residue. The second term describes interactions of the ionizable group with net neutral polar groups in the protein. In most cases, these stabilize the charged form of the residue reflecting the fact that ionized states make stronger

TABLE 1 Experimental pK_a values for the ground state of bR

	pK_a^*
Arg-82	N.A.
Asp-85	~2.5
Asp-96	9.5
Asp-115	9.5
Asp-212	<2.5
Schiff base	13.3

* (Druckman et al., 1982; Engelhard et al., 1985; Braiman et al., 1988; Gerwert et al., 1989; Miercke et al., 1991; Metz et al., 1992.)

TABLE 2 Electrostatic interactions in bacteriorhodopsin

	$\Delta\Delta G^{rf}$	$\Delta\Delta G^{dip}$	Charge-charge interactions (kcal/mol)								calc. pK _a
			Arg-82	Asp-85	Asp-96	Asp-115	Asp-212	Lys-216	Glu-9	Glu-204	
bR_{out}(ε)											
Arg-82	7.8	-1.7	-	-3.4	-0.1	-1.1	-3.8	1.3	-2.8	-5.1	21
Asp-85	7.5	0.1	-3.4	-	0.5	1.9	6.3	-4.2	0.7	1.5	?
Asp-96	4.6	1.8	-0.1	0.5	-	0.5	0.6	-1.2	0.0	0.1	9
Asp-115	7.2	-0.1	-1.1	1.9	0.5	-	1.5	-2.8	0.3	0.8	10
Asp-212	9.0	-3.5	-3.8	6.3	0.6	1.5	-	-5.2	0.9	1.9	?
Schiff base	4.1	1.5	1.3	-4.2	-1.2	-2.8	-5.2	-	-0.5	-1.0	1
bR_{in}(ε)											
Arg-82	9.3	0.6	-	-14.3	-0.3	-1.6	-7.7	2.5	-1.3	-2.5	27
Asp-85	10.8	0.4	-14.3	-	0.5	2.1	6.0	-6.5	0.7	1.5	4
Asp-96	4.8	1.3	-0.3	0.5	-	0.5	0.7	-0.7	0.0	0.1	9
Asp-115	6.8	-0.6	-1.6	2.1	0.5	-	1.6	-3.5	0.3	0.8	9
Asp-212	11.4	-4.2	-7.7	6.0	0.7	1.6	-	-6.9	0.9	1.8	-3
Schiff base	3.3	1.3	2.5	-6.5	-0.7	-3.5	-6.9	-	-0.5	-0.9	15
bR_{out}(wat)											
Arg-82	8.5	-3.2	-	-3.7	-0.1	-1.1	-3.0	1.2	-2.9	-5.5	20
Asp-85	10.1	-7.5	-3.7	-	0.5	2.2	6.9	-4.9	0.6	1.3	3
Asp-96	4.6	1.7	-0.1	0.5	-	0.5	0.7	-1.2	0.0	0.1	9
Asp-115	7.3	-0.2	-1.1	2.2	0.5	-	1.7	-2.9	0.2	0.8	10
Asp-212	10.3	-8.1	-3.0	6.9	0.7	1.7	-	-6.1	0.8	1.7	-2
Schiff base	4.2	2.9	1.2	-4.9	-1.2	-2.9	-6.1	-	-0.4	-0.9	21
bR_{in/cis}(ε)											
Arg-82	9.2	1.3	-	-11.1	-0.3	-1.8	-16.4	5.7	-1.3	-2.1	28
Asp-85	9.4	2.3	-11.1	-	0.4	2.2	5.8	-4.5	0.9	1.4	13
Asp-96	7.1	-0.9	-0.3	0.4	-	0.3	0.4	-0.7	0.1	0.1	8
Asp-115	7.3	-3.7	-1.8	2.2	0.3	-	1.4	-3.3	0.3	0.9	8
Asp-212	10.7	-7.8	-16.4	5.8	0.4	1.4	-	-9.3	0.9	1.5	-10
Schiff base	5.2	3.3	4.2	-4.5	-0.7	-3.3	-9.3	-	-0.5	-1.0	6
bR_{out/cis}(wat)											
Arg-82	9.4	-2.3	-	-1.9	-0.1	-1.5	-2.9	1.6	-3.0	-13.7	25
Asp-85	10.8	-4.5	-1.9	-	0.5	2.2	6.2	-5.2	0.6	1.2	12
Asp-96	7.2	-0.5	-0.1	0.5	-	0.5	0.6	-1.1	0.0	0.1	9
Asp-115	7.6	-3.3	-1.5	2.2	0.5	-	1.7	-3.5	0.3	0.9	8
Asp-212	11.4	-15.0	-2.9	6.2	0.6	1.7	-	-8.5	1.0	1.9	-8
Schiff base	5.2	4.4	1.6	-5.2	-1.1	-3.5	-8.5	-	-0.4	-0.9	5

