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Differences between the photocycles of halorhodopsin and the acid purple form of bacteriorhodopsin analyzed with millisecond time-resolved FTIR spectroscopy

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Abstract

At pH 1, bacteriorhodopsin (bR) is thought to function as a halide ion pump, in contrast to its biological function as a proton pump at neutral pH. Despite the apparent similarity in function between this 'acid purple' form of bR and the native form of halorhodopsin (hR), their FTIR difference spectra measured ca. 5 ms after photolysis are significantly different. The most striking difference is the appearance of a positive band at 1753 cm^{-1} and a negative band at 1732 cm^{-1} in the $bR_{acid\,purple}$ difference spectrum. These and other spectral features are similar, but not identical, to those of the $bR \rightarrow O$ difference spectrum measured at neutral pH. The structure of the $bR_{acid\ purple}$ longest-lived product therefore corresponds more closely to the O photoproduct of the bR proton-pumping photocycle, rather than the hL photoproduct seen on a similar time scale in the hR photocycle. The 1753- and 1732-cm^{-1} bands are largely unaffected by the D212N mutation, but both appear to lose a portion of their intensities with either the D85N or D96N mutation. Thus Asp-85 and -96 likely undergo substantial changes in hydrogen-bonding environment during the halide-pumping cycle of $bR_{acid\ purple}$. Our FTIR results deepen the distinctions between the hR and bR photocycles. The mechanism of chloride pumping in hR has been thought not to involve protonation or hydrogen bonding changes of carboxylic acid groups. In $bR_{acid\ purple}$, however, it seems likely that at least one carboxylic acid might play an important role in the mechanism of chloride pumping, leading to an increase in thermodynamic or kinetic stabilization of the O intermediate.

Keywords: Chloride transport; Aspartic acid; Proton transfer

1. Introduction

Under physiological conditions and in the presence of light, bacteriorhodopsin (bR_{568}) and halorhodopsin (hR_{578}) pump protons outward and halide ions inward, respectively, across the halobacterial cell membrane. Both these processes begin with photoisomerization of a retinylidene–lysine protonated Schiff base chromophore from all-*trans* to 13-*cis*, and both are mediated through a photocycle that requires ca. 10 ms for completion.

It is well known that upon acidification, the absorption maximum of bR_{568} is shifted to the red, indicating the formation of a stable blue-colored species, bR^{acid}_{605} [13,12]; the pK for this transition is 2.9. Upon illumination, bR^{acid}_{605} does not exhibit charge transport, indicating that it is incapable of pumping either protons or anions [8]. With the addition of Cl^- (or Br^-), however, the absorption maximum of bR^{acid}_{605} is blue-shifted to 564 nm (or, respectively, 568 nm), corresponding to the formation of a species known

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as $bR_{acid\ purple}$. This form of bR appears to function as an 'inward' halide pump, transporting Cl^- or Br^- anions in the direction opposite to that of proton transport by bR_{568}, and in the same direction as halide transport by hR [9,11].

It has been proposed that the mechanism of halide transport by $bR_{acid\ purple}$ is similar to that of hR. In particular, UV-visible flash spectroscopy measurements demonstrate that the chloride-pumping photocycle of $bR_{acid\ purple}$, like that of hR, lacks an M-like (ca. 410 nm absorbing) intermediate [9,11]. Other results, however, indicate that $bR_{acid\ purple}$ has only red-shifted photoproducts; i.e., no analogue of hL_{550} is formed [19].

