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Site-directed isotope labeling and FTIR spectroscopy: assignment of tyrosine bands in the bR → M difference spectrum of bacteriorhodopsin

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Abstract

Fourier transform infrared difference spectroscopy has been used extensively to probe structural changes in bacteriorhodopsin and other retinal proteins. However, the absence of a general method to assign bands to individual chemical groups in a protein has limited the application of this technique. While site-directed mutagenesis has been successful in special cases for such assignments, in general, this approach induces perturbations in the structure and function of the protein, thereby preventing unambiguous band assignments. A new approach has recently been reported (Sonar et al., Nature Struct. Biol., 1 (1994) 512–517) which involves cell-free expression of bacteriorhodopsin and site-directed isotope labeling (SDIL). We have now used this method to re-examine bands assigned in the bR → M difference spectrum to tyrosine residues. Our results show that out of 11 tyrosines in bR, only Tyr 185 is structurally active. This work further demonstrates the power of SDIL and FTIR to probe conformational changes at the level of individual amino acid residues in proteins.

Keywords: Isotope labeling; FT-IR spectroscopy; Bacteriorhodopsin; Tyrosine; Cell-free protein synthesis; M intermediate

1. Introduction

Since the discovery of bacteriorhodopsin, the light-driven proton pump from *Halobacterium salinarium*, in the early 1970's by W. Stoeckenius and co-workers [1], it has become one of the most intensively studied membrane proteins. Much of this interest in bR stems from its unusual properties including:

- — the existence of a photocycle consisting of a series of intermediates with different visible absorptions [2];
- — the ability to produce large quantities of bacteriorhodopsin; its stability at very high temperature [3], and the ability to regenerate into a structurally intact and functional protein from its denatured form [4,5] or proteolytic fragments [6,7];
- — the self-assembly of bacteriorhodopsin into a 2-dimensional hexagonal crystalline lattice [1].

Abbreviations: FTIR, Fourier transform infrared; bR, bacteriorhodopsin; SDIL, site-directed isotope labeling; cf-bR, bacteriorhodopsin expressed in a cell-free protein synthesis system

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A key goal in understanding how bacteriorhodopsin and other retinal proteins function is to obtain a detailed picture of the structural changes which occur in the protein. For this purpose, FTIR-difference spectroscopy has been extensively used as represented by several papers in this issue (for a recent review see [8] and references therein). The technique derives its usefulness from the ability of FTIR-difference spectroscopy to detect bands arising due to the vibrations of individual *residues and chemical groups* in a protein which undergoes conformational and/or chemical change.

However, a key problem in the use of FTIR-difference spectroscopy is *the assignment of bands to individual amino acid residues*. Only after such assignments are made can information be derived about changes in specific components of the protein. In the past, assignments have been made on the basis of two different approaches illustrated in Fig. 1: uniform isotope labeling of amino acids and site-directed mutagenesis. While these methods led to the assignment of several specific bands [9–16], many of these assignments are still tentative while most bands have not yet been assigned. The essential problem is that mutations, especially those of amino acid residues in the active site of a protein, can *alter the structure and function of a protein* thereby preventing unambiguous assignments of individual bands.

We recently introduced a third method (Fig. 1) for assigning bands in FTIR-difference spectra [17] based on a technique which we have termed site-directed isotope labeling (SDIL). As summarized in Fig. 2, a key element in SDIL is the use of a suppressor tRNA aminoacylated with an isotopically labeled amino acid. This tRNA is targeted to insert the isotopic amino acid at the proper position in the nascent protein by using an amber codon at the corresponding position in the gene. Cell-free synthesis (in vitro translation) and exogenous addition of the aminoacylated suppressor tRNA prevent aminoacylation of non-suppressor tRNAs with the isotopic amino acid, similar to the approach used for site-directed non-native amino acid replacement (SNAAR) [18–21].