($\Delta\Delta G^{rf}$, $\Delta\Delta G^{dip}$ and charge-charge interactions are in kcal/mol.)

hydrogen bonds than neutral ones. In general, the bR_{out}(wat) structure has more positive values for $\Delta\Delta G^{rf}$ and more negative values for $\Delta\Delta G^{dip}$ than does bR_{out}(ε). This is simply because the buried cavities present in bR_{out}(ε) contain waters that are treated as a continuum. The high dielectric located in the cavity regions contribute favorably to $\Delta\Delta G^{rf}$. However, the explicit water molecules in bR_{out}(wat) are treated as low dielectric and displace the high dielectric in the cavities. This makes the $\Delta\Delta G^{rf}$ term more positive but provides stabilizing dipolar interactions that contribute favorably to $\Delta\Delta G^{dip}$.

Table 2 lists the magnitude of charge-charge interactions among the functionally important residues and interactions between these groups and Glu-9 and Glu-204, which are close to the Arg-82 side chain in several structures. These values provide useful insights into the magnitude of pairwise electrostatic interactions within bR, which are quite large between groups in and around the active site. It is clear that the ionization states of these groups are strongly coupled, a

fact that produces the highly anomalous titration curves described below. In essence, the low dielectric environment in the protein interior produces a large desolvation penalty, but also serves to shield the pairwise interactions from the high dielectric solvent, strengthening them in comparison to those that occur on the surface of globular proteins.

bR_{out}(ε)

For all the residues studied in this work, the loss of solvation described by the reaction field term is larger than the stabilizing interactions provided by polar groups. Due to their decreased solvation in the protein, the pK_a values of Arg-82 and the Schiff base are lowered while those of the aspartic acids are raised. Although the Schiff base and the side chains of Asp-85 and Asp-212 are positioned near cavities that are treated as high dielectric regions in this structure, this does not compensate for the loss of interaction with the bulk solvent. The effect is somewhat larger for Asp-212. However,

Asp-212 forms hydrogen bonds with hydroxyl hydrogens on Tyr-57 and Tyr-185, which provides some compensation for the loss of solvation. In contrast, there are no obvious hydrogen bonding interactions available to Asp-85. Moreover, repulsive interactions on the order of 1 kcal/mol with main chain atoms of Arg-82, Tyr-83, and Ala-81 and the side chain of Trp-86 further destabilize the charged form of Asp-85. The net effect is for both aspartic acids to remain neutral until about pH 13 and to have nearly identical titration curves.

The calculated pK_a values are strongly affected by charge-charge interactions. The titration curves predicted for the $bR_{out}(\epsilon)$ structure are plotted in Fig. 6. Asp-96 and Asp-115 do not interact strongly with other charged residues. Their titration curves have a standard shape although their pK_a values are shifted to about 9 due to large uncompensated desolvation penalties (Table 2). The large desolvation penalty for Arg-82 is nearly compensated by attractive interactions with Asp-85 and Asp-212. That its pK_a is shifted to about 21 is due to additional large stabilizing interactions with Glu-9 and Glu-204. Glu-204 lies near the extracellular surface and is in position to form a salt bridge with Arg-82 when it is extended as in the $bR_{out}(\epsilon)$, $bR_{out}(wat)$, and $bR_{out/cis}(wat)$ structures. The Schiff base pK_a is shifted down to 1, i.e., the neutral form is highly favored in this structure. This might seem somewhat surprising because the Schiff base undergoes large attractive interactions with Asp-85 and Asp-212 that should stabilize its charged form. However, these groups are predicted to remain neutral until about pH 12; hence, they are unavailable to stabilize a charge on the Schiff base at lower pH values.

