A Large Photolysis-Induced pK_a Increase of the Chromophore Counterion in Bacteriorhodopsin: Implications for Ion Transport Mechanisms of Retinal Proteins

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ABSTRACT The proton-pumping mechanism of bacteriorhodopsin is dependent on a photolysis-induced transfer of a proton from the retinylidene Schiff base chromophore to the aspartate-85 counterion. Up until now, this transfer was ascribed to a >7-unit decrease in the pK_a of the protonated Schiff base caused by photoisomerization of the retinal. However, a comparably large increase in the pK_a of the Asp-85 acceptor also plays a role, as we show here with infrared measurements. Furthermore, the shifted vibrational frequency of the Asp-85 COOH group indicates a transient drop in the effective dielectric constant around Asp-85 to ~2 in the M photointermediate. This dielectric decrease would cause a >40 kJ-mol^{-1} increase in free energy of the anionic form of Asp-85, fully explaining the observed pK_α increase. An analogous photolysis-induced destabilization of the Schiff base counterion could initiate anion transport in the related protein, halorhodopsin, in which aspartate-85 is replaced by Cl^- and the Schiff base proton is consequently never transferred.

INTRODUCTION

Attempts to explain the driving force for proton transfer in bacteriorhodopsin (bR) (Henderson et al., 1990; Mathies et al., 1991; Ebrey, 1993; Lanyi, 1993; Rothschild, 1992; Krebs and Khorana, 1993) have focused on a large drop in the pK_a of the Schiff base following chromophore photoisomerization, from 13.5 in the unphotolyzed state (Sheves et al., 1986; Govindjee et al., 1994) to 7 or less afterward (Kalisky et al., 1981). It has been concluded that the *all-trans* to 13-*cis* isomerization carries the Schiff base nitrogen from a position where it participates in hydrogen-bonding interactions with one or a few water molecules (Maeda et al., 1994; Deng et al., 1994; Bashford and Gerwert; 1992) to a less hydrogen-bonded state that destabilizes the positive charge and thus makes the protonated Schiff base much more acidic (Brown et al., 1994).

It is widely accepted that Asp-85 serves as the acceptor for the Schiff base proton in the M photoproduct of bR. This conclusion was based on the presence of a positive absorption band at 1762 cm^{-1} in the photolysis-induced bR→M infrared (IR) difference spectrum (see Fig. 1 *A*), which has been assigned to the COOH carbonyl stretching frequency ($\nu_{C=O}$) of this residue (Rothschild et al., 1981; Engelhard et al., 1985; Braiman et al., 1988). At an early stage of analysis (Rothschild et al., 1981), it was concluded that the 1762 cm^{-1} frequency means that the pK_a of this Asp in the M state is near 2.5, based on a published correlation between carbonyl stretching frequencies ($\nu_{C=O}$) of several carboxylic acids and their pK_a values (Bellamy, 1968). Since then, the idea that Asp-85 has a pK_a of ~2.5 throughout the photocycle has been contradicted only in very general terms. For example, recently an increase of unspecified size in the pK_a of Asp-85 has been suggested as occurring in the L state concomitantly with a large Schiff base pK_a drop to raise the former to within 0.5 pH units of the latter, and thus to promote a transient proton transfer (Brown et al., 1993; Lanyi, 1993).

Below we demonstrate that the pK_a of Asp-85 is far from constant. In fact, it must increase to at least 10.5 during the photocycle, because Asp-85 is fully protonated in the M state and—in the D96N mutant at least—simultaneously capable of hydrogen/deuterium (H/D) exchange in the dark with an external buffer, even at this high pH. Furthermore, there are strong, but not conclusive, indications that this >8-unit pK_a increase of Asp-85 occurs even as the pK_a of the Schiff base drops by a similar amount, with the logical result that a photoisomerization-induced conformation change associated with formation of the long-lived M intermediate transiently raises the pK_a of the former to at least 4 units above that of the latter.

MATERIALS AND METHODS

All bR used was in the form of purple membranes prepared from *Halobacterium salinarium* (formerly designated *H. halobium*). Time-resolved FTIR spectra were obtained on a Nicolet Instruments (Madison, WI) 60SXR spectrometer using a stroboscopic method (Braiman et al., 1991; Mitrovich et al., 1995). Samples contained 50–80% water by weight, according to the relative sizes of water and protein absorption bands.

Static FTIR difference spectra of bR at different pH values were measured on a Nicolet 740 spectrometer, using an attenuated total reflection (ATR) technique as reported previously (Marrero and Rothschild, 1987). An important exception is that our commercially-obtained ATR cell (Foxboro, East Bridgewater, MA) was opaque to ambient light. Solutions containing 35 mM $CaCl_2$ and sufficient HCl to produce the desired pH values in the range 2–3 were alternately flowed into the ATR cell, allowing the buffers to contact the bR-coated surface of the Ge ATR plate. For a visible spectral measurement of the same titration, bR was incorporated in

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a 10% polyacrylamide gel. Two pieces cut from a single gel were soaked overnight in the buffers used for the IR measurement before measuring their visible spectra (each referenced to the appropriate buffer) using a Shimadzu UV-265 spectrometer.

Deuterium exchange experiments were performed using the D96N mutant expressed as purple membrane in *H. salinarium* (Sasaki et al., 1992). FTIR spectra were measured by using the static ATR-IR technique described above, but with a custom modified ATR cell cover that permitted fiberoptic illumination of the purple-membrane-coated Ge plate through a Plexiglas window mounted into it. The sample cell was filled with a buffer (500 mM NaCl plus 200 mM sodium carbonate, pH 10.5) and allowed to sit in the spectrometer for several hours until the FTIR spectral baseline was stable. Then spectra were measured immediately before, and at 1-min time intervals after, a brief (1-min) period of illumination that was followed immediately by exchange of the buffer for an identical deuterated buffer. In control experiments, the illumination and spectral difference measurements were carried out without any buffer exchange.