In our initial study, we used SDIL to label Tyr 147 and Tyr 185 with L-[ring-$^2H_4$]. bR → K FTIR-difference spectra recorded for these samples along with unlabeled and uniformly labeled bR showed that out of 11 tyrosines, only Tyr 185 is structurally

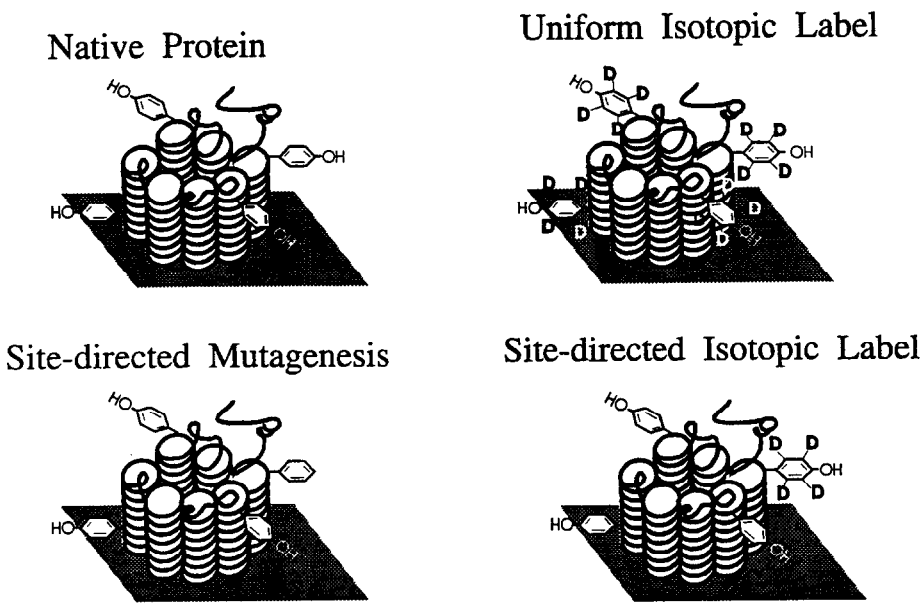

Fig. 1. Schematic drawing of different methods for band assignments in FTIR difference spectra. Top left: native protein; top right: uniform isotope labeling of a specific amino acid (tyrosine); bottom left: site directed mutagenesis of a specific tyrosine; bottom right: site directed isotope labeling of a specific tyrosine. (Figure adapted with permission from *Nature Struct. Biol.* (August 1994 issue)).