The complex titration behavior of Asp-85 and Asp-212 precludes a definitive assignment of their pK_a values. Desolvation raises their pK_a values higher, and strong repulsive interactions between the charged form of these residues results in a further shift causing these groups to remain neutral up to very high pH values. They begin titrating as a pair above pH 12, exhibiting a curve that goes through a plateau region where the two groups effectively share one proton between them.

The predictions obtained from the $bR_{out}(\epsilon)$ structure are clearly inconsistent with experimental results. Because the pK_a values of the two anomalous aspartic acids are very high, the resulting Schiff base pK_a is much too low. However, it is possible to shift the pK_a values of Asp-212 and Asp-85 into the experimental range by introducing additional positive charge into the active site or by stabilizing their ionized states through additional hydrogen bonding interactions with polar groups.

$bR_{in}(\epsilon)$

As discussed above, a modified Arg-82 position was defined in which its side chain was rotated into an internal cavity near the retinal binding site situated below Asp-212 and Asp-85 (Fig. 3). In this location, a salt bridge is formed with Asp-85. The calculated pK_a values (Fig. 6 and Table 2) are in good agreement with experiment. The titration curves of Asp-96

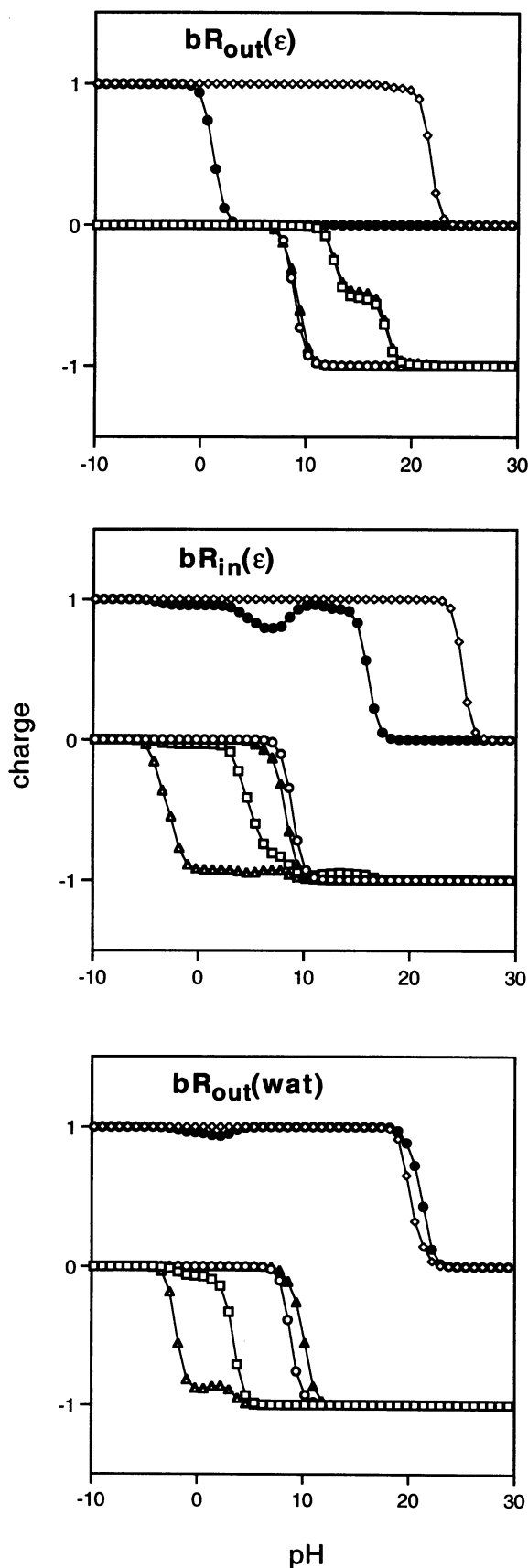


FIGURE 6 Calculated titration curves for $bR_{out}(\epsilon)$, $bR_{in}(\epsilon)$, and $bR_{out}(wat)$. Arg-82 (\diamond); Asp-85 (\square); Asp-96 (\circ); Asp-115 (\blacktriangle); Asp-212 (\triangle); Schiff base (\bullet).

and Asp-115 are essentially unchanged from the $bR_{out}(\epsilon)$ structure and have a shape that is characteristic of standard titration curves. Those of Asp-85, Asp-212 and the Schiff base are not completely smooth due to the strong interactions between them.

