Estimated Acid Dissociation Constants of the Schiff Base, Asp-85, and Arg-82 during the Bacteriorhodopsin Photocycle

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ABSTRACT The pK_a values of D85 in the wild-type and R82Q, as well as R82A recombinant bacteriorhodopsins, and the Schiff base in the D85N, D85T, and D85N/R82Q proteins, have been determined by spectroscopic titrations in the dark. They are used to estimate the coulombic interaction energies and the pK_a values of the Schiff base, D85, and R82 during proton transfer from the Schiff base to D85, and the subsequent proton release to the bulk in the initial part of the photocycle. The pK_a of the Schiff base before photoexcitation is calculated to be in effect only 5.3–5.7 pH units higher than that of D85; overcoming this to allow proton transfer to D85 requires about two thirds of the estimated excess free energy retained after absorption of a photon. The proton release on the extracellular surface is from an unidentified residue whose pK_a is lowered to about 6 after deprotonation of the Schiff base (Zimányi, L., G. Váró, M. Chang, B. Ni, R. Needleman, and J. K. Lanyi. 1992. *Biochemistry.* 31:8535–8543). We calculate that the pK_a of R82 is 13.8 before photoexcitation, and it is lowered after proton exchange between the Schiff base and D85 only by 1.5–2.3 pH units. Therefore, coulombic interactions alone do not appear to change the pK_a of R82 as much as required if it were the proton release group.

INTRODUCTION

Photoexcitation of bacteriorhodopsin causes the all-*trans* to 13-*cis* isomerization of the retinal chromophore; the initial state recovers in a reaction cycle which results in the electrogenic translocation of a proton across this membrane-spanning protein (1–4). The transport mechanism is based on sequential changes in the pK_a values of the retinal Schiff base and vectorially arranged protonatable groups in the protein. The initial events occur in the extracellular region, and consist of proton transfer from the Schiff base to D85 (5, 6) and an ensuing release of a proton to the aqueous phase from a so-far unidentified group we had termed XH (7). Considerations of the location of R82 (5, 8), interaction of R82 and D85 (9, 10, this report), and strongly delayed proton release when R82 is replaced with glutamine or alanine (11, 12, and L. S. Brown, Y. Cao, R. Needleman, and J. K. Lanyi, manuscript in preparation) make R82 a candidate for XH. These initial steps are followed by the reprotonation switch which changes access of the deprotonated Schiff base from the extracellular to the cytoplasmic side (8, 13–15), protonation of the Schiff base from D96 (6, 16–18), reprotonation of D96 from the cytoplasmic aqueous phase and reisomerization of the retinal (6, 13, 18), and finally proton transfer from D85 to X and full recovery of the initial state.

Spectroscopic titrations had revealed that in bacteriorhodopsin the pK_a of the Schiff base is near 13 (19) and that of D85 about 2.5 (9). The pK_a of a buried arginine in proteins is expected to be high because the guanidinium pK_a in water is 12.5 and the free energy penalty for immersing the delocalized charge of the $NH{=}C\text{-}NH_3^+$ group in a hydrophobic environment is not large. Thus, it would seem that considerable changes in proton affinities of these groups are required after photoexcitation if the required proton transfers are to take place. There are two major problems: 1) The approach of the pK_a values of the Schiff base and D85 to within 0.6 pH units, as suggested by kinetic data (14), needs a free energy input of 56 kJ/mol. The excess enthalpy of the first easily measured stable intermediate (state K) is only 50 kJ/mol (20), and a large entropic contribution to ΔG is unlikely in K which arises in the subnanosecond time range. 2) The pK_a of the proton release group XH is lowered to 5.8 after deprotonation of the Schiff base (7). If XH were indeed R82, this would represent a very low proton affinity for a guanidinium group and imply the transient creation of a highly unusual dipole environment.

This report is on the proton affinities of the Schiff base, D85, and R82 and calculations on how they might interact with one another during the photocycle to generate the observed proton transfer reactions. By using the recombinant bacteriorhodopsins D85N, D85T, R82Q, R82A, and D85N/R82Q[1] to simulate hypothetical states in which D85 or R82, or both, are uncharged, we can calculate from the measured pK_a values the free energy changes for the relevant protonation reactions with and without coulombic effects from the other residues. The assumption we make is that the R → Q or A and the D → N or T residue replacements have the same effect on the other residues as deprotonation of R82 and protonation of D85, respectively. Since D212, a residue also