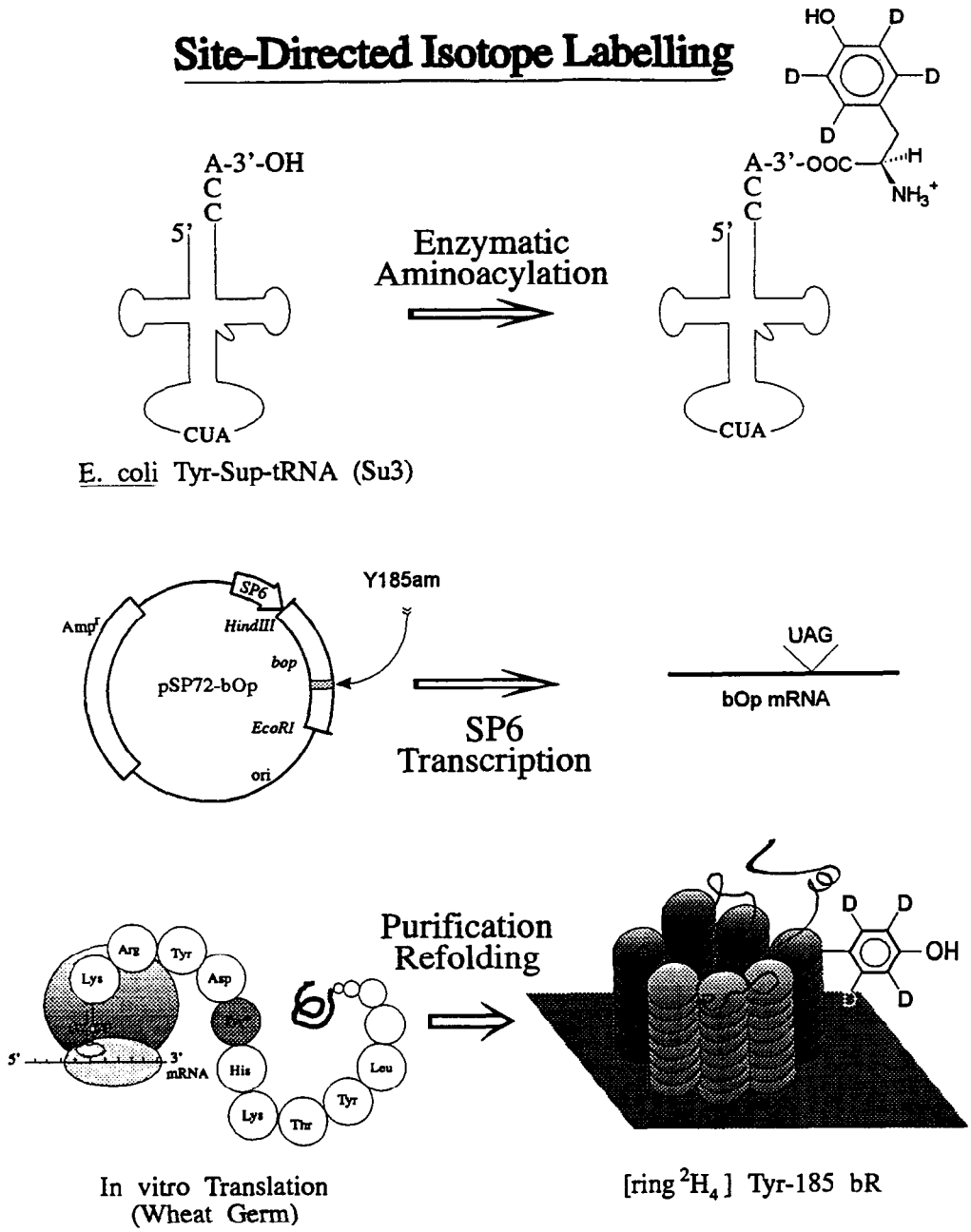

Fig. 2. Major steps in site directed isotope labeling of a protein. Top: *E. coli* tyrosine sup-tRNA is enzymatically aminoacylated with a deuterated tyrosine; middle: using recombinant DNA methods an amber codon is inserted in the gene at the position of the residue to be isotope labeled followed by nRNA synthesis using run off transcription; bottom: the mRNA is translated in a cell-free protein synthesis system in the presence of the aminoacylated suppressor tRNA. The resulting SDIL analog is then purified and refolded. (Figure adapted with permission from *Nature Struct. Biol.* (August 1994 issue)).

active during this transition [17]. In this paper, we have extended this work by labeling an additional residue, Tyr 57, and by measuring the bR → M difference spectra for all three SDIL samples. Our results clearly show that Tyr 185 is the only tyrosine which is structurally active during bR → M and most likely during the entire photocycle. This also demonstrates that Tyr 57 does not change its protonation state although it is located in the active site of the protein.

2. Materials and methods

SDIL analogs of bR, including L-[ring-$^2H_4$] Tyr 57, L-[ring-$^2H_4$] Tyr 147, L-[ring-$^2H_4$] Tyr 185 and the cell-free expressed non-isotope-labeled bR were prepared as described previously [17,22]. Briefly, the tyrosine codons (TAC) of Tyr 57, Tyr 147 and Tyr 185 were replaced by the amber codons (TAG) in the synthetic *bop* gene using cassette mutagenesis to obtain Y57am, Y147am and Y185am constructs, respectively. Run-off transcripts yielding corresponding mRNAs were obtained by in vitro transcription after placing these genes under control of an SP6 or T7 promoter [22]. Cell-free expression of these mRNAs were carried out in wheat germ extracts as described [22] in the presence of *E. coli* tyrosine suppressor tRNA (*E. coli* suppressor $tRNA^{Tyr}_{CUA}$), aminoacylated by L-[ring-$^2H_4$] Tyr (Sigma Chemical Co., St. Louis, MO) using a mixture of *E. coli* aminoacyl tRNA-synthetases [23].

Bacteriorhodopsin, where all tyrosines are labeled by L-[ring-$^2H_4$] Tyr, was kindly provided by J. Herzfeld (Chemistry Department, Brandeis University, Waltham, MA) which was isolated from *Halobacteria salinarium* grown in a stringent medium containing L-[ring-$^2H_4$] Tyr as described previously [24,25].