The results for the $bR_{in}(\epsilon)$ structure suggest that the experimentally observed pK_a values can be understood if it is assumed that the active site is electrically neutral. This result is in complete agreement with the previous finding of Bashford and Gerwert (1992). However, as will be discussed below, the $bR_{in}(\epsilon)$ structure defined here appears inconsistent with a number of experimental observations. Thus, it is worth considering other mechanisms for shifting the calculated pK_a values into the proper range.

$bR_{out}(wat)$

The inclusion of explicit waters has a strong effect on the calculated pK_a values. The results are very similar to those obtained for the $bR_{in}(\epsilon)$ structure and are also in good agreement with experiment. In the $bR_{in}(\epsilon)$ structure, the charged Arg-82 stabilizes the ionized forms of Asp-85 and Asp-212, which in turn stabilize the protonated form of the Schiff base. In the $bR_{out}(wat)$ structure, the water dipoles make strong hydrogen bonds with the two carboxylates, thus lowering their pK_a values. This can be seen clearly in Table 2 where the polar interactions nearly compensate for the desolvation penalty. In essence, the water dipoles in the $bR_{out}(wat)$ structure have the same effect as the presence of the Arg-82 side chain in the protein interior in $bR_{in}(\epsilon)$.

Flattened regions are present in the titration curves for Asp-85 and Asp-212 between pH -1 and 3, arising from their strong repulsion (Fig. 6). Asp-212 deprotonates at a lower pH than Asp-85 due to greater hydrogen bonding interactions with protein groups that stabilize its charged form (see Discussion). In this pH range, the two carboxyls cannot lose their protons simultaneously because the growing charge on Asp-212 prevents the deprotonation of Asp-85. In effect, one proton is distributed between Asp-85 and Asp-212 in the ratio of about 9:1.

$bR_{in/cis}(\epsilon)$

Trans-cis isomerization has a profound effect on pK_a values. Relative to the $bR_{in}(\epsilon)$ structure, the Schiff base interacts more strongly with Arg-82 and Asp-212 in $bR_{in/cis}(\epsilon)$. However, its charge-charge interaction with Asp-85 is diminished. The repulsion between Asp-85 and Asp-212 is lessened, and the interaction between Arg-82 and Asp-85 is also weakened. The most significant change in the titration curves obtained for this structure (Fig. 7) is that the Schiff base pK_a is shifted below that of Asp-85 so that it loses its proton while Asp-85 remains neutral. Above about pH 8, the active site is electrically neutral. Because there is no positive charge available to stabilize Asp-85, its pK_a is shifted to 13.

$bR_{out/cis}(wat)$

This bR structure contains retinal in the 13-*cis* form as described above. In addition, five water molecules are placed in the largest internal cavity. The charged states of the ionizable groups and their titration curves are similar to those described above for the $bR_{in/cis}(\epsilon)$ structure (Fig. 7). Internal waters in $bR_{out/cis}(wat)$ are located in the same space previously occupied by Arg-82, which faces inward in $bR_{in/cis}(\epsilon)$. They effectively stabilize the charges on other titratable residues to the same extent as does the guanidinium group on Arg-82.