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[1] *Abbreviations used:* recombinant bacteriorhodopsins are denoted as D85N, R82A, etc. where the first letter and number stand for the residue in wild-type and the second letter for the substitution. Bis-Tris propane, 1,3-bis[tris(hydroxymethyl)methylamino]propane; CAPS, 3-cyclohexylamino-1-propanesulfonic acid.

near the Schiff base, remains unprotonated at pH low enough to protonate D85 (21), its pK_a must be low enough to exclude it from affecting the results. The results indicate that in the proton exchange between Schiff base and D85 the effective pK_a difference is only 5.3–5.7 pH units, which corresponds to 30–33 kJ/mol. Furthermore, the results are inconsistent with the lowering of the pK_a of R82 to 5.8 after proton transfer from the Schiff base to D85. The calculated pK_a of R82 under these conditions is above 11. Unless the changes in protein conformation and/or the isomeric state of the retinal during the photocycle can considerably further decrease the proton affinity of R82, this residue cannot be the extracellular proton release group XH.

MATERIALS AND METHODS

Halobacterium halobium strains containing recombinant bacteriorhodopsin genes were constructed with a shuttle vector (22, 23) derived from the plasmid pHV2. The D85N residue replacement is described elsewhere (15); the D85T, R82Q, R82A, and D85N/R82Q mutations were produced in a similar way. Purple membranes containing either these or the wild-type protein were purified according to a standard method (24).

All spectra were measured in the dark at room temperature with a Shimadzu UV-250 double-monochromator spectrophotometer connected to a desktop computer. Spectra were obtained with the purple membranes both when encased in polyacrylamide gels, and equilibrated with 100 mM Na_2SO_4 containing 50 mM phosphate, 50 mM Bis-Tris propane, and 50 mM Bis-Tris propane *plus* 50 mM 3-cyclohexylamino-1-propanesulfonic acid (CAPS) buffers as in the pH ranges 1–4, 6–9, and 6–12, respectively, and in suspensions under the same ionic conditions. Essentially the same pK_a values were found in either case, as well as in light-adapted and dark-adapted samples. In all but the blue chromophores the light adaptation event was ascertained by the approximate 5–6-nm red shift after the illumination. Where wavelength maxima are given they are for the light-adapted chromophore. NaCl was not used in order to avoid binding of chloride to the Schiff base in some of the recombinant proteins (25).

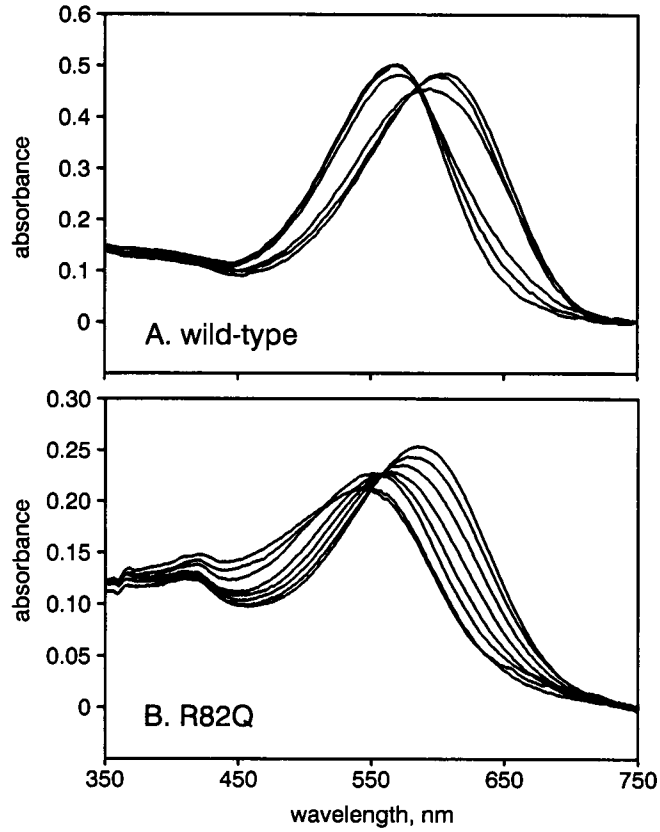

FIGURE 1 Spectroscopic titration of D85 in wild-type (*A*) and R82Q (*B*) bacteriorhodopsins. In *A* the purple membrane was encased in polyacrylamide gel to prevent aggregation, in *B* suspensions were used. The spectra were measured at the following pH values (in the direction of shift toward lower wavelengths): *A*, 1.5, 2.0, 2.5, 3.0, 3.5, and 3.97; *B*, 5.75, 6.31, 6.8, 7.26, 7.72, 8.28, 8.84, and 9.27.