FTIR-difference spectra were recorded at 2 cm^{-1} on a 740 Nicolet spectrometer. Approximately 25–30 μg of purple membrane or SDIL samples suspended

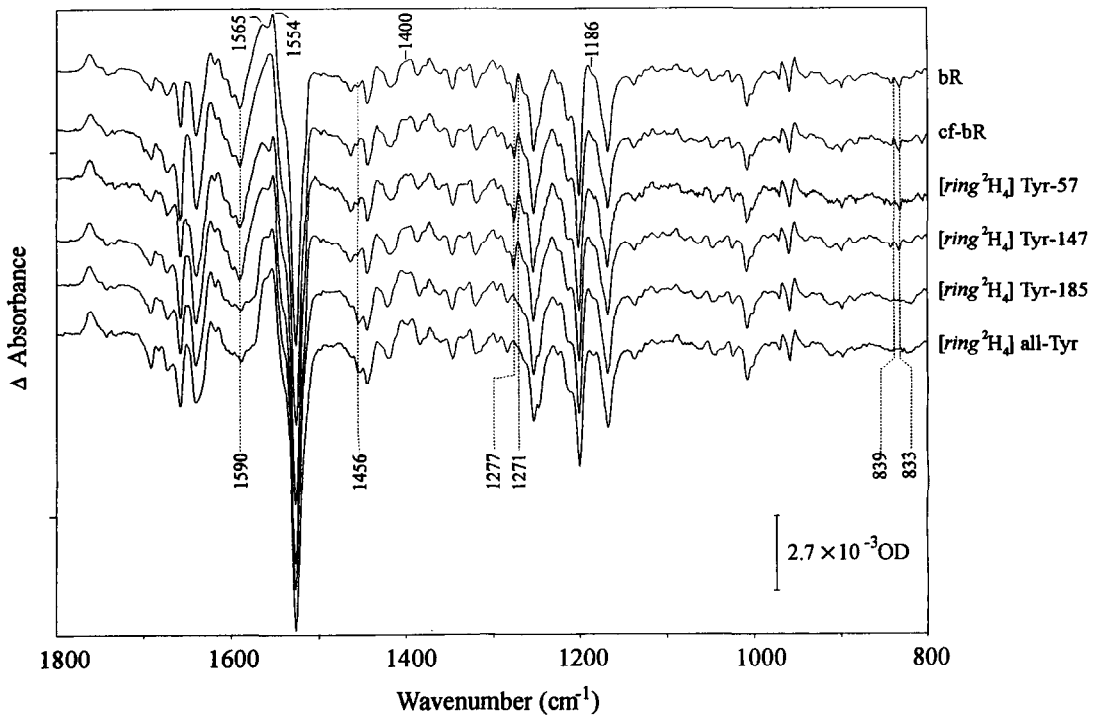

Fig. 3. Comparison of FTIR difference spectra for bR → M transition of bacteriorhodopsin and SDIL analogs of bR. Each spectrum was recorded at 2 cm^{-1} resolution and at 250 K under conditions described in the text. The scale corresponds to the FTIR difference spectrum of bR.

in distilled water was placed on an AgCl window and was allowed to dry overnight in a dry-box purged with air at −150° C dewpoint. The window with the sample was then rehydrated and placed in a specially designed cell, light adapted and cooled to 250 K as previously described [25,26].

3. Results

3.1. L-[ring-$^2H_4$] Tyr 57 and L-[ring-$^2H_4$] Tyr 147

Fig. 3 shows the bR → M difference spectra of bR, cell-free expressed bR (cf-bR), the three SDIL analogs of bR and bR containing uniform labels at all tyrosines (L-[ring-$^2H_4$] all-Tyr). In the cases of L-[ring-$^2H_4$] Tyr 57 and L-[ring-$^2H_4$] Tyr 147 only small differences are found between these spectra and unlabeled bR, such as those near 1554, 1400 and 1186 cm^{-1} which are attributable to variations in the small level of the N-intermediate accumulated under steady-state illumination at 250 K [27–30]. However, all of the bands previously assigned to tyrosine vibrational modes on the basis of uniform isotope labeling remain unaltered as seen in Fig. 4. For example, a negative band at 833 cm^{-1} previously assigned to a characteristic Fermi resonance of tyrosinate [24,31], negative/positive bands at 1277/1271 cm^{-1} assigned to the CO^- stretch of tyrosinate, a positive band at 1456 cm^{-1} assigned to a tyrosine vibrational mode and a negative band at 1590 cm^{-1} possibility due to a tyrosinate ring mode all appear [24,25]. Thus, we conclude on the basis of these results that *Tyr 57 and Tyr 147 do not undergo a significant change in their environment or protonation state during the bR → M transition.*