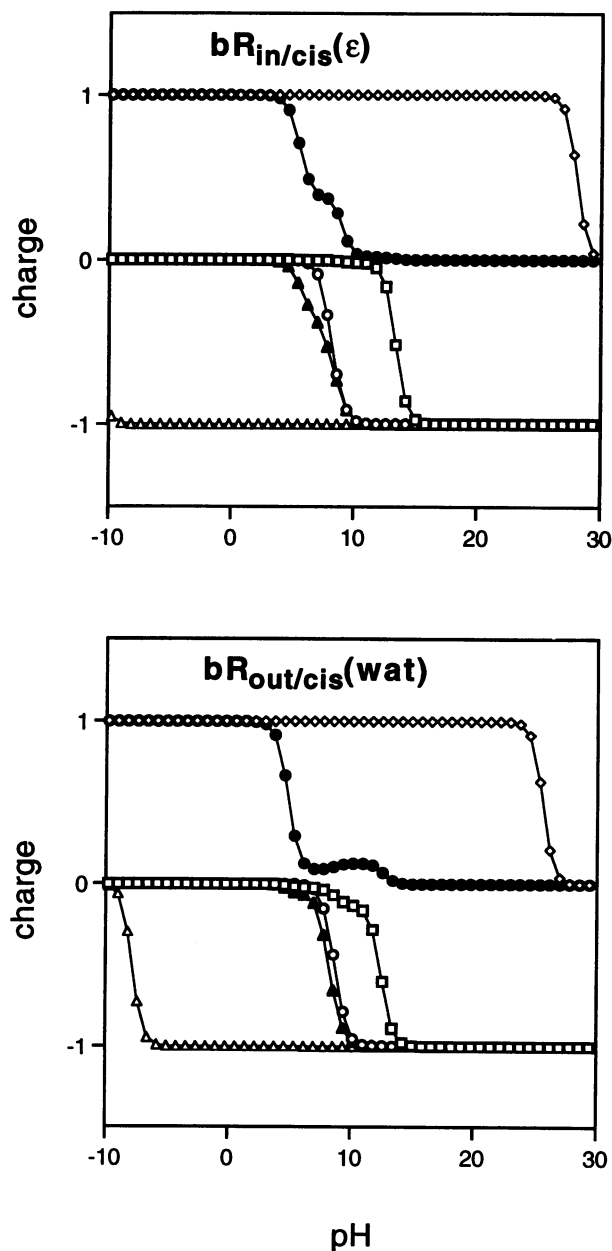


FIGURE 7 Calculated titration curves for $bR_{in/cis}(\epsilon)$ and $bR_{out/cis}(wat)$. Arg-82 (\diamond); Asp-85 (\square); Asp-96 (\circ); Asp-115 (\blacktriangle); Asp-212 (\triangle); Schiff base (\bullet).

DISCUSSION

Many of the calculated titration curves have a complex appearance, containing fluctuations and plateau regions that deviate from the normal sigmoidal shape. This behavior is due to the strong interactions between two or more ionizable residues resulting from their proximity in a low dielectric environment. Such groups tend to exhibit strong coupling of their ionization states. For instance, Asp-85 and the Schiff base interact with -6.5 kcal/mol in $bR_{in}(\epsilon)$, which causes the titration curves for both of these residues to fluctuate slightly between pH 5 and 10 (Fig. 6). The Schiff base begins titrating as low as pH 5 because its ionized form is destabilized by desolvation and by unfavorable polar contributions. At this pH, Asp-212 is fully charged and Asp-85 is approximately 60% deprotonated. In addition, the electrostatic repulsion between these aspartates is 6 kcal/mol. These factors force the Schiff base to remain protonated, stabilizing the charged states of the aspartic acids. As a result, between pH 8 and 10, the fractional protonation of the Schiff base increases in response to the growing negative charge in the region, despite the increasing solution pH. This unusual behavior may be a basic feature of proton transfer in membrane proteins. Such effects are not seen in globular proteins because the interactions are generally smaller due to greater solvent screening. Anomalous titration behavior also has been obtained in studies involving strongly interacting ionizable groups (Bashford and Gerwert, 1992; Yang et al., 1993).

It is worth emphasizing the uncertainties in the numerical results obtained in this work. Based on the studies reported so far (Bashford and Karplus, 1990; Bashford and Gerwert, 1992; McGrath et al., 1992; Yang et al., 1993), pK_a calculations on well defined crystal structures can be expected to yield values within one or two pK units from experiment. However, this assumes that no significant conformational variation results from a change in protonation state. Because this is unlikely to be the case in the interior of a membrane protein, our results tend to overestimate pK_a shifts, as will be discussed further below. The low resolution of the bR structure introduces additional uncertainties. A further problem arises from the fact that water positions were built into cavities where they are expected to reside, despite the lack of data as to their specific locations. Finally, we have not included the effects of the low dielectric environment of the membrane, but instead have assumed that the entire protein is surrounded by bulk water.