RESULTS

Spectroscopic detection of the deprotonation of the Schiff base and the protonation of D85 is based on specific shifts of the main absorption band of the retinal chromophore. Deprotonation of the Schiff base results in a strong blue shift of the absorption maximum from 550–610 nm to about 410 nm, while protonation of D85 results in a red shift to 590–610 nm (the maxima depend on whether the wild-type or one of the mutated proteins is used). In addition, deprotonation of R82 should result in a blue-shift, because the maxima of the R82Q, R82A, and D85N/R82Q chromophores are blue-shifted by 15, 13, and 34 nm, from their respective wild-type and D85N counterparts (cf. below).

Spectra showing the titration of D85 in wild-type and in the R82Q protein are given in Fig. 1, *A* and *B*. Deprotonation of D85 produces the expected blue shift in both proteins, from 604 to 568 nm in the wild-type, and from 586 to 553 nm in R82Q. Removal of the positive charge of R82 raises considerably the pH at which the blue shift occurs. In R82Q there is an additional blue shift above pH 8, with an isosbestic point at 516 nm; such a shift in this pH range seems to be characteristic of the R82Q residue replacement (cf. below).

Spectra showing the deprotonation of the Schiff base in the D85N and D85N/R82Q proteins are given in Fig. 2, *A* and *B*. While the appearance of an absorption band near 400 nm with increasing pH clearly indicates the formation of unprotonated Schiff base in both systems, progressive blue shifts in the main absorption band complicate the spectra. At pH 6 the maximum of D85N is at 616 nm (Fig. 2A), which is strongly red-shifted from wild-type evidently because the anionic D85 which functions as the main component of the counterion to the Schiff base (9) is removed. With increasing pH the main band shifts to about 570 nm, roughly concurrently with the appearance of the deprotonated Schiff base. Most of the amplitude at 400 nm arises below pH 10, but its constant value between pH 10 and 11 followed by a second rise at higher pH, as well as the persistence of absorption near 570 nm at pH as high as 11.7, indicates that in this protein the Schiff base deprotonation has two components. In contrast, in D85N/R82Q the 400-nm band arises as a simple function of pH between 9 and 12. The main band at 582 nm (Fig. 2 *B*) is red-shifted from wild-type but not as much as in D85N, because partial compensation of the effect of removing D85 by removal of the positively charged R82. With increasing pH the main band shifts to about 570 nm with an

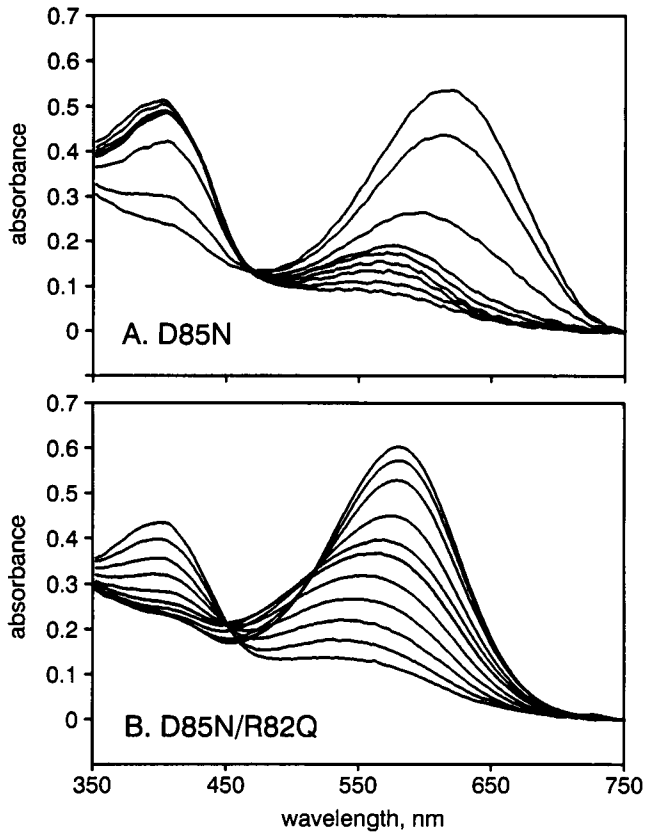

FIGURE 2 Spectroscopic titration of the Schiff base in D85N (*A*) and D85N/R82Q (*B*) bacteriorhodopsins. Purple membrane suspensions were used. The spectra were measured at the following pH values (in the direction of the decreasing amplitude of the main absorption band): *A*, 6.23, 7.56, 8.86, 9.76, 10.16, 10.58, 11.01, 11.38, and 11.67; *B*, 7.11, 7.53, 8.02, 8.54, 8.93, 9.24, 9.7, 10.12, 10.90, and 11.33.