RESULTS

The pK_a of Asp-85 in the M state

A pK_a above 11 for Asp-85 in the M state is clearly suggested by the presence of the positive Asp-85 $\nu_{C=O}$ band at 1762 cm^{-1} in the bR→M difference spectrum measured at pH = 10.5 (Fig. 1 *B*), which is as large as the band measured at pH 7 (Fig. 1 *A*). This band is in a frequency range typically seen for protonated carboxylic acids, and never for their deprotonated forms (Bellamy, 1968). In the bR→M difference spectra of Fig. 1, *A* and *B*, the entire region from 1800–1000 cm^{-1} is largely pH-independent. It is therefore unlikely that their 1762 cm^{-1} bands arise from different residues or from completely different photoproduct states.

To test directly whether the Asp-85 COOH group was in proton-transfer equilibrium with the bulk, we attempted to measure whether its COOH proton could exchange with deuterons added to the external medium. The fastest we could replace the buffer in our ATR-IR cell in situ in the spectrometer was about 1 min; obtaining a spectrum with an adequate signal/noise ratio subsequently required another 1-min period.

Therefore, to detect exchange at Asp-85 in the photoproduct, we used the D96N mutant of bR, which has a very long-lived deprotonated Schiff base intermediate called M_N (Sasaki et al., 1992). In Fig. 1 *D*, we show the bR→M_N difference spectrum of this mutant measured in buffer at pH = 10.5 (uncomplicated by H/D exchange). As noted previously (Sasaki et al., 1992), this difference spectrum is intermediate between the bR→M and bR→N spectra of wild-type bR. (Fig. 1, *B* and *C*). The 1758 cm^{-1} positive band of Fig. 1 *D* demonstrates that in the M_N, Asp-85 still becomes transiently protonated as in the wild-type M and N species. Repeated scanning of the difference spectrum after the light was turned off showed that this 1758 cm^{-1} absorbance change decays at room temperature with a time constant of 5 min (spectral data not shown). This is comparable to the 2-min decay time previously measured for the M_N→bR decay at pH = 10 (Sasaki et al., 1992).

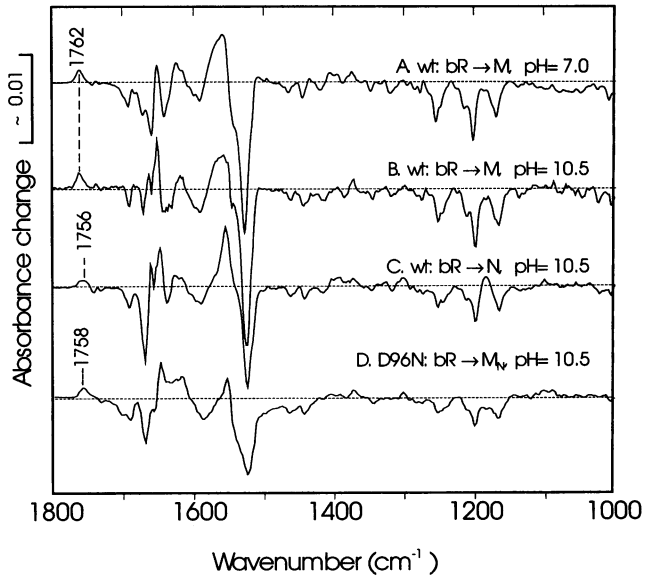

FIGURE 1 (*A–C*) Time-resolved FTIR difference spectra obtained at room temperature using a stroboscopic time-resolved technique on a Nicolet 60SXR spectrometer (Braiman et al., 1991). The resulting spectra correspond to the following species: (*A*) M photoproduct minus unphotolyzed bR at 25°C and pH 7.0. The effective time range from which the M spectrum was calculated was ~0.1 − 0.5 ms after photolysis, and the photolysis repetition rate was ~1 s^{-1}. (*B*) M minus bR at 3°C and pH 10.5, calculated from a data set covering a time range of 17–500 ms after photolysis. (*C*) N minus bR, calculated from the same data set as (*B*). To obtain *B* and *C*, singular value decomposition and multiexponential kinetic fitting programs were used as described previously (Chen and Braiman, 1991) to separate spectral contributions from different intermediates. The fitted time constant for the M→N decay process in this data set was 250 ± 100 ms; the fitted time constant for the N→bR process was 3 ± 1 s. The same time constants and spectral bandshapes for the peaks at 1762 cm^{-1} (*B*) and 1756 cm^{-1} (*C*), were obtained when the kinetic fit was restricted to the range 1780–1745 cm^{-1}. (*D*) M_N minus bR difference spectrum of the D96N mutant, obtained at room temperature and pH 10.5 in an ATR cell using a Nicolet 740 spectrometer in its normal scanning mode (~2 scans/s). After 1 h of dark adaptation inside the spectrometer, a reference spectrum was measured for 6 min in the dark, then the sample was exposed to 1 min of continuous white-light illumination from a projector lamp, and finally the sample spectrum was collected for 1 min in the dark.