Bashford and Gerwert have carried out similar calculations on the ground state of bR using methods and approximations similar, but not identical, to those used here (Bashford and Gerwert, 1992). They utilize different charge sets in their work. In addition, certain calculations treated the Schiff base as protonated throughout so that its pK_a was not calculated directly, whereas other calculations adjusted the intrinsic pK to a value that would give a final pK_a near 12. Also, the effects of the bilayer were taken into account by treating it as a low dielectric slab. The similarity between

these two sets of results gives credence to the general conclusions of both studies.

When ionizable groups change their protonation state, the protein will generally relax to accommodate the new electrostatic interactions and to generate a new hydrogen bonding pattern. The effects are difficult to predict, particularly if the changes extend beyond residues in the immediate environment of the titratable group. We have used energy minimization to account for local rearrangement of side chains, but this will tend to underestimate the stabilizing effects resulting from relaxation of the entire protein. The result is a tendency to favor the ionization state present in the ground state of bR. For example, the high pK_a calculated for Arg-82 is likely to be an overestimate because conformational changes that favor the neutral form have not been fully accounted for.

Despite these limitations, our calculations allow us to reach a number of general conclusions about the nature of electrostatic interactions and proton affinities in bR. Although the pK_a values we obtain are only approximate, our calculations contain a significant amount of information. This includes the effects of the local and global electrostatic environment on the ionization state of principal residues involved in the proton transfer pathway. In addition, general patterns of interactions between these residues are discernible in all of the structures studied. Finally, as will be discussed further below, this information sheds light on the central problem of the proton pumping mechanism of bR: What drives the pK_a changes?

We find that Asp-96 and Asp-115 have pK_a values near 9, in agreement with experimental observation. This increase in pK_a relative to the reference state is due to the reduction of the reaction field term, which is essentially uncompensated by interactions with permanent dipoles or with other titratable groups. We find, in agreement with Bashford and Gerwert (1992), that Asp-85 cannot be ionized in $bR_{out}(\epsilon)$. Although they found Asp-212 to be charged, this is likely because they adjusted the pK_a of the Schiff base to 12. Its positive charge could then stabilize the negative charge on Asp-212 leading to the prediction that the latter residue is also ionized. It is interesting that removing the restriction that the Schiff base be charged leads to an active site where no groups are ionized. Apparently, the overall desolvation penalties for the Schiff base, Asp-85, and Asp-212 are so large that they cannot be overcome even by favorable charge-charge interactions between them.

The pK_a of Asp-85 falls into the expected range when the Arg-82 side chain is positioned into the protein interior as in $bR_{in}(\epsilon)$. The pK_a of Asp-85 is also lowered in the $bR_{out}(wat)$ structure where the Arg-82 side chain faces the solvent and five water molecules occupy the site instead. The assignment of low dielectric to the water molecules when they are treated explicitly in $bR_{out}(wat)$ increases the desolvation penalty of Asp-85 by about 3 kcal/mol. However, polar contributions are enhanced by approximately 7 kcal/mol relative to the $bR_{out}(\epsilon)$ structure due to direct hydrogen bonds between the carboxylates and the water molecules. It may appear

surprising that explicit waters treated as having a low dielectric constant have a larger stabilizing effect on an ionized group than the same waters treated as a dielectric continuum with a high dielectric constant. However, the electrostatic potential of a strongly oriented dipole is greater than that of a polarized continuum. This effect has been noticed previously in pK_a calculations on an ion pair in T4 lysozyme (Yang et al., 1993). The large effects found here for ordered water molecules support the suggestion of Sheves and co-workers that structured water plays an important role in determining pK_a values in bR (Gat and Sheves, 1993).

