Photoactivation Perturbs the Membrane-embedded Contacts between Sensory Rhodopsin II and Its Transducer*

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The photoactivation mechanism of the sensory rhodopsin II (SRII)-HtrII receptor-transducer complex of Natronomonas pharaonis was investigated by time-resolved Fourier transform infrared difference spectroscopy to identify structural changes associated with early events in the signal relay mechanism from the receptor to the transducer. Several prominent bands in the wild-type SRII-HtrII spectra are affected by amino acid substitutions at the receptor Tyr199 and transducer Asn74 residues, which form a hydrogen bond between the two proteins near the middle of the bilayer. Our results indicate disappearance of this hydrogen bond in the M and O photointermediates, the likely signaling states of the complex. This event represents one of the largest light-induced alterations in the binding contacts between the receptor and transducer. The vibrational frequency changes suggest that Asn74 and Tyr199 form other stronger hydrogen bonds in the M state. The light-induced disruption of the Tyr199-Asn74 bond also occurs when the Schiff base counterion Asp75 is replaced with a neutral asparagine. We compared the decrease in intensity of difference bands assigned to the Tyr199-Asn74 pair and to chromophore and protein groups of the receptor at various time points during the recovery of the initial state. All difference bands exhibit similar decay kinetics indicating that reformation of the Tyr199-Asn74 hydrogen bond occurs concomitantly with the decay of the M and O photointermediates. This work demonstrates that the signal relay from SRII to HtrII involves early structural alterations in the deeply membrane-embedded domain of the complex and provides a spectroscopic signal useful for correlation with the downstream events in signal transduction.

Sensory rhodopsin II (SRII) functions as a phototaxis receptor for blue light avoidance in several halophilic archaea (1–4). This protein belongs to a growing family of microbial rhodopsins, seven-helix retinylidene membrane proteins now found in all domains of life (5–8). The SRII receptor forms a tight intermolecular complex with its cognate transducer protein HtrII in the cell membrane. The transducer, like its homologous methyl-accepting chemotaxis transducers, possesses two transmembrane helices and a large cytoplasmic domain that binds at its distal end a His-kinase that phosphorylates a flagellar motor switch regulator (1, 9).

Interactions of HtrII with the SRII receptor are localized to the transmembrane and membrane-proximal domains (10, 11). A crystal structure of SRII bound to an N-terminal HtrII fragment containing the transmembrane helices (TM1 and TM2) shows tight van der Waals interaction and three hydrogen bonds between TM2 and SRII helices F and G (12). An atomic structure of the membrane-proximal domain of the transducer is not available; however, fluorescent probe accessibility and Förster resonance energy transfer measurements show interaction of this domain with the cytoplasmic E-F loop of the receptor (13).

The signal relay mechanism from SRII to HtrII in the complex has become a focus of interest in the past several years, in part because of its importance to the general understanding of interaction between integral membrane proteins. In accord with the unified model for transport and signaling by microbial rhodopsins (14, 15), the key event in the transducer activation is an outward tilt of the receptor helix F during the lifetime of its M intermediate, which has been directly detected by EPR of paramagnetic probes in the free receptor (16) and in its complex with transducer (17). In a response to the helix F movements, the TM2 of HtrII is displaced from its initial position (17). Additional structural changes were observed in the cytoplasmic membrane proximal region by fluorescent probe accessibility measurements (13).

Fourier transform infrared (FTIR) difference spectroscopy has been used extensively in the past to elucidate structural changes in the photocycles of several microbial rhodopsins (18–27). This technique measures changes in the infrared absorption of protein groups and enables studies of light-induced conformational changes without the necessity of introducing potentially structure-perturbing probes. Because of its sensitivity to small changes in hydrogen bonding, it is especially well suited for the study of interactions of polar protein groups. Here we have applied time-resolved FTIR difference spectroscopy to examine the changes in interaction between SRII Tyr199 and HtrII Asn74 during the light-activated photocycle. These residues form a hydrogen bond in the dark state that functions as an interhelical bridge between helix G of SRII and TM2 of HtrII embedded near the middle of the membrane interior (12). Assessing the environment of this hydrogen bond would answer whether the movements of helix F and TM2 previously detected near the cytoplasmic region extend more deeply into the membrane interior domain of the complex.

