Accelerated Publications

Site-Directed Isotope Labeling and ATR-FTIR Difference Spectroscopy of Bacteriorhodopsin: The Peptide Carbonyl Group of Tyr 185 Is Structurally Active During the bR → N Transition†

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ABSTRACT: The largest secondary structural change occurs in the bacteriorhodopsin (bR) photocycle during the M → N transition. In this work site-directed isotope labeling (SDIL) and attenuated total reflection Fourier transform infrared (ATR-FTIR) difference spectroscopy were used to investigate this conformational change. L-Tyrosine containing a ^{13}C isotope at the carbonyl carbon was selectively incorporated at Tyr 57, Tyr 147, and Tyr 185 by SDIL. This involves the cell-free expression of bR in the presence of *Escherichia coli* suppressor $tRNA^{Tyr}_{CUA}$ aminoacylated with L-[1-^{13}C]Tyr. ATR-FTIR difference spectroscopy reveals that of the 11 tyrosines, only the peptide carbonyl group of Tyr 185 undergoes a significant structural change during the bR → N transition. Along with other spectroscopic evidence, this result suggests that the Tyr 185−Pro 186 region of the protein is structurally active and may function as a hinge which facilitates the tilt of the cytoplasmic portion of the F-helix in bacteriorhodopsin during the M → N transition.

Bacteriorhodopsin (bR)[1] is a 26 000 MW protein which mediates light-driven proton transport across the purple membrane of *Halobacterium salinarium* (formerly known as *Halobacterium halobium*) (Stoeckenius & Bogomolni, 1982; Rothschild & Sonar, 1995). Upon light absorption, bR undergoes a cyclic series of steps involving several metastable intermediates with distinct visible absorptions:

$$bR_{570} \rightarrow K_{630} \rightarrow L_{550} \rightarrow M_{412} \rightarrow N_{550} \rightarrow O_{640} \rightarrow bR_{570}$$

While an increasingly accurate picture has been obtained from both resonance Raman and solid-state NMR spectroscopy of the structural changes of retinylidene chromophore during this photocycle (Lugtenburg et al., 1988; Mathies et al., 1991), much less is known about changes in the protein backbone structure.

Several recent studies indicate that the largest change in the bR secondary structure occurs during the M → N transition.[2] An intense negative band at 1670 cm^{-1} appears in the amide I region of the time-resolved FTIR difference spectrum of bR during formation of the N intermediate (Bousché et al., 1991, 1992; Braiman et al., 1991) and disappears during N decay (Bousché et al., 1992; Hessling et al., 1993). Similar results are also obtained from low-temperature steady-state measurements, where the N intermediate is stabilized by either high pH (Pfefferle et al., 1991; Ormos et al., 1992) or by protein modifications such as Thr-46 → Asp (Rothschild et al., 1993).

Changes in the secondary structure of bR have also been detected by circular dichroism (Draheim & Cassim, 1985), X-ray (Koch et al., 1991), and neutron diffraction experiments (Dencher et al., 1989). Recently, electron diffraction studies of the mutant Asp-96-Gly revealed that the cytoplasmic portion of the F- and G-helices undergoes a small tilt away from the membrane normal (Subramaniam et al., 1993).

The goal of the present work is to determine which portions of the bR structure are involved in the conformational changes detected by FTIR difference spectroscopy during the M → N transition. For this purpose site-directed isotope labeling (SDIL) (Sonar et al., 1994) has been used for the first time to incorporate isotope labels at specific positions along the polypeptide chain. In contrast to earlier biophysical studies based on uniform isotope labeling, this approach in conjunction with FTIR allows probing of

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[1] Abbreviations: FTIR, Fourier transform infrared; ATR, attenuated total reflection; PM, purple membrane; bR, bacteriorhodopsin; SDIL, site-directed isotope labeling.

[2] This step may involve subspecies (Varo & Lanyi, 1990; Sasaki et al., 1992).