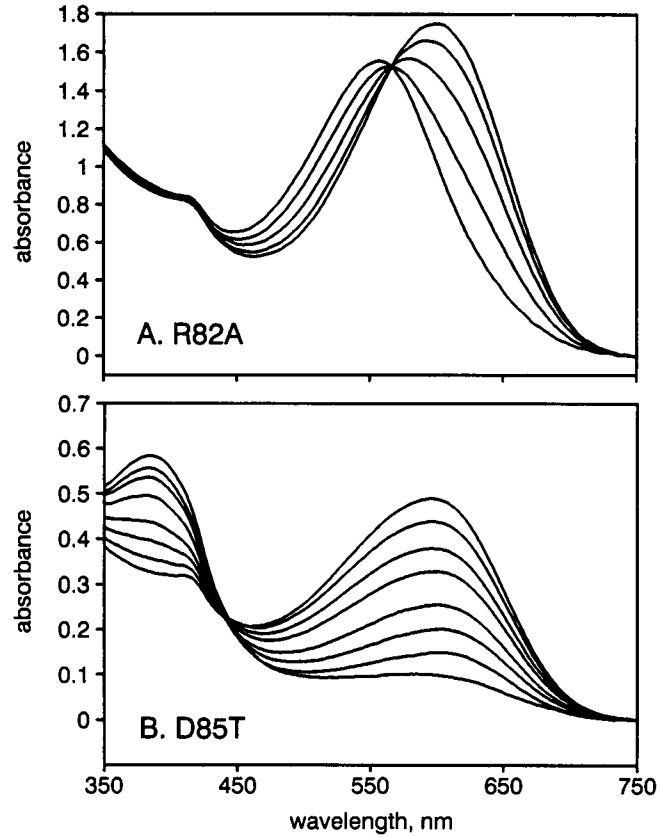

FIGURE 3 Spectroscopic titration of D85 in R82A (*A*) and the Schiff base in D85T (*B*) bacteriorhodopsins. Purple membrane suspensions were used. The spectra were measured at the following pH values (in the direction of the decreasing amplitude of the main absorption band): *A*, 5.85, 6.37, 6.91, 7.49, and 8.09; *B*, 6.16, 6.55, 7.22, 7.57, 8.25, 8.71, 9.11, and 9.59.

isosbestic point at 518 nm before deprotonation of the Schiff base begins at pH 8.5, in a manner similar to the shift in this pH range in R82Q (Fig. 1 *B*). This is followed by a further shift to about 540 nm concurrently with the appearance of the 400-nm band (Fig. 2 *B*). The separation of the two transitions is shown by the presence of two isosbestic points. The blue shift of the maximum before deprotonation of the Schiff base and band broadening have been observed in the wild-type protein also, but at pH > 10 (19, 26, 27). It was interpreted as production of an intermediate species near 480 nm (27). The origin of this transformation of the protonated Schiff base is not known. The R82Q residue replacement apparently lowers the pH at which it occurs.

Fig. 3 *A* shows spectra during the titration of D85 in the R82A protein. The spectral transition resembles those in wild-type and R82Q (Fig. 1), except that the blue shift with increasing pH is from 601 to 555 nm. Fig. 3 *B* gives spectra for the deprotonation of the Schiff base in D85T. In contrast to the data for D85N, this titration appears simple and no blue shift of the main band occurs.

The absorption changes in Figs. 1 (*A* and *B*) and 3 *A* were used to calculate titration curves for the deprotonation of D85 in the wild-type and R82Q and R82A proteins. Fig. 4, *A* and *B*, show these to be simple transitions with pK_a values of 2.7, 7.2, and 7.2, under the conditions used, respectively. The noninteger number of protons used in fitting the titration curve for wild-type (and for several other mutants, cf. legends to the figures) has been attributed to effects of the large negative surface potential on surface pH for this protein (25, 28).