With FTIR spectroscopy, it is possible to detect deuterium exchange at the Asp-85 group of M_N. For example, after 1 day of equilibration with deuterated pD = 10.5 buffer, the bR→M_N difference spectrum of D96N shows a COOD carbonyl stretch vibration due to Asp-85 at 1748 cm^{-1} (Fig. 2 *B*). The deuterium exchange did not significantly alter the single-exponential decay time of 5 min for this band. The 10-cm^{-1} deuteration-induced downshift is similar to that seen for the 1762 cm^{-1} band in the wild-type bR→M difference spectrum (Engelhard et al., 1985). The same 1748 cm^{-1} difference band is observed even when the D96N sample is photolyzed to form M_N in undeuterated buffer, and exposed to deuterated medium only after being blocked from light (Fig. 2 *C*). This demonstrates that within the lifetime of the protonated Asp-85 in the M_N species, this group's protons are in exchange equilibrium with protons (deuterons) in the bulk solution at pD = 10.5.

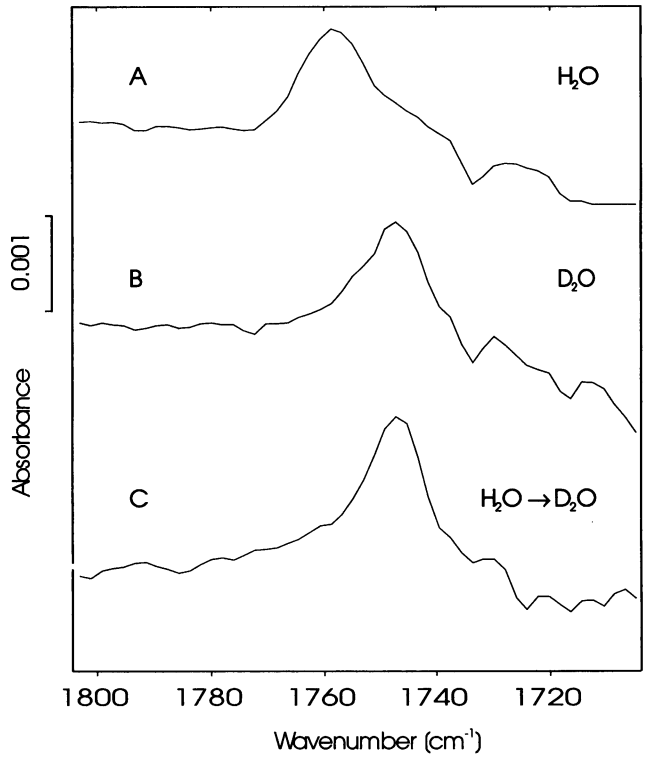

FIGURE 2 FTIR difference spectra of the long-lived M_N photoproduct of the D96N mutant in the C=O stretching region of carboxylic acids, measured in an ATR cell using a Nicolet 740 spectrometer. All sample spectra were obtained as in Fig. 1 *D*, but were measured during the second minute after illumination was turned off. This permitted the exchange of deuterated for undeuterated buffer during the intervening minute, for spectrum (*C*). All reference spectra were scanned for the period between 60 and 66 min after the sample spectrum to reduce baseline changes due to fast deuterium exchange of peptide backbone groups in (*C*), while still permitting the M_N to decay fully.

Asp-85 remains protonated in the long-lived N state above pH 10.5

Doubts about whether transiently protonated Asp-85 ever reaches equilibrium with the external pH 10.5 buffer in the wild type can be reduced, because its COOH vibration (measured by the absorbance increase at 1760 cm^{-1}) actually has a lifetime that is >12-fold greater than the measured apparent M decay time. This occurs because Asp-85 remains protonated in the photointermediate following M, known as N. During the 250-ms M→N decay process, the IR-difference band due to the Asp-85 COOH group decreases somewhat in intensity, and its peak frequency downshifts to 1756 cm^{-1}, as seen in Fig. 1 *C*. A definitive assignment of the 1756 cm^{-1} N band through site-directed mutagenesis is lacking, because mutations at Asp-85 tend to perturb this part of the photocycle very strongly. Nevertheless, site-directed mutagenesis has shown that in other photoproducts with protonated Schiff base groups (e.g., O_{acid}), a positive band at ~1755 cm^{-1} is indeed characteristic of Asp-85 COOH (see below). This tends to support the assignment of this band to Asp-85 in N as well. Furthermore, there is no other carboxylic acid to which the 1756 cm^{-1} band in N can reasonably be assigned.

Thus, the >3 s lifetime of the 1756 cm^{-1} difference band at pH 10.5 and its equal relative intensity in bR→N difference spectra measured at pH 10.5 (Fig. 1 *C*), 9.2 (Braiman et al., 1991), and 7 (Pfefferlé et al., 1991) provide strong evidence that Asp-85 remains fully protonated in an N state that has reached proton-transfer equilibrium with the high-pH buffer, and only deprotonates when N decays back to bR.

Measurement of $\nu_{C=O}$ in unphotolyzed bR

In the unphotolyzed (bR) state, Asp-85 appears to have a "normal" COOH frequency near 1723 cm^{-1}. A negative band at this frequency is of course not observed in bR→M difference spectra, because of the predominance of the unprotonated form at pH = 7 (4–5 pH units above the pK_a of Asp-85). However, as shown in Fig. 3 *A*, a $\nu_{C=O}$ of 1723 cm^{-1} is observed in protonation-induced difference spectra obtained near the pK_a of Asp-85. Visible difference spectra obtained between the same pair of pH values (Fig. 3 *B*) show that this pH jump covers a substantial portion of the transition range between normal purple membrane (bR, λ_{max} = 568 nm) and its blue form ($bR_{acid\ blue}$, λ_{max} = 605 nm). This transition in the visible absorption spectrum arises because of protonation of the Asp-85 counterion of the retinylidene Schiff base chromophore (Subramaniam et al., 1990; Metz et al., 1992). We limit ourselves to a portion of the Asp-85 titration range to reduce complications from the 14 water-exposed carboxylate groups at the membrane surfaces of bR. These groups are expected to titrate with a typical aspartate pK_a near 4.5, and also have a $\nu_{C=O}$ band near 1723 cm^{-1} (Gerwert et al., 1987). The 1723 cm^{-1} positive COOH band in Fig. 3 *A*, and the 1578 and 1396 $cm^{-1}COO^-$ bands, are thus largely due to titration of Asp-85.