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individual peptide groups in the backbone of bacteriorhodopsin and other proteins.

We also report the first use of ATR-FTIR difference spectroscopy to measure the M → N transition. In contrast to earlier FTIR studies, where the IR beam was directed through a thin, partially hydrated purple membrane film, ATR-FTIR allows for measurements of samples in aqueous media, whose pH and ionic composition can be accurately controlled (Marrero & Rothschild, 1987a,b; Yang et al., 1987).

Our results demonstrate that the structural changes observed during the M → N transition involve changes in the environment of the Tyr 185 carbonyl group. In contrast, no changes are observed in the carbonyl groups from all other tyrosines, including six that are embedded in the membrane. Along with recent polarized ATR-FTIR measurements of the bR → N transition (C. F. C. Ludlam and K. J. Rothschild, unpublished results) as well as earlier studies based on site-directed mutagenesis (Rothschild et al., 1990), these results suggest that the Tyr 185–Pro 186 region may serve as a hinge around which changes in the F-helix orientation occur.

MATERIALS AND METHODS

ATR-FTIR Difference Spectroscopy. Sample solutions of either native purple membrane or an SDIL-bR analog were prepared (2 μM bR, 1 mM NaPi, 1 mM KCl, 0.4 mM $MgCl_2$, 0.6 mM $CaCl_2$, and 50 mM NaCl), and 25 μL of each of these samples was dried onto a germanium crystal internal reflection element (l = 50 mm; w = 20 mm; d = 2 mm) using a slow stream of argon gas. This procedure results in a purple membrane film which remains tightly bound to the Ge crystal, even upon exposure to a flowing solution (Marrero & Rothschild, 1987a,b). The crystal was then mounted in a setup consisting of a temperature-controlled ATR cell (MEC-1TC) with a quartz window for sample illumination (TMP-V-DI4, Harrick Scientific Corp., Ossining, NY). Sample illumination was from a 100-W tungsten lamp filtered with a 505 nm long pass filter and optical fiber (Dolan-Jenner Industries, Inc., Lawrence, MA).

The sample film was equilibrated with a pH 9.0 buffer (25 mM NaPi titrated with 0.5 M NaOH) at 3 °C. ATR-FTIR spectra were recorded on a Bio-Rad FTS-60A spectrometer (Bio-Rad, Cambridge, MA) equipped with an MCT detector using attenuated total reflection. Each spectrum is the average of 480 scans at 2-cm^{-1} resolution. Successive spectra were recorded with the light alternately on and off. Approximately 50 light–dark differences were then averaged to obtain the final difference spectrum.

Site-Directed Isotope Labeling. ^{13}C labeling at the C1 position of the peptide bond at Tyr 57, Tyr 147, and Tyr 185 in bacteriorhodopsin was accomplished using methods previously reported for the incorporation of perdeuterated ring label at these positions (Sonar et al., 1994). 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 PCR and cassette mutagenesis to obtain Y57am, Y147am, and Y185am constructs, respectively (Sonar et al., 1994). mRNAs were obtained by *in vitro* transcription by placing these genes under the control of an SP6 or T7 promoter (Sonar et al., 1993b). Cell-free expression of these mRNAs was carried out in wheat germ extracts as described (Sonar et al., 1993b). The