In a similar way, the amplitude changes at 400 nm in Figs. 2, *A* and *B*, and 3 *B* were used to calculate the fractions of deprotonated Schiff base as functions of pH. As expected from the spectra the titration of the Schiff base is described by two components in D85N, but by a single component in D85T and D85N/R82Q (Fig. 5). The first pK_a in D85N and the single pK_a in D85T are both much lower than the pK_a in D85N/R82Q, indicating that below pH 9 the two proteins with single replacement of D85 are not equivalent to the double mutant. We conclude from this that in these proteins the charge state of residue 82 is not the same as in D85N/R82Q; i.e., it is protonated. The second transition in D85N is in the same pH range as the single transition in D85N/R82Q however, arguing that during the titration these two proteins become similar, i.e., that in the higher pH region R82 becomes deprotonated. If this is so, the shift of the main band in D85N from 616 nm to about 570 nm (Fig. 2 *A*) will have

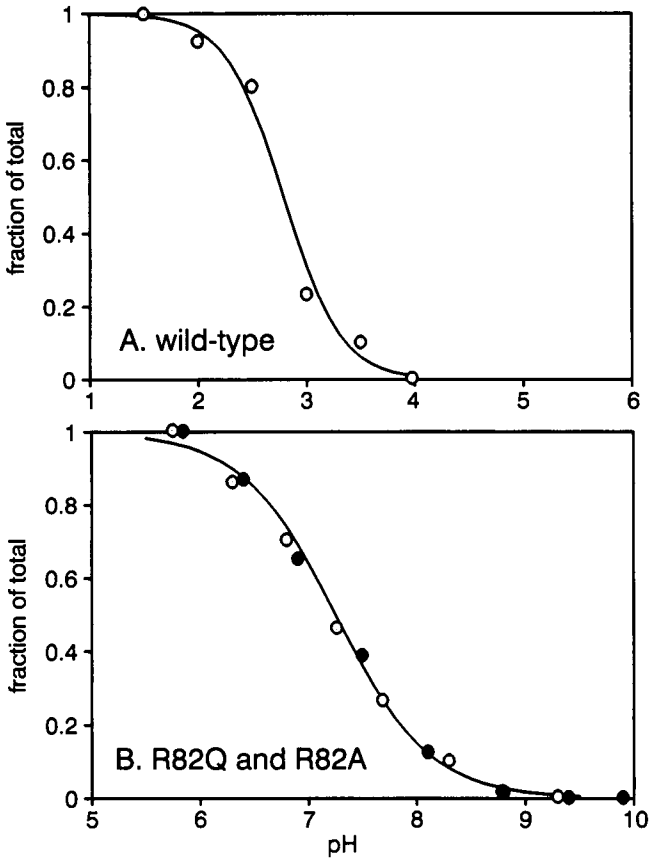

FIGURE 4 Deprotonation of D85 in wild-type (*A*) and R82Q and R82A (*B*) bacteriorhodopsins. The points are calculated from data in Figs. 1 (*A* and *B*) and 3 *A*. In *B* the open circles refer to R82Q, the close circles to R82A. The lines are the best fits of simple deprotonation equilibria. The results are (including the apparent numbers of protons): for wild-type, pK_a = 2.7 (n = 1.70); for R82Q and R82A, pK_a = 7.2 (n = 1.00).