A question of considerable importance that still has not been resolved is whether the guanidinium group of Arg-82 is in the active site, as in the $bR_{in}(\epsilon)$ structure, or pointing towards the membrane surface, as in the $bR_{out}(\epsilon)$ and $bR_{out}(wat)$ structures. Our finding that experimental pK_a values can be obtained even if Arg-82 faces outward removes the restriction found in previous work that the active site be electrically neutral (Bashford and Gerwert, 1992). It is of interest to consider other evidence in light of our results. The mutation of Arg-82 to alanine shifts the pH at which the purple to blue transition occurs by 4.6 units from 2.6 in the wild type to 7.2 in the mutant (Balashov et al., 1993; Brown et al., 1993). Because this transition is believed to result from protonation of Asp-85, the observed pK_a shift provides a direct measure of the electrostatic interaction between Arg-82 and Asp-85 (Balashov et al., 1993). The magnitude of the interaction in the $bR_{out}(\epsilon)$ and $bR_{out}(wat)$ structures is approximately -3.5 kcal/mol, which is equivalent to 2.5 pK units. The same interaction in $bR_{in}(\epsilon)$ is about 11 pK units. Neither result is in agreement with the observation, although the pK_a shift calculated for the bR_{out} structures is within a reasonable range. Small changes in the position of Arg-82 in the $bR_{out}(\epsilon)$ structure could easily increase the calculated shift. As noted above, cavities are not found in the active site of the $bR_{in}(\epsilon)$ model. That such waters are observed argues against the $bR_{in}(\epsilon)$ structure used in this work. However, recent reports using molecular dynamics claim it is possible to generate a structure that can accommodate both Arg-82 and a number of water molecules in the neighborhood of the Schiff base (Zhou et al., 1993; Humphrey et al., 1994). This possibility has not been considered in the present study, however, because we have examined models that are as close as possible to the experimentally derived structure (Henderson et al., 1990).

An observation of considerable interest is the differential effects of Asp-85 and Asp-212 on the properties of bR. The replacement of Asp-85 with a neutral residue red shifts the absorption maximum of the chromophore significantly more than the mutation of Asp-212 (Otto et al., 1990; Brown et al., 1993). In addition, the Asp-212 replacement has almost no effect on the pK_a of the Schiff base, whereas the Asp-85 substitution lowers it to between 7 and 8.4 (Otto et al., 1990; Brown et al., 1993). This appears somewhat surprising because both Asp-85 and Asp-212 undergo strong electrostatic interactions with the Schiff base that are approximately equal in magnitude.

To determine the effects of mutations, we have replaced both Asp-85 and Asp-212 with asparagine in the $bR_{in}(\epsilon)$ and $bR_{out}(wat)$ conformations. For each structure, the asparagine was placed in the original aspartic acid position, then minimized along with all residues within a 15-Å radius using DISCOVER. The Asn-212 mutation in the $bR_{in}(\epsilon)$ structure results in a calculated Schiff base pK_a of 13, which is shifted down by 2 pK units from that calculated for the wild-type structure. A downward shift in the Schiff base pK_a is to be expected in the mutant because a large (6 kcal/mol) attractive interaction is no longer present. However, this appears to be compensated by a change in hydrogen bonding interactions between the Schiff base and polar groups when Asp-212 is removed. In the mutant, the polar contributions to the Schiff base are -3.1 kcal/mol compared with 1.3 kcal/mol in the original $bR_{in}(\epsilon)$ structure, which offsets the loss of the charge-charge interaction with Asp-212. In contrast to the Asp-212 mutation, the replacement of Asp-85 with asparagine in $bR_{in}(\epsilon)$ is predicted to lower the Schiff base pK_a to about 5. Because Asp-85 lacks protein hydrogen-bonding groups, its substitution has the effect of removing a critical negative charge without providing alternative compensation that might stabilize a protonated Schiff base. It is interesting that when the $bR_{out}(wat)$ structure is used only a minor pK_a shift is calculated for both the Asp-85 and Asp-212 mutations. In this case, the removal of the negative charge is compensated by a significant reorientation of buried waters. Because the small pK_a shift for Asp-85 is in conflict with experiment, it is clear that the $bR_{out}(wat)$ structure allows for greater reorientation of buried waters than is actually possible.