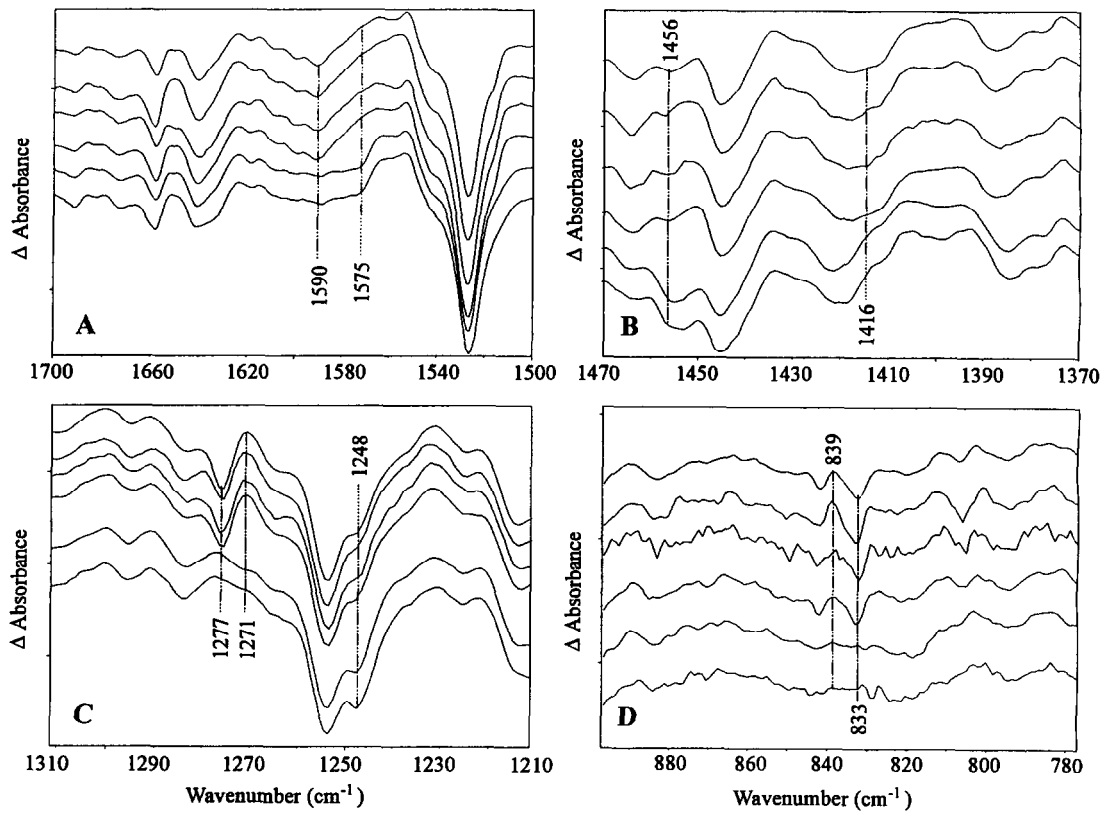

Fig. 4. Expansion in selected regions of the data shown in Fig. 3.

3.2. L-[ring-$^2H_4$] Tyr 185

In contrast to L-[ring-$^2H_4$] Tyr 57 and L-[ring-$^2H_4$] Tyr 147, the bR → M difference spectrum of L-[ring-$^2H_4$] Tyr 185 exhibits significant changes compared to unlabeled bR (Fig. 3). Furthermore, these changes are very similar to those produced by uniform L-[ring-$^2H_4$] labeling of bR. In particular, bands characteristic of tyrosine or tyrosinate vibrations including the 1590, 1456, 1277 and 833 cm^{-1} bands disappear (Figs. 3 and 4), whereas new bands appear in both spectra at 1575 cm^{-1}, 1416 cm^{-1}, and 1240–1250 cm^{-1} which were tentatively assigned on the basis of model compound studies to L-[ring-$^2H_4$] Tyr (or tyrosinate) vibrational modes [24,25]. This is especially clear in the case of the negative/positive pair at 1277/1271 cm^{-1} (Fig. 4) previously attributed to a change in the environment of a tyrosinate residue. This demonstrates that all of the spectral changes due to uniform [ring-$^2H_4$] Tyr labeling arise exclusively from the isotope labeling of Tyr-185. Thus, we conclude that *Tyr-185 is the only tyrosine residue which is structurally active during the bR → M transition of the bR photocycle.* Since similar results were also obtained for the bR → K [17], bR → L and bR → N difference spectra (unpublished results, data not shown), it appears likely that Tyr-185 is the only tyrosine which is structurally active during the entire bR photocycle.