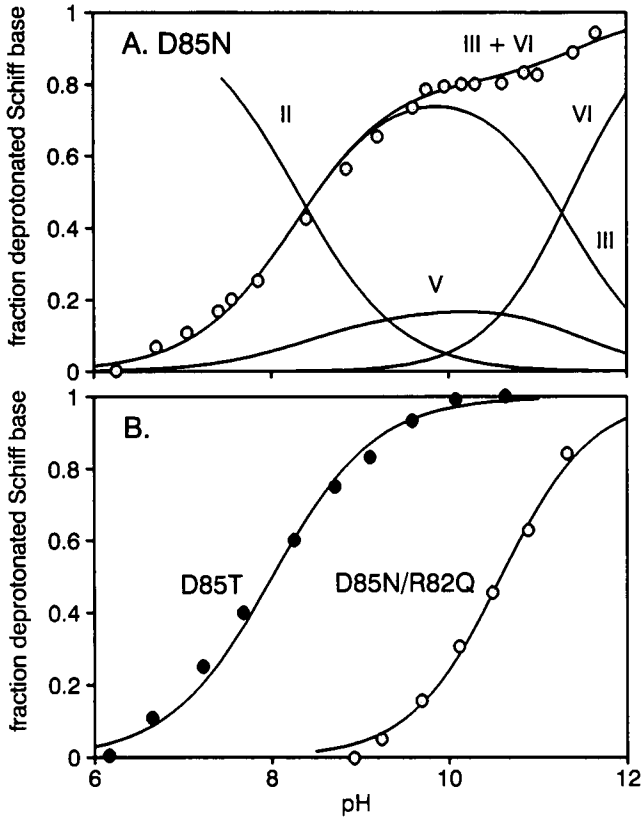

FIGURE 5 Deprotonation of the Schiff base in D85N (*A*) and D85N/R82Q and D85T (*B*) bacteriorhodopsins. The points are calculated from data in Figs. 2 (*A* and *B*) and 3 *B*. The lines are the best fits of simple deprotonations in *B* but a three-state equilibrium in *A*, as explained in the text. Also shown in *A* are calculated concentrations for several of the postulated states (labeled according to the model in Fig. 6). The results are (including the apparent numbers of protons): D85N/R82Q, pK_a = 10.6 (n = 0.85); D85T, pK_a = 8.0 (n = 0.75); D85N, pK_a = 8.4 for II → III, (n = 0.75), pK_a = 9.3 for II → V (n = 0.80), and pK_a = 10.6 for V VI (n = 0.85).

originated both from the loss of the positive charge of R82 upon deprotonation and the blue shift of unidentified origin in R82Q mutants in general. In contrast, D85T shows neither a second pK_a nor the blue shift, suggesting that if R82 deprotonates in this protein this does not occur before the Schiff base deprotonates. The titration curve for D85N in Fig. 4 *A* was therefore evaluated in terms of a model with step-by-step protonation of both the Schiff base (SB) and R82: [SB $R82H^+$] ↔ [SBH^+ $R82H^+$] ↔ [SBH^+ R82] ↔ [SB R82]. These correspond to states III, II, V, and VI in Fig. 6, respectively, as discussed below. The points in Fig. 5 *A* represent the total Schiff base deprotonation, calculated from the absorption increase at 400 nm in Fig. 2 *A*. They are well approximated by the calculated line for the sum of the concentrations of states III and VI if the pK_a values of the reactions designated in Fig. 6 as II → III, II → V, and V → VI are chosen to be 8.4, 9.3, and 10.6, respectively. The titration of D85T (Fig. 5 *B*) fits this scheme also since a simulation (not shown) indicates that if the pK_a of the II → III reaction is lowered to 8.0, as observed (Fig. 5 *B*), while the other two are unchanged the second deprotonation and the production of the blue-shifted state designated as V becomes hardly perceptible during the titration.

The second titration curve (labeled D85N/R82Q) in Fig. 5 *B* is from the absorption increase at 400 nm in Fig. 2 *B*. It represents the single-step deprotonation of the Schiff base with D85 replaced with asparagine and R82 replaced with glutamine, corresponding to the V → VI reaction in Fig. 6.

Thus, D85, R82, and the Schiff base strongly influence the proton affinities of each other. Under the conditions used the pK_a of D85 is 2.7 in the wild-type protein but it is raised to 7.2 in R82Q and R82A where the charge of R82 is removed. The pK_a of the Schiff base in the D85N/R82Q protein, where neither D85 nor R82 is charged, is lowered to 10.6 from the reported 13 in wild-type. It is lowered further to 8.4 in D85N where R82 is charged but D85 is not, and a second deprotonation component appears at 10.6. D85T should be equivalent to D85N, but in D85T the Schiff base deprotonation is represented by a single pK_a of 8.0 instead, which is somewhat lower than in D85N. While the pattern of these pK_a values is similar to those reported earlier for mutated bacteriorhodopsins expressed in the *Escherichia coli* system (25), the numerical values of the pK_a values are considerably different and the titration of the *E. coli*-expressed D85N protein had