A striking result determined by our calculations is the subtle interplay found between the protonation states of the Schiff base and Asp-85. In both bR_{cis} structures the pK_a values of these two groups effectively switch, with the pK_a of the Schiff base reduced to about 6 while that of Asp-85 is raised to about 12. This accounts for the remarkable observation that a group in the protein with a pK_a near 13 can transfer a proton to one with a pK_a below 3 as a consequence of a *trans-cis* isomerization. The shifts in pK_a values that occur are because these two residues act as a coupled pair; small variations in the pK_a of either Asp-85 or the Schiff base can lead to a preference for one state over the other. This is because the ionization states where both groups are charged or both are neutral are approximately equal in energy. Small changes in the environment of either group can shift the balance between the two states. More specifically, isomerization of the retinal decreases the intrinsic pK of the Schiff base. If the Schiff base is neutral, the ionized state of Asp-85 is no longer stabilized by a positive charge on the retinal, so its pK_a is shifted to much higher values. The same coupling is not seen for Asp-212, whose ionized state is significantly more stabilized by interactions with hydrogen bonding groups and does not depend as strongly on the protonation state of the Schiff base.

Although our results provide an explanation for the transfer of a proton from the Schiff base to Asp-85 in the M state,

they do not reveal how this might lead to further proton release. For example, we have not obtained evidence for a state in which Arg-82 is neutral, as would be required if it is involved in proton release. Indeed, no energetically feasible mechanism has been proposed that would account for arginine losing its positive charge. It is evident from Table 2 that Arg-82 is strongly desolvated in all of the proposed conformations. This could produce, in principle, a large decrease in its pK_a . However, as is also shown in Table 2, there are a number of acidic residues in its vicinity to always provide significant stabilization of its charged state.

A number of mechanisms for various features of the proton pumping pathway have been proposed (Váró and Lanyi, 1991; Lanyi, 1992; Cao et al., 1993). These have been primarily structural in nature in that various proton donors and acceptors are associated with different steps observed in the photocycle. However, a detailed understanding of the mechanism requires that the driving forces for each of the postulated events be identified. Our calculations explain how proton transfer from the Schiff base to Asp-85 might occur, but the interactions that drive the remaining proton transfer events have not yet been defined. Ultimately, a satisfactory model must not only describe the pathway for proton transfer, but must also provide an explanation, in structural terms, of the forces that drive the unidirectional flow of protons. pK_a calculations, as described in this work, provide a framework for the construction of physically meaningful mechanistic models.

CONCLUSIONS

We calculate extremely strong electrostatic interactions between ionizable groups within the active site of bR. This leads to strong coupling between the protonation states of the various residues and the possibility of unusual titration behavior. The $bR_{out}(\epsilon)$ model (Henderson et al., 1990) of bR in which Asp-85, Asp-212, and the Schiff base are all ionized is inherently unstable in the absence of additional stabilizing interactions. The positive charge of Arg-82 or highly directed hydrogen bonds with buried waters are capable of providing the necessary interactions. These groups can be accommodated in a large cavity located near the Schiff base in the reported structure.

Despite approximately equivalent electrostatic interactions with the Schiff base, mutation of Asp-85 is known to have a much larger spectroscopic effect on the retinal and to induce a larger pK_a shift on the Schiff base than Asp-212. This can be accounted for if the groups that form hydrogen bonds with Asp-212 reorient upon its mutation and stabilize the protonated Schiff base. Asp-85 forms no such hydrogen bonds with the protein, and when replaced, there are no such possibilities for compensating interactions. On the other hand, we find that structured waters can play the same role in Asp-85 mutants, a result that is contradicted by experiment. The problem may be due to uncertainties in the detailed structure of the binding site and in the number of water molecules it can accommodate.

The fact that Asp-212 forms hydrogen bonds with the protein whereas Asp-85 does not may be a crucial factor in the proton transfer processes that occur in the early stages of the photocycle. Asp-212 is stabilized by hydrogen bonds that lower its pK_a below that of Asp-85. In contrast, the ionized state of Asp-85 is stabilized only when the Schiff base is protonated. The ionization states of the two groups are found to be coupled closely. Environmental effects that destabilize the protonated Schiff base by just a small amount can shift its pK_a down by many units while concomitantly raising the pK_a of Asp-85 to a similar extent. This effect would appear to account for the fact that a *trans-cis* isomerization, which results in only a minor charge displacement, can cause a group with a pK_a of around 13 to transfer a proton to a group with a pK_a of about 3.

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