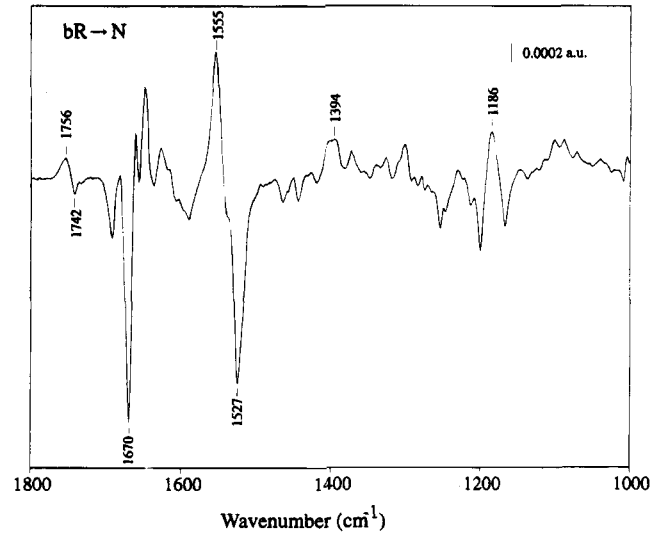

FIGURE 1: bR → N ATR-FTIR difference spectroscopy of native bacteriorhodopsin: Averaged difference spectrum of bR recorded at 276 K and pH 9.0 (see Materials and Methods for details).

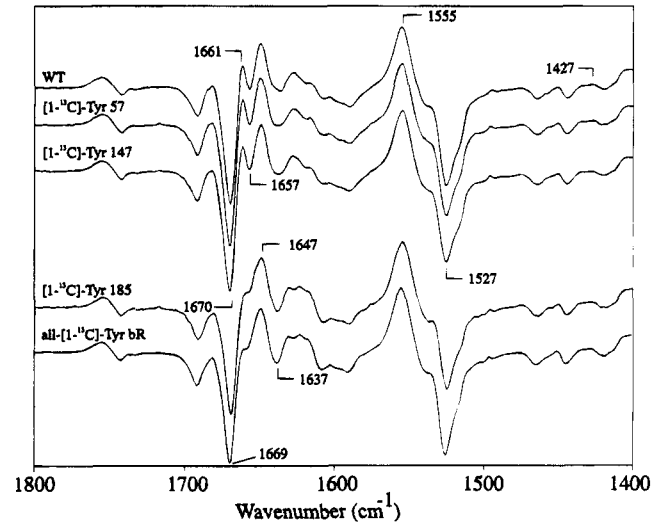

FIGURE 2: bR → N ATR-FTIR difference spectroscopy of bacteriorhodopsin and SDIL analogs of bR: Comparison of the difference spectra of native bR, [1-^{13}C]Tyr 57, [1-^{13}C]Tyr 147, [1-^{13}C]Tyr 185, and all-[1-^{13}C]Tyr bR.

Escherichia coli tyrosine suppressor tRNA (*E. coli* suppressor $tRNA^{Tyr}_{CUA}$), which has previously been shown to have a high *in vivo* suppression efficiency, was aminoacylated by L-[1-^{13}C]Tyr (Cambridge Isotope Laboratories, Woburn, MA) using a mixture of *E. coli* aminoacyl tRNA synthetases (Seong & RajBhandary, 1987).

Uniform Isotope Labeling. L-[1-^{13}C]Tyr was purchased from Cambridge Isotope Laboratories, Woburn, MA, and incorporated into *Halobacterium salinarium* R1 using biosynthetic methods previously reported (Rothschild et al., 1986). On the basis of analysis of the incorporation of trace L-[ring-$^{2}H_6$,^{3}H]Tyr, 50–80% of the tyrosine residues were found to be labeled, without any scrambling to other sites.

RESULTS

Figure 1 shows the ATR-FTIR difference spectrum of native purple membrane measured at 3 °C and pH 9.0. Except for intensity differences due to polarization effects (Marrero & Rothschild, 1987b), the spectrum is very similar