In agreement with earlier workers (Gerwert et al., 1987), we conclude that the residue that protonates in the purple-blue transition (i.e., Asp-85) has a $\nu_{C=O}$ near 1723 cm^{-1} in the unphotolyzed state. This is nearly 40 cm^{-1} lower than in M. Whereas it is possible that the protonation of this group results in a protein conformational change that substantially alters its environment from that in bR at physiological pH, this is somewhat unlikely because the purple/blue transition is very rapid and completely reversible in the dark, and simultaneously shifts the absorption bands of both 13-*cis* and *all-trans* components of dark-adapted bR.

Light-induced changes in $\nu_{C=O}$ at acid pH

Addition of high Cl^- concentrations to $bR_{acid\ blue}$ leads to formation of a third species, $bR_{acid\ purple}$, that has visible and resonance Raman spectra almost identical to that of the physiological form of bR, indicating a very unperturbed chromophore environment. Such spectra have been inter-

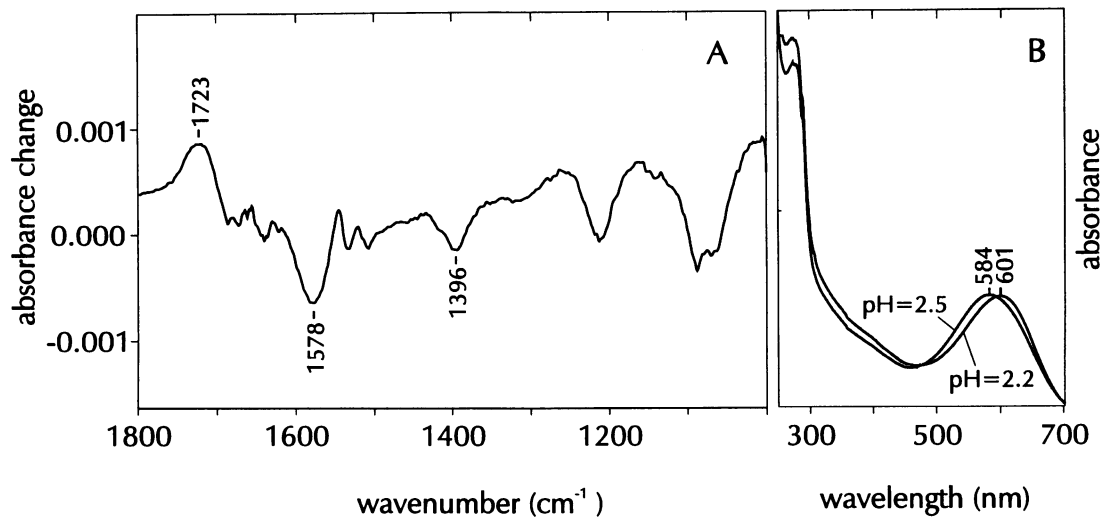

FIGURE 3 The effects of a pH shift between 2.2 and 2.5 on the IR (*A*) and visible (*B*) absorption spectra of bR. In the IR range, only the difference spectra between the two states is shown with positive absorbance bands corresponding to the lower pH. This IR difference spectrum is an average calculated from eight full cycles of raising and lowering the pH.

preted to indicate Cl^- binding in place of the titrated Asp-85 counterion. Acid-base titrations of bR in the presence of constant high Cl^- concentrations are also consistent with an Asp-85 COOH frequency near 1725–1730 cm^{-1} (data not shown).

Furthermore, a negative band at 1731 cm^{-1} and positive band at 1755 cm^{-1} in the photolysis-induced difference spectrum of $bR_{acid\ purple}$ (Fig. 4 *A*) are due to Asp-85. The positive bands are due to the photoproduct known as O_{acid} (Mitrovich et al., 1995). The $bR_{acid\ purple} \rightarrow O_{acid}$ difference spectra in Fig. 4 *A* directly demonstrate a large photolysis-induced change in $\nu_{C=O}$, consistent with a dielectric constant decrease around this residue, as described below.

It is noteworthy that the photolysis-induced perturbation on the environment around residue 85 is preserved even when the COOH group is mutated to $CONH_2$ (Fig. 4 *B*). That is, the trough at 1731 cm^{-1} and peak at 1755 cm^{-1} in *A* are replaced by a trough at 1681 cm^{-1} and a peak at 1700 cm^{-1} in *B*. This indicates that all four of these spectral features are due to C=O stretching vibrations of the residue at position 85, either Asp-85 or Asn-85. The frequency increase between trough and peak in each of the two spectra is probably due to a photolysis-induced decrease in polarity and/or hydrogen-bonding character of the environment around residue 85. The ~50-cm^{-1} frequency decrease between corresponding peaks in the spectra of proteins containing Asp-85 and Asn-85 reflects the different characteristic carbonyl frequencies of COOH and $CONH_2$ groups.

It is also noteworthy that nearly the same $\nu_{C=O}$ frequency (~1755 cm^{-1}) is observed for Asp-85 in the O_{acid} species produced at pH 1 and in the N species produced at pH 10.5. From this result, it seems clear that the environment around Asp-85 is much more perturbed by photolysis than by any pH-induced protein conformational changes.