To examine further the possible mechanisms of chloride transport by $bR_{acid\ purple}$, we have analyzed its Fourier-transform infrared (FTIR) difference spectrum with 0.1–45 ms time resolution at 4° C. In agreement with earlier transient visible spectroscopy [19], the transient FTIR difference spectra are generally consistent with the presence of a single photoproduct on the ms time scale. The structure of this photoproduct corresponds closely (but not exactly) to the O photoproduct of the bR proton-pumping photocycle, rather than to the hL photoproduct seen on a similar time scale in the hR chloride-pumping photocycle. Notably, in the light-induced difference spectrum of $bR_{acid\ purple}$, there are strong IR difference bands between 1730 and 1760 cm^{-1}, attributable to COOH groups of the protein undergoing structural changes during the photocycle. These bands indicate that one or two aspartic or glutamic acid residues, probably Asp-85 and Asp-96, are strongly perturbed when $bR_{acid\ purple}$ pumps anions. The results indicate that although these residues remain protonated throughout the halide-pumping photocycle of $bR_{acid\ purple}$, the environment around both of them becomes transiently less hydrogen-bonding on the ms time scale.

2. Materials and methods

2.1. Sample preparation

Purple membrane was prepared from *Halobacterium salinarium* using standard bacterial growth media and isolation procedures [3]. Strains expressing D85N, D96N, and D212N mutant forms of the protein were gifts of Janos Lanyi and Richard Needleman. Identical procedures were also used to prepare hR from a bR^-, hR-overproducing strain of *H. salinarium* provided by Richard Needleman. All of these *H. salinarium* mutants produce substantial amounts of bR or hR in the form of high-density membrane fragments that could be isolated using the same procedures as for wild-type bR.

To prepare protein samples for FTIR spectroscopy, a 1–3 mg quantity of purple membrane fragments was washed several times in a buffer containing either 1.0 M KCl with concentrated HCl added to a pH of 1.0, or 1.0 M KCl and 10 mM HEPES pH = 7.0. A portion of the pellet obtained from a final centrifugation ($5000 \times g$) was squeezed between two CaF_2 windows as described previously [5]. Visible absorption spectra taken of wild-type and D85N, D96N, and D212N mutant samples, taken immediately before FTIR spectroscopy, all showed an optical density of 0.3–0.6 at an absorption maximum of 568 ± 3 nm.

2.2. Infrared spectroscopy

Time-resolved FTIR difference spectra were obtained using a stroboscopic method on a Nicolet 60SXR spectrometer [5,6]. Interferograms were compiled from data digitized over a brief time range after each flash. In general, between 512 and 8192 data points were digitized during each time slice, corresponding to slices of between 5 and 90 ms in duration, as indicated in the figure legends. The longest values permitted the collection of a complete interferogram, with a spectral resolution of 1 cm^{-1}, during each individual mirror scan. This also required reducing the bandwidth of the spectrum to ca. 4000 cm^{-1} by using a long-pass interference filter. The start of digitization was delayed until at least 0.1 ms after photolysis, to avoid contributions from early photoproducts. The sample holder was thermostated at 4° C.

3. Results

3.1. Millisecond FTIR difference spectrum of the $bR_{acid\ purple} \rightarrow O_{acid}$ transition

The photoproduct difference spectrum of $bR_{acid\ purple}$, obtained at 4° C using data collected between

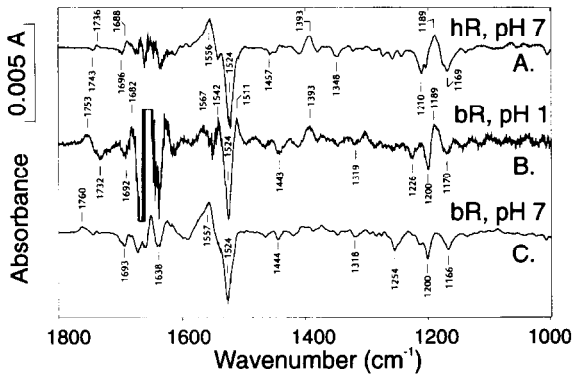

Fig. 1. FTIR difference spectra taken at 4° C with a spectral resolution of 1 cm^{-1}. (A) hR at pH 7.0, interferograms compiled from points taken 1–91 ms after photolysis. Data from $5 \cdot 10^5$ flashes were averaged together. (B) bR at pH 1.0, 0.1–5 ms after photolysis; $2 \cdot 10^5$ flashes. (C) bR at pH 7.0, 1–91 ms after photolysis; $5 \cdot 10^5$ flashes.