to the bR → N difference spectrum recorded by both static (Ormos, 1991; Pfefferle et al., 1991) and time-resolved FTIR spectroscopy (Bousché et al., 1991, 1992; Braiman et al., 1991; Hessling et al., 1993). Bands characteristic of the N intermediate are found at 1756 cm^{-1} (+) (Asp 85 carboxyl C=O stretch) and 1742 cm^{-1} (−) (Asp 96 carboxyl C=O stretch), 1670 cm^{-1} (−) (α-helix amide I); 1390−1400 cm^{-1} (+) (carboxylate C=O stretch (Bousché et al., 1991) and C_{11}−H in-plane bending (Maeda et al., 1992)); and 1186 cm^{-1} (+) (C−C stretch of retinal (Fodor et al., 1988)). A reduction also occurs in the intensity of the negative 1527-cm^{-1} band (ethylenic C=C stretch) relative to the bR → M difference spectrum (data not shown) (Bousché et al., 1991). The latter effect is due to cancellation of the negative C=C ethylenic stretch by light-adapted bR (bR_{570}) at 1527 cm^{-1} with the positive C=C ethylenic stretch mode of N at 1555 cm^{-1} (Fodor et al., 1988).

In Figure 2 the bR → N difference spectra of native purple membrane and of all-[1-^{13}C]Tyr bR (i.e., all tyrosines are labeled) are compared to those of the three SDIL analogs of bR: [1-^{13}C]Tyr 57, [1-^{13}C]Tyr 147, and [1-^{13}C]Tyr 185. While the bR → N spectra of [1-^{13}C]Tyr 57 and [1-^{13}C]Tyr 147 are almost identical to that of native purple membrane, the spectrum of the [1-^{13}C]Tyr 185 bR analog shows distinct changes in the amide I region which are almost identical to the spectrum of the all- [1-^{13}C]Tyr bR sample (lower trace). The most noticeable change (see Figure 3) is the drop in intensity and the small frequency shift of the 1670-cm^{-1} band in both the [1-^{13}C]Tyr 185 and the all-[1-^{13}C]Tyr bR sample. Changes are also found at 1680 cm^{-1} (+), 1661 cm^{-1} (+), 1657 cm^{-1} (−), and 1647 cm^{-1} (+) and in the 1600−1635-cm^{-1} region, where several new bands appear. These results indicate that of the 11 tyrosine carbonyl groups in bR, only the vibrations of the Tyr 185 carbonyl group contribute to structural changes reflected in the amide I region of the bR → N difference spectrum.[3]

DISCUSSION

FTIR difference spectroscopy has been used extensively to investigate protein structural changes which occur during the bR photocycle (Rothschild et al., 1981; Bagley et al., 1982; Rothschild & Marrero, 1982; Siebert & Maentele, 1983; Braiman & Rothschild, 1988; Gerwert, 1992; Rothschild, 1992; Cao et al., 1993). While most studies have focused on protonation and hydrogen bonding of amino acid side chains, several bands in the amide I region of the bR → K, L, M, and N difference spectra have been assigned to peptide group vibrations [see Rothschild (1992) and references therein]. Most notable is an intense negative band at 1670 cm^{-1} which appears during the M → N transition (*e.g.*, only a weak band is observed in the bR → M difference spectrum) (Bousché et al., 1991; Braiman et al., 1991) and has been assigned to the amide I mode of buried α-helical structure (Rothschild et al., 1993).

In this work we have utilized ATR-FTIR difference spectroscopy to measure the bR → N difference spectrum.

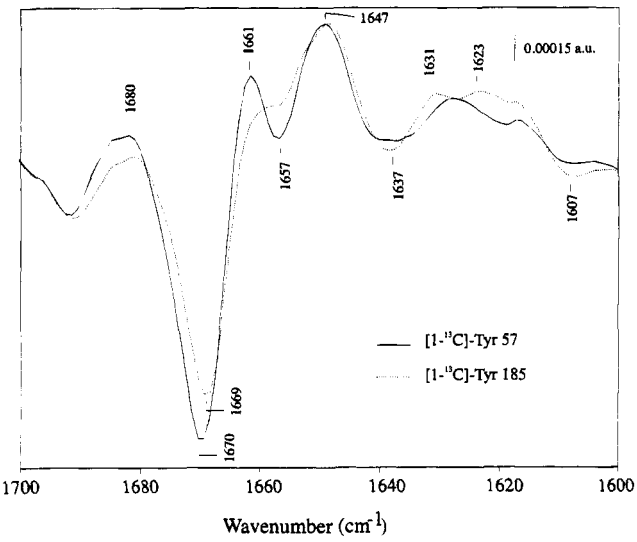

FIGURE 3: bR → N ATR-FTIR difference spectroscopy of [1-^{13}C]-Tyr 57 and [1-^{13}C]Tyr 185: Comparison of the difference spectra for [1-^{13}C]Tyr 57 and [1-^{13}C]Tyr 185 in the region between 1600 and 1700 cm^{-1}.