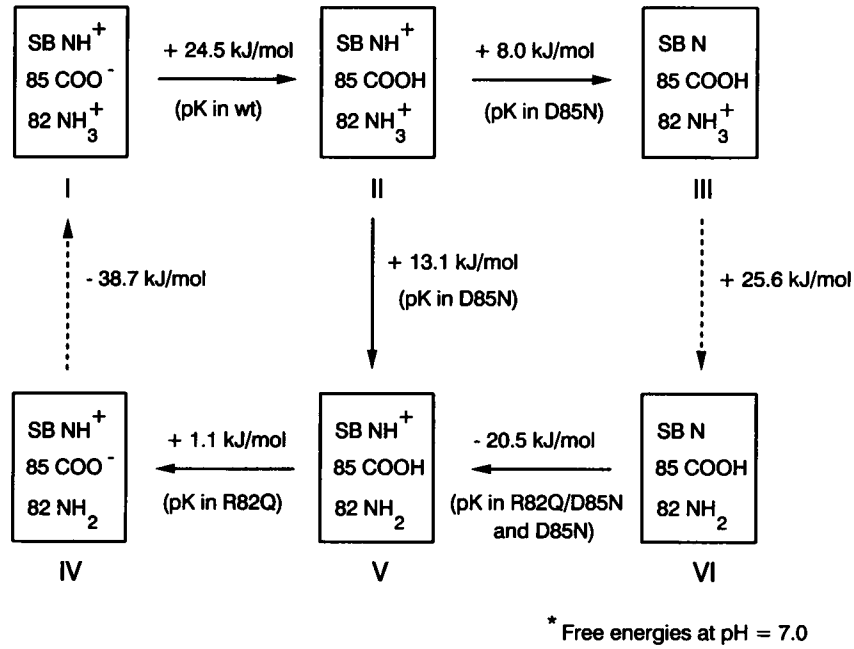

FIGURE 6 Thermodynamic cycle for the protonation reactions involving the Schiff base, D85, and R82. Solid arrows indicate transitions for which the free energies are calculated from the experimentally determined pK_a values (with the source of the information in *parentheses*); dashed arrows refer to those transitions for which the values are calculated. The reference pH is 7.0.

only one component. These differences are caused to some degree by the somewhat different ionic conditions used, but most likely also by the fact that the protein in our samples is in the purple membrane lattice rather than in detergent micelles, and the lipids of the purple membrane contribute strongly to a negative surface charge.

DISCUSSION

The free energy changes of protonation reactions inaccessible to direct measurement were estimated by considering a thermodynamic cycle (Fig. 6) in which the initial state containing charged Schiff base, D85, and R82 (state I) is converted by one possible route of step-wise proton exchanges with the aqueous phase to a state in which all three groups are uncharged (state VI), and then returned via a different route. The mutated proteins differ from states II through VI only in that instead of protonating the carboxyl group of D85 the residue is replaced by asparagine or threonine, and instead of deprotonating the guanidinium group of R82 the residue is replaced by glutamine or alanine. *If the pK_a values are influenced solely by mutual through-space coulombic interaction of the three groups, five of the protonation steps in Fig. 6 (solid arrows) correspond to the titrations of the mutant proteins in Figs. 1–5, and the missing pK_a values can be calculated.*

The evidence suggests that assuming the equivalence of the mutated proteins and the single-step protonation states in Fig. 6 is not quite justified but the discrepancies are minor. Although shifts in the chromophore absorption maximum and in the pK_a of the Schiff base are not always strictly correlated (29), it is reasonable to expect that the charge environment of the Schiff base be reflected to some degree in the chromophore absorption maxima (30–32). State II should be equivalent to the blue form of the wild-type protein at low pH, D85N, as well as to D85T and the blue form of D85E (33). The maxima of these are at 604, 616, 595, and 615 nm, respectively. Likewise, state V should be equivalent to the D85N/R82Q protein, the blue form of R82Q, as well as to the blue form of R82A. The maxima of these are at 582, 588, and 601 nm, respectively. Thus, some differences exist within each group but those which correspond to state II are distinctly blue-shifted relative to those corresponding to state V. Comparisons of the titration curves of different D85 and R82 residue replacements are even more clear-cut. The titration of the Schiff base in D85T produced a pK_a differing only by 0.4 units from that of D85N (Fig. 5). The pK_a values of the titration of D85 in R82Q and R82A coincide exactly (Fig. 4 *B*). Thus, in the residue interactions involving D85 asparagine and threonine are nearly equivalent as replacements, and glutamine and alanine are exactly equivalent as replacements for R82.

Since the net change in free energy over each of the two cycles I → II → V → IV → I, and II → III → VI → V → II, is by definition zero, the five measured ΔG values define the ΔG values of the I → IV and III → VI reactions (dashed arrows) as well. Thus, the missing pK_a values of R82 in the initial and fully deprotonated states, respectively, can be calculated. In these calculations we used a reference state with a pH of 7.0 (as long as it is uniform, the choice of this pH does not affect the conclusions). All interconversions were assumed to be simple protonation reactions, for which the relationship $\Delta G = -2.3RT(\text{p}K_a - \text{pH})$ applies. The measured pK_a of D85 in the wild-type corresponds to deprotonation of D85 with the other two groups protonated, and gives ΔG = +24.5 kJ/mol for the I → II transition. The measured pK_a of D85 in R82Q and R82A corresponds to deprotonation of D85 with R82 deprotonated, and gives −1.1 kJ/mol for the IV → V reaction. The pK_a values of the Schiff base in D85N and D85T correspond to the deprotonation of the Schiff base with