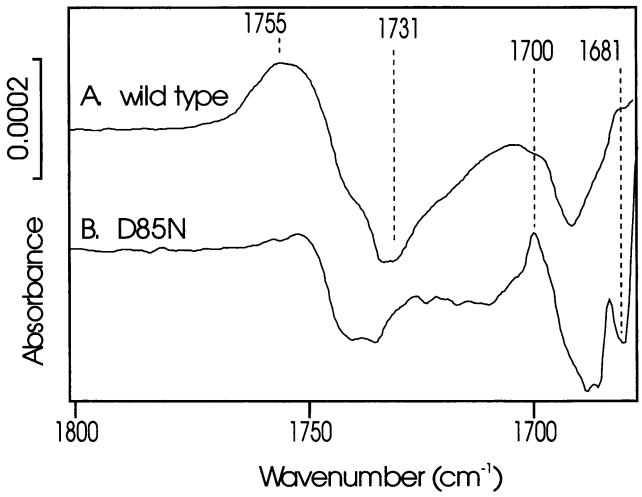

FIGURE 4 Time-resolved FTIR difference spectra of the $bR_{acid\ purple} \rightarrow O_{acid}$ for the wild type (*A*) and the D85N (*B*) mutant. Spectra were obtained of samples in 1 M KCl at pH 1 and 4°C, over a time range of 0.5–15 ms after photolysis, as described previously (Mitrovich et al., 1995).

DISCUSSION

The M and N states are metastable photointermediates of bR. It might be suspected that a COOH group, detectable in such species only with time-resolved spectroscopy, cannot be interpreted in terms of a pK_a, which is a concept based on equilibrium thermodynamics. However, most molecules that have been assigned pK_a values are also metastable, even if their lifetimes are typically measured in years instead of milliseconds. The important criterion in deciding if a pK_a can be defined is whether a molecule has a lifetime much longer than required to reach proton-transfer equilibrium with a bulk aqueous medium at a well-defined pH.

The measured lifetime of the ~1762 cm^{-1} band in the bR→M spectrum of Fig. 1 *B* is at least 10-fold greater than has been described previously in the presence of liquid water with a defined pH. Previous time-resolved IR spectral measurements at neutral pH and room temperature showed a lifetime for this band of ~20 ms (Braiman et al., 1991; Gerwert et al., 1991). This left considerable doubt as to whether this band reflected a COOH group that could have reached proton-exchange equilibrium with external buffer. The dissociation rate constant of a general acid in aqueous solution is $k_{deprot} \simeq 10^{10-pK_a}$ (s^{-1}) (Eigen, 1964; Kalisky et al., 1981). Thus, for example, if Asp-85 had a pK_a of 8 and an environment with lots of water around it, it would be expected at neutral pH to have a dissociation rate constant a bit faster than the 50 s^{-1} decay rate of the Asp-COOH group, and an association rate constant only 10-fold larger. However, these rate estimates are valid only for aqueous environments; in a non-hydrogen-bonded environment inside a protein, one would expect these rate constants to be up to several orders of magnitude slower. It is therefore uncertain whether proton-transfer equilibrium could be attained within the 10- to 20-ms lifetime of M at neutral pH.

The long lifetime of the deprotonated Schiff base at high pH (as measured in visible spectra), as well as several time-resolved IR measurements at elevated pH values (Bousché et al., 1991; Pfefferlé et al., 1991) have indicated that the Asp-85 COOH band probably decays much more slowly at high pH. However, until now, no kinetic constants of the Asp-85 COOH band at elevated pH have actually been published, and no one has concluded that it would be appropriate to extrapolate kinetics measured in the visible spectrum to the decay of the Asp-85 COOH group. In fact, the quarter-second lifetime that we measure for the 1762 cm^{-1} band in Fig. 1 seems longer than should be required for simple proton transfers from Asp-85 to bulk water, at least for a group with a pK_a of 8 or 9, if we use the formula for k_{deprot} given above. This result alone indicates that Asp-85 probably has a definable pK_a of at least 8.

The question remains, whether it would be expected theoretically for an Asp-85 with an even higher pK_a (e.g., 10 or 11) to reach equilibrium with an external buffer during this 250-ms lifetime. Closed lipid vesicles containing bR require up to ~1 min to reach pH equilibrium across the hydrophobic membrane (Lind et al., 1981). However, this slow time reflects the fact that bR molecules make up only a tiny fraction of the vesicle surface area, and the predominating lipids are, comparatively speaking, nearly impermeable to protons. Any individual bR molecule in an open sheet would be expected to equilibrate much more quickly with the surrounding medium. In fact, Asp-85 lies even closer to the membrane surface than the Schiff base (Henderson et al., 1990), and H/D exchange measurements indicate that in the unphotolyzed state, protons can diffuse from the bulk to a water molecule situated between the Schiff base and Asp-85 in ~2 ms (Deng et al., 1994). In the M state, there is probably no such water molecule contacting Asp-85 (see below), but it would still be surprising if pH equilibration with the bulk were as slow as 250 ms.

However, none of the preceding arguments is a direct experimental proof that Asp-85 can reach proton equilibrium with the bulk. To demonstrate this experimentally, it is important to detect proton exchange between the COOH group and bulk buffer. We are not yet able to perform sufficiently fast and sensitive buffer-exchange FTIR difference spectra to make such a measurement within the lifetime of the M or N intermediate of wild-type bR; however, it is possible with the long-lived M_N photoproduct of the D96N mutant of bR. Within a 2-min mixing and measurement time following exposure to a bulk medium at pD = 10.5, the Asp-85 COOH group of M_N undergoes complete deuterium exchange (see Fig. 2). Therefore, Asp-85 achieves a proton-transfer equilibrium with bulk buffer at pH 10.5.