In contrast to FTIR transmission methods, which are restricted to thin, hydrated films, ATR allows FTIR measurements of purple membrane and other membrane systems in a bulk aqueous medium, whose pH and ionic composition can be accurately controlled (Brauner et al., 1987; Marrero & Rothschild, 1987a,b). This is possible, because the infrared light penetrates only a small distance (1 μm) from the Ge internal reflection crystal into the purple membrane film (Fringeli et al., 1989). Our results show that the 1670-cm^{-1} band not only is detected in FTIR difference spectra of thin, hydrated films but also is observed in the presence of bulk aqueous solution. The only significant changes found between the ATR and transmission bR → N difference spectra were alterations in band intensity, including an increase of the negative 1670-cm^{-1} band due to the dichroism of this band.

A second important aspect of this work is the utilization of the SDIL technique to incorporate isotopes at specific atomic positions in the bR backbone. This approach provides a general, nonperturbing method for assigning bands to specific peptide groups or to specific regions of the protein in the case of coupled modes. In contrast, uniform isotope labeling, such as the ^{13}C labeling of all lysine carbonyls of bR (Takei et al., 1994), cannot in most cases provide definitive band assignments to a specific peptide group. In addition, site-directed mutagenesis (SDM) cannot be used for such a purpose, since it only allows substitutions of the amino acid side chains. In addition, SDM is known to perturb structure and/or function when key amino acid residues are mutated (Sonar et al., 1993a). Our results show that the amide I region of the bR → N difference spectrum includes significant contributions from the Tyr 185 carbonyl group. Hence, we conclude that this group undergoes a significant change in its structure during this transition.

A comparison of the changes induced by the [1-^{13}C]Tyr 185 label in bR and recent studies on synthetic polypeptides also indicates that the Tyr 185 carbonyl is likely to be part of an α-helical structure in light-adapted bR. The isotope shift induced by 1-^{13}C labeling at specific backbone carbonyls in a 25-residue soluble polypeptide was found to be ap-

[3] Recent measurements also show that smaller changes occur in the amide I region in the the bR → K transition (S. Sonar, X.-M. Liu, J. Herzfeld, C. P. Lee, U. L. RajBhandary, and K. J. Rothschild, unpublished results). This indicates that the Tyr 185 carbonyl responds directly to the chromophore isomerization as early as the bR → K transition.

proximately 30−40 cm^{-1} (Tadesse et al., 1991; Martinez et al., 1994) in agreement with normal mode calculations.[4] A similar ^{13}C isotope induced shift (~40 cm^{-1}) was recently observed in the truncated 26-residue hydrophobic fragment of phospholamban (C. F. C. Ludlam, S. Arkin, X.-M. Liu, S. Smith, D. Engelman, and K. J. Rothschild, unpublished data). In this case an intense band at 1655 cm^{-1} assigned to the amide I mode of α-helical structure undergoes a drop in intensity and a small downshift in frequency (2 cm^{-1}), while a small band assigned to the ^{13}C labeled carbonyl appears near 1613 cm^{-1}. In the case of bacteriorhodopsin, the similar downshift and drop in intensity of the 1670-cm^{-1} (−) band assigned to α-helical structure (Rothschild et al., 1993) and the appearance of a new negative band at 1637 cm^{-1} indicate that the Tyr 185 carbonyl group also participates in an α-helical structure in the bR_{570} state.[5] The spectral changes in the amide I band can be attributed to vibrational uncoupling of the ^{13}C-labeled carbonyl group from the rest of the α-helix amide I mode which involves the coupled vibrations of multiple carbonyl groups (Parker, 1983, and references therein).