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The abbreviations used are: SRII, sensory rhodopsin II; TM, transmembrane; FTIR, Fourier transform infrared.
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MATERIALS AND METHODS

Plasmid Construction and Site-directed Mutagenesis—The wild-type construct FP120 encoded a fusion protein in which full-length SRII and the 120 N-terminal residues of HtrII containing an additional His tag at the C terminus are joined by the flexible linker ASASNGASA. The fusion gene was placed in plasmid pET21d (Novagen) under control of the T7 promoter. The plasmid was transformed into the Escherichia coli BL21 (DE3) strain. Single amino acid substitutions were performed using QuikChange II site-directed mutagenesis kit (Stratagene). The mutagenesis primer sequences were 5′-GGCGCTATGTCTTCCCTGACCTCGTTGCAC-3′ and 5′-GTTGACGGAGTTGAAAGCAGTAAACGCTC-3′ for Tyr199→Phe mutation, 5′-CCTGCTCCGGATCGACCTCGGGCTCGTTGC-3′ and 5′-GCAACGGCCCGAGGTCGATCCGGATCGACCTCGGGCTCGTTGC-3′ for Asn34→Asp mutation, and 5′-GCCTCCGGTGATACACCATC-3′ for Asp75→Asna mutation. The double mutant D75N/Y199F was constructed by two-step mutagenesis.

Protein Expression and Purification—The cells were grown in LB medium with ampicillin, 50 µg/ml, to an absorbance at 600 nm of ~0.4, and the protein synthesis was induced by addition of 1 mM isopropyl-β-D-thiogalactopyranoside and 5 mM all-trans-retinal. After the induction period, the cells were centrifuged at 1000 × g, resuspended in 50 mM Tris/HCl, pH 7.0, 5 mM MgCl2, 1 mM phenylmethylsulfonyl fluoride buffer and disrupted by a microfluidizer (Microfluidics Corp., Newton, MA), and the membranes were then harvested by ultracentrifugation. The membranes were solubilized in 300 mM NaCl, 10 mM imidazole, 50 mM potassium phosphate, pH 7.6, 1.5% octyl glucoside. After centrifugation of the solubilized membranes, the supernatant was incubated with nickel-nitrotriacetic acid-agarose (Qiagen), and the His-tagged protein was eluted with a gradient of imidazole in 50 mM potassium phosphate, pH 7.6, 300 mM NaCl, 0.5% octyl glucoside in a Biologic DualFlow system (Bio-Rad).

Protoplastome Reconstitution—Purified proteins were reconstituted in phospholipids by the dialysis procedure previously described (28). In the work reported here the protein-to-lipid ratio was 1;6.5 (w/w). Extraction of halobacterial phospholipids used in the reconstitution procedures was performed as described previously (22).

FTIR Difference Spectroscopy—The sample preparation and FTIR measurement procedures have been described previously (27). Rapid-scan time-resolved FTIR spectroscopy was performed using a BRUKER IFS 66 v/s FTIR spectrometer (Bisherics, Billerica, MA) operating at 4 cm⁻¹ spectral resolution and 240 kHz scanner velocity corresponding to a data acquisition window of 18 ms.