0.1 and 5 ms after photolysis, is shown in Fig. 1B. Comparisons of this spectrum to others taken at subsequent 5-ms spacings (not shown) indicate no changes, other than a uniform decrease in magnitude of the difference peaks that is consistent with the decay of a single species on this time scale. This species, which we will call O_{acid}, corresponds to the O-like species observed as the unique long-lived photoproduct of $bR_{acid\ purple}$ observed previously with time-resolved visible spectroscopy [19].

3.2. Comparisons to millisecond difference spectra of physiological photoproducts of bR and hR

The $bR_{acid\ purple} \rightarrow O_{acid}$ difference spectrum is compared to photoproduct difference spectra of hR and bR taken on similar time scales in Figs. 1 and 2. The large negative band at 1732 cm^{-1} and positive band at 1753 cm^{-1} are the most striking differences between the $bR_{acid\ purple}$ spectrum and the neutral pH difference spectra of the other photoreactions. In the 1800–1700 cm^{-1} spectral region, the hR → hL difference spectrum (Fig. 2B) shows only a weak differential band with a positive peak at 1737 cm^{-1} and a negative peak at 1741 cm^{-1}. This difference band has been attributed to the analog of bR residue Asp-115 undergoing a small perturbation when hL is formed [15]. The bR → M difference spectrum (Fig. 2D) shows a similar small difference band, as well as a larger positive peak at 1760 that is due to the proton transfer to Asp-85 from the Schiff base [4].

In order to see if the unusual COOH-region bands in the $bR_{acid\ purple} \rightarrow O_{acid}$ spectrum were simply a pH-induced perturbation of normal difference bands in this spectral region, we examined the time-resolved FTIR difference spectrum of hR under identical acidic conditions. The bands at 1753 and 1732 cm^{-1} were clearly not present (Fig. 2A). Furthermore, the entire spectrum (not shown) was perturbed from a normal hR → hL difference spectrum (Fig. 1A). The signal/noise ratio in the remaining portion of the spectrum was poor, because the sample bleached irreversibly after a relatively small number of flashes. Therefore it is not possible to state with great certainty whether bands indicative of an O-like photoproduct were present.

The 1732 and 1753 cm^{-1} bands are strong evidence that the $bR_{acid\ purple}$ principal photoproduct is not exactly analogous to L- or M-like photoproducts. In fact, this bandshape differs significantly from those observed for this spectral region in difference spectra of bR and its K, L, M, or N photoproducts [4,6], or in hR → hL difference spectra [7]. Other unique features of the $bR_{acid\ purple} \rightarrow O_{acid}$ difference spectrum include the positive bands at 1567, 1542, and 1511 cm^{-1}, and the negative band at 1226 cm^{-1}. One or more of the former trio are likely to be due to C=C stretching vibrations of the photoproduct's chromophore. The assignment of the 1226-cm^{-1} vibration is unclear, but is probably due to a protein group since it does not correspond to any

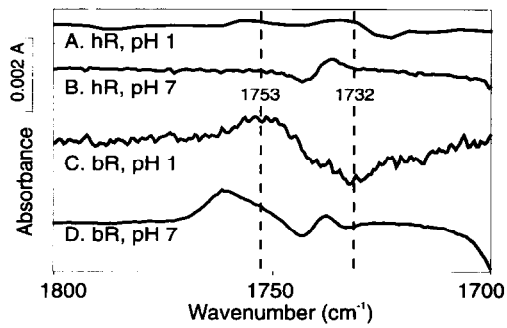

Fig. 2. Expansion in the COOH carbonyl stretching region of FTIR difference spectra taken at 4° C. (A) hR at pH 1.0, measured 0.2–5 ms after photolysis with a spectral resolution of 4 cm^{-1}; $8 \cdot 10^4$ flashes. (B)–(D) as in Fig. 1.

known vibration of an all-*trans* retinal protonated Schiff base. These spectral features, unique to the $bR_{acid\ purple} \rightarrow O_{acid}$ difference spectrum, show that the structures of both chromophore and protein in the photoproduct differ significantly from those present in the hL, L, M, and N intermediates of the hR and bR photocycles.