In water, carboxylic acids achieve fast proton-transfer equilibrium via a mechanism involving transient deprotonation (Eigen, 1964). However, it has been shown that in nonaqueous media, concerted exchange mechanisms involving solely neutral species may be faster than the rate of carboxylic acid deprotonation (Gerritzen and Limbach 1984). Indeed, in the very nonpolar environment that we propose for Asp-85 in the M and N intermediates (see below), it would not be surprising if a concerted exchange mechanism dominated, i.e., the half-time of Asp-85 deprotonation were longer than our measured <2-min H/D exchange time.

Still, the rate of approach to any equilibrium is determined by the *sum* of the rate constants for the forward and reverse reactions. For this simple reason, one cannot conclude that, just because the deprotonation rate of Asp-85 is probably slower than the measured H/D exchange rate, so must be its approach to pH equilibrium with the external medium. In particular, an acid dissociation constant and a corresponding free-energy difference between protonated and unprotonated forms of Asp-85 in the M_N state can reasonably be asserted to exist, and their values experimentally constrained, if *either* deprotonation or protonation of this residue from the bulk is significantly faster than the decay of the photointermediate state itself.

In fact, the latter reverse-reaction rate, corresponding to the protonation of ionized Asp-85 from the bulk, is not likely to be slower than our measured rate of H/D exchange, even if the observed exchange is principally due to a concerted mechanism. This is because, in contrast to the *deprotonation* of a carboxylic acid in a nonpolar environment, carboxylate *protonation* involves charge recombination rather than separation inside the relatively low-dielectric medium of the protein. On simple physical grounds, carboxylate protonation would therefore be expected to occur at least as fast as a charge-neutral concerted exchange involving the same particles (protons and deuterons) moving across the same local energy barriers (the double-well potentials of transiently-formed H-bonds). There is in fact no experimental or theoretical precedent for the protonation

rate of a carboxylate to be slower than the rate of COOH/COOD exchange of the corresponding acid with bulk solvent, even though there is precedent for the *deprotonation* rate to be slower (e.g., Gerritzen and Limbach, 1984).

We can apply the preceding logic and our measured H/D exchange rate for Asp-85, to reach a firm lower bound on its pK_a as follows: 1) For the M_N state, the measured COOH/COOD exchange rate with pH = 10.5 buffer puts an experimental lower bound on the rate of protonation, from the bulk, of the small amount of ionized Asp-85 that must transiently be made during the lifetime of M_N. As discussed above, this lower bound is quite firm, even if it is assumed that, because of an incredibly slow Asp-85 deprotonation rate (e.g., 1 day^{-1}), only a few thousandths of the bR molecules in the sample actually deprotonate at Asp-85 during the 5-min lifetime of M_N, and a concerted mechanism can therefore dominate the overall H/D exchange process. 2) This lower bound on the protonation rate of Asp-85 from the bulk ($k_{prot}[H^+] \geq 1\ min^{-1}$) is several-fold faster than the measured decay rate of the COOH group at pH 10.5. 3) Therefore, the rate of deprotonation of Asp-85 to the bulk (k_{deprot}) must be slower than $k_{prot}[H^+]$ at pH = 10.5. (Otherwise, k_{deprot} would also have to be faster than the COOH group's decay rate, in which case there would be time for this group to reach equilibrium with the bulk within the lifetime of M_N, and the COOH band would be observed to decay faster than M_N itself.) 4) Because it is always true that $pK_a = pH - \log (k_{deprot}/k_{prot}[H^+])$ (Eigen, 1964), and we have concluded that the fraction in the second term on the right is less than 1 when pH = 10.5, we know that $pK_a > 10.5$.

Thus, in the D96N mutant at least, we have shown that Asp-85 almost certainly has a definable pK_a that is transiently raised to >10.5 by a photolysis-induced conformational change. There is no good reason to believe that this should be a phenomenon specific to this particular mutant. The D96N mutation is located in the intracellular proton channel and therefore should not affect accessibility of protons to Asp-85 through the extracellular channel. That is, the Asp-85 COOH group in wild-type M is still probably capable of exchanging protons with the external medium through this channel. Furthermore, the D96N mutation produces only a small shift in the Asp-85 COOH vibrational frequency relative to wild type (Fig. 1), indicating the protein structure around Asp-85 is very similar in the wild type and D96N mutant. We conclude that the pK_a of Asp-85 in the wild-type M is probably also well above 10.5.

Previous workers have calculated that the pK of Asp-85 could be raised by as much as 6- to 7-pH units as a consequence of deprotonation of the Schiff base group, which eliminates a positive charge stabilizing the aspartate anion (Sampogna and Honig, 1994). However, this explanation is insufficient to rationalize the continued high pK_a of Asp-85 in N, which differs from M by the transfer of a proton from Asp-96 to the Schiff base group (Henderson et al., 1990; Mathies et al., 1991; Ebrey, 1993; Lanyi, 1993; Rothschild, 1992; Krebs and Khorana, 1993). The stability of the protonated Schiff base group in N formed at pH 10.5 suggests that this group's pK_a might have returned almost to its prephotolysis value of 13.5 (Brown et al., 1993; Govindjee et al., 1994), even while the Asp-85 pK_a remains perturbed by >8 units above its initial value. (Note, however, that in the D96N mutant at high pH, the long-lived M_N photointermediate decays without producing significant quantities of a species having Asp-85 and the Schiff base simultaneously protonated. We have also not yet been able to measure external deuterium exchange with the protonated Schiff base within the lifetime of wild-type N. It is therefore still conceivable that, despite its >3-s lifetime, the Schiff base in the wild-type N is not in proton-exchange equilibrium with the external medium at pH = 10.5.) Furthermore, N-like intermediates, having normal IR difference spectra including a 1755 cm^{-1} band indicative of protonated Asp-85, have been observed in the photocycles of several bR mutants lacking an M state (e.g., D212N (Cao et al., 1993)). Thus the 8-unit transient rise in the pK_a of Asp-85 during the photocycle is probably not as dependent on Schiff base deprotonation as previously indicated (Sampogna and Honig, 1994). In fact, Asp-85 protonation can more likely be counted as a cause, rather than purely an effect, of Schiff base deprotonation in the M state.