4. Discussion

Earlier assignments of tyrosine bands in the bR → M difference spectrum of bacteriorhodopsin were made using a combination of uniform isotope labeling [24,25,32,33] and site-directed mutagenesis [10]. In the latter case, spectra were obtained for a set of Tyr → Phe mutants corresponding to all 11 tyrosines in the bR sequence. This resulted in the tentative assignment of several bands to protonation changes of Tyr 185 [10]. However, this work was hampered by changes induced in the structure and function of several tyrosine mutants. In particular, Tyr 57 → Phe and Tyr 83 → Phe exhibited reduced activity which made it difficult to determine whether these residues contributed to the native bR → M difference spectra. In addition, it has recently been found that changes induced in the bR → M spectra of the Tyr 185 → Phe mutant are not limited to changes arising only from tyrosine vibrations. In particular, new bands appear due to the presence of the O intermediate along with the normal light-adapted bR (bR_{570}) [34–36].

In contrast, the current work demonstrates that tyrosine band assignments can be made using essentially unperturbed samples [1] that are specifically labeled at individual residues, thus avoiding the problems of site-directed mutagenesis. Furthermore, it was not necessary in this case to analyze spectra from all 11 SDIL tyrosine analogs since shifts induced in one (L-[ring-$^2H_4$] Tyr 185) resulted in shifts very similar to the uniform [L-ring-$^2H_4$] all-Tyr sample.

Our results show that Tyr 57 does not undergo significant structural activity including protonation changes during the bR → M transition. Measurement of the bR → K FTIR difference spectrum of L-[ring-$^2H_4$] Tyr 57 (unpublished data) also confirmed our original conclusion that this residue is not structurally active during the bR → K transition [17]. These results are interesting in light of the finding that substitution of Tyr 57, which is located close to Asp 212 in the active site of bR [14], with Asn or Asp [16,37] blocks the formation of the M intermediate. One likely possibility is that although Tyr 57 is itself not structurally active, it plays a key role through hydrogen bonding with nearby residues in stabilizing the structure of the active site of bR. It also may hydrogen bond directly to a structurally active water molecule as indicated by recent FTIR measurements [38]. Finally, although we do not observe protonation changes of Tyr 57 during the photocycle, it still may deprotonate during high pH production of the alkaline form of bR [16,37].

Most of the bands in the bR → M difference spectrum that are affected by L-[ring-$^2H_4$] labeling are more easily assigned to Tyr 185 tyrosinate modes than tyrosine modes as previously noted [24,25,32,33]. Although, solid-state NMR and UV-resonance Raman experiments have not detected ty-

[1] Earlier studies have shown that the secondary structure, light–dark adaptation and photocycle kinetics are essentially the same in in vitro produced SDIL samples and native bacteriorhodopsin found in purple membrane [17,22].

rosinates in bR or its photoproducts [39–41], this may reflect fractional ionization of Tyr 185, consistent with a recent FTIR study [42]. One possibility is that the observed fractional tyrosine deprotonation/reprotonation events correspond to proton movement through a proton conducting hydrogen bonded network that extends from Asp-96 to the retinylidene Schiff base [43]. Alternatively, these events might reflect a more local change in the hydrogen bonding between Tyr 185 and a second residue such as Asp 212 which is likely to interact with Tyr 185 [13,14] [2].

Importantly, future SDIL/FTIR studies should be able to distinguish between these two models. For example, it should now be possible to systematically probe the structural changes of individual residues during the photocycle including Thr 46, Thr 89 and Asp 212 which have been implicated along with Tyr 185 as participating in a proton conducting wire [43]. Similarly, band assignments and information about protonation changes of key Asp groups including Asp 96, Asp 85, Asp 115 and Asp 212 can also be determined using this approach.

Acknowledgements

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