However, the similarity of the negative features in Figs. 1B and 1C, especially those in the C=C (1500–1600 cm^{-1}) and C–C fingerprint (1100--1200 cm^{-1}) regions, suggests rather similar starting structures for the chromophore in bR and $bR_{acid\ purple}$. This is not surprising, considering the similarity of visible absorption and resonance Raman spectra of these species [18].

3.3. Similarity of $bR_{acid\ purple}$ photoproduct and O

It is also not surprising that the similarity of visible absorption properties between the $bR_{acid\ purple}$ photoproduct and the O photointermediate of the physiological photocycle [19] is reflected in a clear similarity of their IR absorption spectra. Nearly all of the labeled peaks in the $bR_{acid\ purple} \rightarrow O_{acid}$ difference spectrum (Fig. 1B) are almost identical to features observed in previously published spectra of the bR → O transition of the proton-pumping photocycle measured at physiological pH [10]. In particular, the bR → O spectrum showed positive (+) and negative (−) difference peaks at 1755 (+), 1737 (−), 1560 (+), 1524 (−), 1507 (+), 1395 (+), 1199 (−), and 1187 (+) that are similar in frequency and intensity to bands seen in the $bR_{acid\ purple} \rightarrow O_{acid}$ spectrum of Fig. 1B. Similar peaks were also observed in the bR → O difference spectrum of the Y185F mutant of bR [2].

At the same time, there are significant differences between the previously-published bR → O spectra and the $bR_{acid\ purple} \rightarrow O_{acid}$ spectrum (Fig. 1B). For example, the negative peak at 1737 cm^{-1} and the positive band at 1560 cm^{-1} in the bR → O spectrum [10] are significantly different in frequency from analogous peaks in the $bR_{acid\ purple} \rightarrow O_{acid}$ spectrum (Fig. 1B). Additionally, there is no analogue in the $bR_{acid\ purple} \rightarrow O_{acid}$ spectrum to the substantial positive peak seen at ca. 1170 cm^{-1} in the bR → O difference spectrum of wild-type [10] and Y185F mutant [2] samples; instead, there is a clear negative band at 1170 cm^{-1}. Likewise, there is no clear analogue in the O spectrum of the positive peak at 1542 cm^{-1} of the O_{acid} spectrum (Fig. 1B). These differences in strong IR bands in the C=C and C–C stretching regions could indicate that the chromophores have different configurations in the two species.

Finally, there is a positive band at 1682 cm^{-1} in the $bR_{acid\ purple} \rightarrow O_{acid}$ difference spectrum (Fig. 1B) that was not observed in the bR → O difference spectrum of wild-type bR [10]. Interestingly, however, such a band was seen in the bR → O difference spectrum obtained from the Y185F mutant [2]. This band is in the appropriate frequency region for an arginine $C{=}NH^{+}$ vibration, and is reminiscent of a similar band observed at 1688 cm^{-1} in the hR → hL difference spectrum (Fig. 1A). The latter hL band, along with an hR (negative) band at 1696 cm^{-1}, was previously attributed to a transient perturbation of an interaction between the halide ion and Arg-108 of hR [7]. The appearance of an analogous difference band in the $bR_{acid\ purple} \rightarrow O_{acid}$ difference spectrum, but not in the bR → O difference spectrum of wild-type bR, is a further indication of this band's possible importance in the mechanism of halide binding and/or pumping.