The observation of an M intermediate with deprotonated Schiff base at external pH values below 7 (e.g., Fig. 1) strongly suggests that the pK_a of the Schiff base drops this low during the photocycle, as proposed previously (Kalisky et al., 1981; Sampogna and Honig, 1994). This conclusion, when combined with our estimate of a pK_a of >11 for Asp-85, indicates that ΔpK_a between the Schiff base and Asp-85 drops from ~10 in the bR state to below −4 in M.

This would appear to contradict the previous estimate that the ΔpK_a between the Schiff base and Asp-85 is nearly zero in M (Lanyi, 1993; Brown et al., 1994). However, the previous estimate applied to the first of two detected M intermediates (termed M_1), whereas our measurements can only be considered to apply to the later M_2 state, which is though to have undergone an irreversible conformational change relative to M_1 and L (Váró and Lanyi, 1991; Brown et al., 1994). The lifetimes of M_1 and earlier photointermediate states are almost certainly too short to define true pK_a values for the membrane-buried groups in them, so it is reasonable to rely on estimates of ΔpK_a from kinetic measurements. Nevertheless, as we demonstrate here, estimates of ΔpK_a based on individual groups' pK_a values referenced to external pH are possible for longer-lived photoproducts. In fact, the discrepancy between our ΔpK_a value for M_2 and the earlier "kinetic" ΔpK_a measurement for M_1 can be easily rationalized. A drop in ΔpK_a from ~0 in M_1 (and L) to −4 in M_2 would help to explain the known irreversibility of the transition between these states, which is accompanied by a free-energy drop of at least 13 kJ/mol (Váró and Lanyi, 1991; Lanyi, 1993).

In any case, our straightforward observation of a long-lived Asp-85 COOH group with a carbonyl frequency of 1758 cm^{-1} at pH 10.5 in the D96N mutant contradicts

previously published estimates of a $pK_a \simeq 2.5$ for Asp-85 in M (Rothschild et al., 1981; Braiman et al., 1988). As mentioned above, these estimates came from FTIR work that associated a very high COOH frequency of this group with an unusually low pK_a. However, the cited correlation between pK_a and $\nu_{C=O}$ (Bellamy, 1968) applies only to a particular set of α-substituted carboxylic acids whose $\nu_{C=O}$ values, all measured in CCl_4, depend on their "intrinsic" pK_a values, all measured in water. The pK_a differences were not obtained by changing the solvent environment, but by using various α-substituents (e.g., halogens or conjugated π-electron systems) known to be capable of through-bond withdrawal or donation of electrons to the carboxylate group. It is the resulting π-electron density variation that produces correlated changes in pK_a and $\nu_{C=O}$. As has been pointed out previously (Sasaki et al., 1994), this correlation cannot be extrapolated to estimate the pK_a of aspartic acid side chains in various protected environments within a protein. In this case, there are no through-bond effects, only external (noncovalent) perturbations.

In fact, the high $\nu_{C=O}$ frequency of Asp-85 in the M state is entirely consistent with our high estimated pK_a for this group. This can be seen by relating changes in $\nu_{C=O}$ during the photocycle to changes in the polarity and hydrogen bonding capability of the carboxylate environment. It is the latter that tend to determine the pK_a variability when the carboxylate's covalent structure is held constant. Whereas these are not the only solvent interactions that can play a role in shifting $\nu_{C=O}$ of carboxylic acids, they are dominant ones, as shown by the extensive studies of solvent dependence of this vibration for monomeric γ-phenylbutyric acid (Collings and Morgan, 1963) and for acetic and propionic acids (Nyquist et al., 1994; Dioumaev and Braiman, 1995). The most important conclusion from such data is that $\nu_{C=O}$ for the monomer is lowered upon adding carbon-atom substituents to the α-carbon, and also upon increasing either the polarity of the solvent or its ability to form hydrogen bonds.

For carboxylic acids (other than acetic acid), $\nu_{C=O}$ values above $\sim 1750\ cm^{-1}$ are observed only in the monomer state, and then only in solvents (hexane, CCl_4, CS_2) that lack an ability to form hydrogen bonds. (As shown by Haurie and Novak (1967), the $\sim 1754\ cm^{-1}$ band seen for acetic acid in dioxane and other hydrogen-bond-accepting solvents is not a true C=O vibration, but rather a perturbed or mixed mode involving a Fermi resonance with an overtone of the C-C stretch. For all other α-unsubstituted aliphatic carboxylic acids in such solvents, no Fermi resonance occurs, and the C=O stretch is observed at a frequency near or below 1744 cm^{-1} (Dioumaev and Braiman, 1995).) Furthermore, in such hydrophobic solvents there is a strong correlation between $\nu_{C=O}$ and solvent dielectric constant (Dioumaev and Braiman, 1995). When compared to these model R_2HC-CH_2-COOH molecules in various solvents, the 1762 cm^{-1} value for the Asp-85 COOH group of M indicates that it exists in a very non-polar environment, with a dielectric constant between that of hexane ($\epsilon = 1.89$) and CCl_4 ($\epsilon = 2.24$). The drop of the Asp-85 COOH frequency to 1756 cm^{-1} in the N state would be consistent with only a small increase in the dielectric constant to perhaps that of CS_2 ($\epsilon = 2.64$). All 3 of these model solvent environments are significantly less polar than the typical environment around charged residues buried in the interior of globular proteins, for which a dielectric constant of $\epsilon = 4$ has been suggested (Sharp and Honig, 1990).