In contrast, the more complex pattern of isotope-induced spectral changes that occur in positive bands in the bR → N difference spectrum makes it difficult to determine the exact pattern of hydrogen bonding of this group in the N-intermediate without further labeling studies. For example, the presence of isotope-sensitive positive bands at 1680 and 1660 cm^{-1} could be indicative of the participation of the Tyr 185 carbonyl group in β-structure and α-helical hydrogen bonding (Byler & Susi, 1986).

While it is not yet possible to determine whether other regions of the F-helix are involved in this structural change without additional SDIL studies of bR, earlier FTIR measurements involving site-directed mutagenesis of several membrane-embedded proline residues (Rothschild et al., 1990) led to the suggestion that the region centered near Tyr 185−Pro 186 serves as the center of a hinge which allows reorientation of a part of the F-helix. Only a small rotation (less than 30°) of the Tyr 185 ψ torsion angle (Cα−C1) or a combination of small rotations about other peptide bonds including the C−N bond would be sufficient to produce a significant reorientation in the cytoplasmic portion of the F-helix. Electron diffraction measurements (Subramaniam et al., 1993) and a recent study using polarized ATR-FTIR difference spectroscopy of the bR → N difference spectrum are also consistent with the occurrence of a reorientation in a portion of the F-helix of bR (C. F. C. Ludlam and K. J. Rothschild, unpublished results).

Finally, our results exclude all tyrosine carbonyl groups other than Tyr 185 from contributing to the amide I region of the bR → N difference spectrum.[3] This result places some limits on the regions of bR which are involved in the backbone conformational changes which occur during the bR photocycle. In particular, 11 tyrosine carbonyl groups are distributed throughout the bR structure with seven tyrosines (Tyr 26, Tyr 43, Tyr 57, Tyr 83, Tyr 147, Tyr 150 and Tyr 185) buried in intramembrane regions of helices A, B, C, E, and F. There are also four other tyrosines on water-exposed connecting loop regions (Tyr 64, Tyr 79, Tyr 131, and Tyr 135). Excluding Tyr 185, the other tyrosine carbonyls do not appear to undergo significant conformational changes. For example, even small changes in the hydrogen bonding to these tyrosine carbonyls would result in observable band shifts in the amide I region. These changes could arise, for example, from rearrangements in the local protein structure or changes in the extent of water association with these carbonyl groups (Parker, 1983). Thus, the picture that emerges from this work is that the peptide carbonyl group of Tyr 185 undergoes a change in its structure, whereas the peptide carbonyl groups of other tyrosines are relatively static during the photocycle.

Significantly, the introduction of SDIL provides a powerful method for assigning bands in an FTIR difference spectrum of a protein that avoids the problems associated with uniform isotope labeling and site-directed mutagenesis. In the case of bacteriorhodopsin, this approach should allow a more accurate picture to be developed of the conformational changes between the M and N intermediates, regions of the protein involved in proton movement and coupling of chromophore isomerization to protein structural changes. Finally, this approach should be applicable to a wide range of other proteins involved in enzyme catalysis, ion transport, and signal transduction.

[4] The exact downshift induced by labeling a single carbonyl will depend on many factors including the degree of dipole coupling between the individual carbonyl groups (Dwivedi & Krimm, 1984).

[5] This frequency of the amide I band in bacteriorhodopsin is approximately 5−10 cm^{-1} higher than found in most α-helical proteins (Rothschild & Clark, 1979a). Note also that the frequency assigned to the out-of-plane component of the amide I mode in bacteriorhodopsin is close to 1670 cm^{-1} (Rothschild & Clark, 1979b; Earnest et al., 1990).

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