3.4. Analysis of bR mutants

The sizes and frequencies of the $bR_{acid\ purple}$ difference bands at 1753 and 1732 cm^{-1} are reminiscent of bands in FTIR difference spectra of photointermediates in the normal proton-pumping photocycle of bR that have been assigned to Asp-85, -96, and -212. These are three of the four aspartic acid residues located within the transmembrane region of the native protein [4]. It seemed likely that one or more of these residues is responsible for the observed $bR_{acid\ purple} \rightarrow O_{acid}$ difference bands, undergoing proton transfer reaction or a change in hydrogen bonding. More specifically, it has generally been accepted that the ca. 1754 cm^{-1} positive bands in the bR → N and bR → O difference spectra are due to the same residue (Asp-85) that gives rise to the 1761 cm^{-1} band of the bR → M difference spectrum [6,10]. This has been taken as an indication that Asp-85, which picks up a proton during the bR → M photoreaction, remains protonated in the N and O states. The pres-

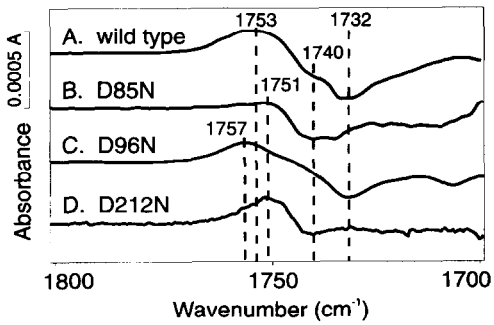

Fig. 3. FTIR difference spectra of wild-type and mutant forms of bR at pH 1.0, as labeled. Spectra were taken as in Fig. 1. (A) $5 \cdot 10^5$ flashes. (B) $2.5 \cdot 10^4$ flashes. (C) $2.7 \cdot 10^4$ flashes. (D) $1.7 \cdot 10^4$ flashes.

ence of a 1753 cm^{-1} band in the $bR_{acid\ purple} \rightarrow O_{acid}$ difference spectrum thus suggested that Asp-85 might be undergoing a structural or environmental change during this photocycle. To test this hypothesis, we studied mutated forms of bR in which single aspartic acid residues were replaced with asparagines (D85N, D96N and D212N).

Somewhat contrary to expectation, at least a portion of both the large positive and negative difference bands in the COOH carbonyl stretching region (1760–1700 cm^{-1}) remains in the time-resolved FTIR difference spectrum of each of the three bR mutants examined under low pH, high Cl^- conditions (Fig. 3). However, there are differences between the mutant spectra that indicate individual components of these bands can be assigned to Asp-85 and Asp-96 vibrations.

For example, the higher-frequency portion of the positive band near 1753 cm^{-1} disappears with the D85N mutation, shifting the peak down to 1751 cm^{-1} (Fig. 3B). Likewise, the low-frequency portion of the negative band at 1732 cm^{-1} is lost, shifting the negative peak up to 1740 cm^{-1}. These changes result in the overall loss of intensity in these difference bands, relative to those between 1400 and 1100 cm^{-1} (Fig. 4). A portion of the difference signal in the 1760–1730 cm^{-1} spectral region is therefore likely due to the Asp-85 COOH carbonyl vibration undergoing a perturbation during the $bR_{acid\ purple} \rightarrow O_{acid}$ transition.

Similarly, with the D96N mutation (Fig. 3C) the 1753 cm^{-1} maximum shifts up to 1757 cm^{-1}; the change in shape of the positive band is consistent with a loss of intensity at 1751 cm^{-1}. At the same time, the change in the shape of the negative band is consistent with a loss of negative intensity near 1740 cm^{-1}. The middle portion of the difference signal in the 1760–1730 cm^{-1} spectral region of the wild type (Fig. 3A) is therefore likely due to Asp-96.