The proposed low-dielectric environment around Asp-85 in M and N is probably quite different from that in the unphotolyzed (bR) state. Electron diffraction measurements (Henderson et al., 1990) indicate that in bR, this environment probably includes the protonated Schiff base, ionized Asp-212 and Arg-82, and one or more water molecules (Bashford and Gerwert, 1992; Humphrey et al., 1994). The very high $\nu_{C=O}$ values for the COOH group in M and N probably indicate the disappearance of all of these charged and polar groups from the immediate surroundings of Asp-85. This would produce a "dielectric destabilization" of the aspartate-85 anion, relative to the uncharged aspartic acid, that would cause this group to become very strongly basic (see below for further details). Thus the high $\nu_{C=O}$ values for the Asp-85 COOH group in M and N should be taken as consistent with an unusually high, rather than low, pK_a.

The two results described above—an 8-unit jump in the pK_a and a 30-cm^{-1} increase in $\nu_{C=O}$ for Asp-85 as a consequence of photolysis—reinforce a single conclusion. They are both consistent with a photolysis-induced "dielectric destabilization" of the chromophore counterion. This conclusion helps to explain the ion transport mechanism not only of bR, but of halorhodopsin (hR) as well.

The magnitude of the anion destabilization ΔG_{anion} can be approximated as the change in the free energy of protonation $\Delta(\Delta G_{prot})$, if we make the commonly accepted assumption that when the solvent dielectric constant is altered, the ensuing free energy change for the ionized species is much larger than that for the neutral species (Sharp and Honig, 1990). In such a case, $\Delta G_{anion} \simeq \Delta\ (\Delta G_{prot}) = 2.303RT\ \Delta pK_a$. The anion destabilization energy ΔG_{anion} must therefore be at least $\sim 40\ kJ\text{-}mol^{-1}$, because our data show that ΔpK_a is at least 8.

A similar value of $\sim 45\ kJ\text{-}mol^{-1}$ for the anion destabilization free energy is obtained if we assume that it arises solely from a change in the Born self-energy of the anion, given by the formula $\Delta E_{anion} \approx e^2/2r_0(1/\epsilon_2 - 1/\epsilon_1)$ (Sharp and Honig, 1990). Here we have estimated that the effective radius r_0 of the carboxylate anion is 4 Å, and that the effective dielectric constant around it decreases from $\epsilon_1 = 4$ to $\epsilon_2 = 2$ during the bR→M photoreaction. It should be noted that this calculation is considerably more sensitive to an erroneous estimate of ϵ_2 than ϵ_1. This independent estimate for ΔG_{anion} is therefore not marred by our inability to correlate a precise value for ϵ_1 from $\nu_{C=O}$ in the unphotolyzed state, because the very high measured $\nu_{C=O}$ in the M state almost certainly means that ϵ_2 is close to 2.

Our experimentally-derived lower bound of 10.5 for the pK_a of Asp-85 in the M and N states of bR makes it one of a select group of Asp residues recently identified as having

pK_a values above 9. For example, it was recently concluded on the basis of FTIR spectral titration that Asp-96 and Asp-115 of unphotolyzed bR both have pK_a values above 11 (Száraz et al., 1994). Furthermore, Asp-26 of reduced thioredoxin from *Escherichia coli* has a $pK_a > 9$ (Wilson et al., 1995). Finally, in model peptides designed with each Asp surrounded by 5 nearby Phe residues, the aspartic acid pK_a is 10.0 (Urry et al., 1994). All of these high-pK_a Asp residues are thought to be situated in very nonpolar environments, such as we have proposed for Asp-85 in the M and N states of bR. Arguments presented above indicate that there should be a correlation between the various pK_a values of these buried Asp residues and their respective $\nu_{C=O}$ vibrational frequencies. However, most of the cited pK_a values actually represent lower bounds, and IR frequencies have not yet been measured for several of the Asp-COOH groups. Therefore the possibility of such a correlation is not yet tested.

In hR, it is thought that externally-provided Cl^- takes the place of the Asp-85 counterion (which is substituted by a threonine at the homologous position in the primary sequence). The environment around this anion is likely to be very similar in bR and hR, given that of the 8 residues nearest to the Asp-85 carboxyl group in the three-dimensional structure of bR (Henderson et al., 1990) Ile-52, Thr-89, and Phe-208 are conservatively replaced by Val, Ser, and Tyr, respectively; and all the others (Arg-82, Trp-86, Tyr-185, Asp-212, and Lys-216 with the chromophore Schiff base) are identical in both proteins. We propose therefore that chromophore photoisomerization in hR induces a protein conformational change similar to that in bR, and leads to a similar dielectric destabilization of the chromophore counterion. The smaller r_0 of the halide, relative to carboxylate, should produce an even larger value of ΔG_{anion}. However, the initial pK_a of Cl^- (approximately -6 under standard aqueous conditions) is apparently so much lower than that of asparate, that proton transfer to it remains energetically unfavorable even after the dielectric destabilization. The Schiff base therefore never deprotonates. Instead, the Cl^- ion is forced to move across the membrane dielectric barrier to a more stable location on the cytoplasmic side. Its release to the external medium must simultaneously be blocked by the transient formation of an even more effective dielectric and/or steric barrier in that direction. The fact that in bR there appears to be no such barrier to protons between Asp-85 and the external medium could reflect their much smaller size and greater mobility, relative to chloride ions.

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