D85 protonated; the data suggest that in D85N, although not in D85T, the titration includes the combined II → III, II → V, and V → VI transitions. The calculated ΔG values from the pK_a values of these reactions in D85N are +8.0, +13.1, and +20.5 kJ/mol, respectively. In D85T the ΔG of the II → III reaction is calculated to be +5.7 kJ/mol, while those of the other two reactions are undefined. If the data from D85N and D85N/R82Q are considered, the five measured ΔG values define the ΔG of the I → IV reaction as +38.7 kJ/mol, which corresponds to a pK_a of 13.8 for R82 with both D85 and the Schiff base charged, and the ΔG of the III → VI reaction as + 25.6 kJ/mol, which corresponds to a pK_a of 11.5 for R82 when both D85 and the Schiff base uncharged. If the data from D85T and D85N/R82Q are considered, the four measured ΔG values define the *difference* between the ΔG values of reactions I → IV and III → VI as 8.6 kJ/mol, which corresponds to a pK_a decrease of 1.5 units for R82 when both Schiff base and D85 lose their charge.

According to the measured and calculated ΔG values therefore the pK_a of D85 is lowered by 4.5 pH units by the positive charge of R82. The pK_a of the Schiff base is raised by 4.6–5.0 pH units by the negative charge of D85, but lowered by 2.2 pH units by the positive charge of R82. The directions and approximate magnitudes of these pK_a shifts are thus as expected from simple coulombic interactions and from results with model retinal compounds (29). They are consistent with the chromophore absorption maxima which are shifted by the D85T and D85N replacements to the red (27 and 48 nm, respectively), and to the blue upon the R82Q and R82A replacements (18 and 13 nm, respectively).

The determined pK_a values may be relevant to the proton transfer reactions during the photocycle. State I corresponds to bacteriorhodopsin before photoexcitation and, except for the isomeric state of the retinal, state III corresponds to the M photointermediate. The combined transition from I to III represents therefore the net proton transfer from the Schiff base to D85. The cycle in Fig. 6 thus provides the means to estimate the ΔpK_a between the Schiff base and D85 in this internal proton transfer. Its value is 5.3–5.7, which is equivalent to 30–33 kJ/mol or about two thirds of the estimated available free energy after absorption of a photon. On this basis the proton transfer from Schiff base to D85 would be energetically feasible. If the extracellular proton release group XH is R82, states III and VI will correspond to what we earlier named M_1 and M_1' (7), and their interconversion results in proton release on the extracellular side. R82 will be an acceptable candidate for XH if its pK_a is higher than 10 in the initial state (I) and becomes about 6 in the M photointermediate (state III). While the initial pK_a of R82 is calculated to be 13.8, the data from D85N suggest that it is lowered to only 11.5 in the M photointermediate. Similarly, the data from D85T suggest a lowering of the pK_a by only 1.5 units. Thus, coulombic effects do not decrease the pK_a of R82 sufficiently to make it a candidate for XH. Braiman et al. (5) had suggested that the origin of the released proton is a water molecule bound to R82. According to recent evidence, in fact, proton release in the photocycle is delayed not only upon the R82Q and R82A substitutions but also in the Y57F protein (L. S. Brown, Y. Cao, R. Needleman, and J. K. Lanyi, manuscript in preparation), consistent with the intriguing suggestion that XH might not be a single residue. The alternative candidate for XH would be a hydrogen-bonded complex of R82, Y57, and a bound water or a hydronium ion.

We wish to emphasize that these pK_a values are calculated from data rather than from inductive models for how buried charges influence one another (34–36). They constitute a description of the system and invite interpretation rather than provide insights to the nature of the measured interactions. The direction and magnitude of the interaction energies found are reasonable and give some confidence that the assumptions are valid. It is possible, however, that protein conformational changes during the photocycle significantly alter the pK_a values by means not evident from the mutated proteins. For example, there are two possible configurations of R82 in the structure (8). A model calculation (36) and the influence of D85 and the Schiff base on R82 both suggest that in the initial state the configuration which orients this residue toward the protein interior is more likely. Reorientation of R82 toward the aqueous interface in the M photointermediate would be a possible way to change its pK_a, but this would raise rather than lower it as required if R82 were group XH. There are no positively charged residues near R82 (8) which are obviously in position to lower its pK_a. However, small changes in the orientation of proton transfer groups can make a large difference in proton affinities (37), and some structural changes near the Schiff base do arise during the photocycle (38–40). Also, the retinal assumes the 13-*cis* rather than the all-*trans* configuration during the photocycle, and the pK_a of R82 might be affected by this. It remains to be seen if such possible noncoulombic influences on R82 can lower its pK_a sufficiently to make it release its proton during the photocycle.

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