The spectral changes in the COOH region seen with the D212N mutation (Fig. 3D) are somewhat similar to those seen for the D85N mutant. Specifically, the high-frequency portion of the positive band near 1753 cm^{-1} is somewhat reduced, as is the negative band near 1732 cm^{-1}. However, this mutant is more perturbed in other spectral regions than is the D85N mutant (e.g. loss of band at 1511 cm^{-1} in Fig. 4), indicating that these spectral changes are more likely due to secondary effects. It would not be surprising, however, if difference bands due to Asp-85 and -212 were to overlap somewhat in the wild-type $bR_{acid\ purple} \rightarrow O_{acid}$ difference spectrum. Based on earlier FTIR difference spectra of acid and chloride titrations of bR [14], both of these residues are

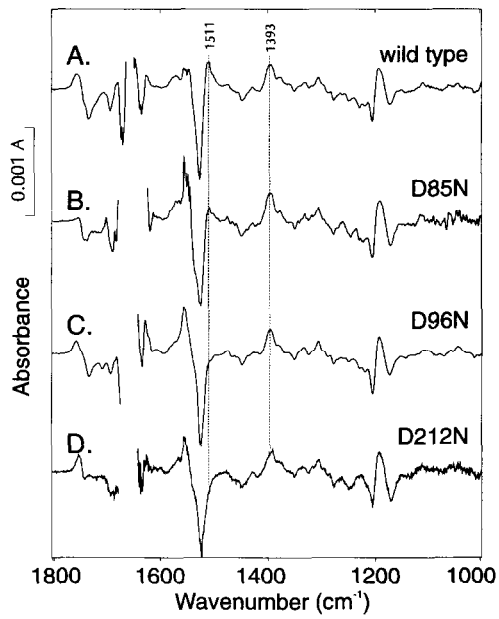

Fig. 4. Full spectral region (1800–1000 cm^{-1}) of the pH 1.0 TR-FTIR difference spectra of wild-type, D85N, and D96N forms of bR shown in Fig. 3.

thought to have frequencies of ca. 1731 cm^{-1} in the unphotolyzed $bR_{acid\ purple}$ state.

The overall results with the mutants suggest the following assignments for the COOH spectral region in the $bR_{acid\ purple} \rightarrow O_{acid}$ difference spectrum. First, Asp-85 contributes a negative band at ca. 1732 cm^{-1} and a positive band at ca. 1757 cm^{-1}. Second, Asp-96 contributes a negative band at ca. 1740 cm^{-1} and a positive band at ca. 1751 cm^{-1}. Asp-212 vibrations probably also contribute to the appearance of this region, but cannot be assigned with as much confidence as those due to the other two aspartic acids.

4. Discussion

Our FTIR spectral results confirm the earlier conclusion [19] that the predominant photoproduct of $bR_{acid\ purple}$ on the ms time scale resembles the O intermediate of the normal proton-pumping photocycle rather than hL, the L-like (or possibly N-like) intermediate that predominates on a similar time scale in the hR photocycle. The hL photointermediate is thought to be present in a chloride-dependent equilibrium with an O-like photoproduct; however, under the high chloride conditions used in the present studies, hR would be expected to make little or none of this hO photoproduct. It is unclear why $bR_{acid\ purple}$, unlike hR, appears to make mostly red-shifted photoproducts. Even under identical low-pH conditions, the hR photoproduct difference spectrum does not greatly resemble that of $bR_{acid\ purple}$ (compare Fig. 2A,B). It seems that channeling of the protein structure into the O-like state at low pH is dictated by a residue (or residues) that is present in bR and not hR. These results deepen the distinctions between the photocycles of hR and $bR_{acid\ purple}$, and indicate that their halide binding and/or transport mechanisms may also differ significantly.

There are signs of important differences in the structures of the O intermediates formed from bR at neutral pH and under acidic conditions. For example, the vibrational bands of O and O_{acid} in the C–C fingerprint region (especially from 1160 to 1190 cm^{-1}) differ substantially, indicating that they do not necessarily have the same chromophore configuration. This is an important possibility to consider, in light of conclusions from resonance Raman spectroscopy that the O intermediate of the bR proton-pumping photocycle has an all-*trans* chromophore [17], whereas the O-like intermediate of the hR chloride-pumping photocycle is 13-*cis* [1]. With FTIR or Raman spectra of $bR_{acid\ purple}$ containing isotopically labeled retinals, it should be possible to determine which of these O-like chromophore structures is adopted by O_{acid}.

Interestingly, despite apparent chromophore structure differences, the hydrogen-bonding environments around Asp-85 in the O (near neutral pH) and O_{acid} species are probably quite similar, as evidenced by similar vibrational frequencies of ca. 1755 cm^{-1}. The Asp-85 frequency of 1755 cm^{-1} is also identical to that reported for the N intermediate, and not very different from the 1760 cm^{-1} frequency seen in M. Furthermore, the Asp-96 COOH frequency of 1751 cm^{-1} in O_{acid} is very similar to that reported for both the L and M intermediates [4,6]. Both of these Asp residues have frequencies at least 8 cm^{-1} lower (≤ 1740 cm^{-1}) in the starting $bR_{acid\ purple}$ state, which has an all-*trans* chromophore, than in these 13-*cis* photoproducts ($\geqslant 1748$ cm^{-1}) [1].

Thus, the COOH vibrational frequencies of Asp-85 and -96 follow a pattern, in that they are always higher in the intermediates with 13-*cis* chromophores than in the all-*trans* starting state. The pattern appears to prevail regardless of the protonation state of the Schiff base (see, however, footnote 1). This suggests that the environment around these Asp residues is quite tightly coupled to chromophore configuration, i.e. that when the chromophore is 13-*cis*, Asp-85 and -96 are in less hydrogen-bonded environments, giving rise to higher carbonyl vibrational frequencies.

An apparent exception to the rule is the O intermediate of the physiological (proton-pumping) photocycle, which is assigned as all-*trans* [1] but has

[1] It should be noted that the Asp-96 COOH vibrational frequency in M has recently been reported as 1736 cm^{-1} [16]. This would make it slightly *lower* than that of the parent bR, contradicting our generalization. This assignment was made, however, using an M state trapped at 230 K at pH 10, for which the FTIR spectrum is somewhat different than under physiological conditions. We therefore continue to rely on the previous assignment of the Asp-96 COOH frequency at ca. 1748 cm^{-1} [4].

high ('13-*cis*-like') frequencies for its Asp-85 and -96 COOH vibrations. A determination of the chromophore configuration of O_{acid} is necessary to see whether it is also an exception to rule linking high COOH vibrational frequencies of these 2 residues to a 13-*cis* configuration.

In any case, our work demonstrates that during the $bR_{acid\ purple}$ photocycle, strong transient perturbations occur in the vicinity of residues 96 and — to an even greater extent — 85, even though these residues have a fixed protonation state. It is likely that intramembrane perturbations at these specific sites play a role in moving halide ions across the membrane at low pH, and protons under physiological conditions. FTIR results on hR [7] are consistent with a model in which the chloride starts out in the location where (in bR) an ionized Asp-85 is normally located, and is translocated to a new site during the hR → hL transition. This translocation also results in a decrease in the hydrogen-bonding of the environment of Arg-108 (the homolog of Arg-82 in the bR structure), which forms part of the anion binding site. If a chloride ion and Arg-82 occupy a similarly-configured site in $bR_{acid\ purple}$, then it is not surprising that photolysis leads to a strong perturbation in the vicinity of Asp-85 that would decrease its hydrogen bonding in the O_{acid} state. Why Asp-96 should also be perturbed is as yet less clear. An intriguing possibility is that the chloride ion ends up transiently in the vicinity of this residue when O_{acid} is formed.

Acknowledgements

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