RESULTS

Spectral Changes in the SRII-HtrII Complex Due to Tyr199→Phe and Asn74→Asp Substitutions—Difference spectra of the receptor-transducer complex were recorded using rapid scan FTIR difference spectroscopy and averaged over a 30–85 ms period after photoexcitation (Fig. 1). This time range is associated predominantly with accumulation of the M intermediate (24), which is a signaling state for the transducer activation (29). Several prominent bands appear at 1163 (v(CO) of a tyrosine side chain (31), whose frequency is sensitive to the hydrogen-bonding environment (32). We therefore assign the 1694 cm⁻¹ induced by the Y199F substitution. This indicates that a positive band near this frequency also arises from vibrations of Tyr199 in the M intermediate of wild-type pigment. The inferred 1265/1275 (−/+) cm⁻¹ bands are clearly seen in the double difference spectra obtained by subtraction of the Y199F mutant from the wild-type spectra. The apparent frequency upshift of the Tyr199 vibration upon photoactivation indicates that a change in the hydrogen bonding strength of Tyr199 occurs during formation of M (see “Discussion”).

(i) A prominent negative band near 1518 cm⁻¹ has reduced intensity in N74D and disappears in the Y199F spectrum. A very strong ring mode vibration, which is sensitive to the protonation state of tyrosine, appears at 1518 cm⁻¹ in the spectra of tyrosine recorded in HCl and near 1480 cm⁻¹ in the spectra of tyrosinate recorded in NaOH (32). From the frequency of this vibration, we conclude that Tyr199 is protonated in the receptor dark state. Note that the position of this band in the difference spectra may be affected by spectral overlap with other peaks in this region.

(ii) A negative peak seen in the double difference spectrum (Fig. 2) near 1694 cm⁻¹ was previously identified in the SRII-HtrII complex but not in the receptor alone (27). It is also absent in both mutant spectra. As noted previously (27), its frequency is typical for the C=O stretching mode of an asparagine side chain. This vibration is found near 1678 cm⁻¹ in the model compound spectra and depending on the hydrogen bonding environment can shift significantly, with the lower frequency corresponding to a stronger hydrogen bond (33). We therefore assign the 1694 cm⁻¹ vibration to the C=O stretching mode of the asparagine side chain.
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Interaction between Tyr\textsuperscript{199} and Asn\textsuperscript{74} in the D75N Mutant of the SRII-HtrII Complex—We studied the effect of replacing the Schiff base counterion Asp\textsuperscript{74} with Asn on light-induced conformational changes in the SRII-HtrII transmembrane domain. The neutralization of the negative charge near the Schiff base strongly perturbs the photocycle (35, 36). However, the D75N mutant is still capable of activating the transducer (13, 37).

The spectrum of the D75N SRII-HtrII complex (Fig. 3, top trace) recorded within the first 50 ms shows predominant formation of the red-shifted O-like state instead of M as indicated by a strong positive band at 1192 cm\textsuperscript{-1} (36). Despite the photocycle differences, a comparison of the D75N and D75N/Y199F samples (Fig. 3, bottom traces) reveals that the spectral changes, which we have assigned to disruption of the interaction between Tyr\textsuperscript{199} and Asn\textsuperscript{74} in the wild-type complex (see above), also appear in the D75N/Y199F mutant. Note, however, that the negative band assigned to Asn\textsuperscript{74} at 1694 cm\textsuperscript{-1} in the wild-type complex is at a lower frequency near 1688 cm\textsuperscript{-1} indicating an altered hydrogen bonding environment due to the D75N mutation. This peak is superimposed on a pair of intense positive/negative bands at 1700/1685 cm\textsuperscript{-1} assigned to Asn\textsuperscript{75} in the D75N mutant receptor (36).

The Kinetics of Difference Bands Arising from the Tyr\textsuperscript{199}/Asn\textsuperscript{74} Interaction and the Receptor during the Recovery of the Initial State—To test whether the return of HtrII to the initial position occurs with different kinetics than the decay of the SRII signaling state, we compared changes in the amplitude of individual difference bands measured between 50 and 250 ms (Fig. 4). In this time window all peaks exhibit a gradual decrease in intensity reflecting the return of complex to the unphotolyzed state. The majority of peaks including the negative retinal vibrational modes at 1200, 1240, and 1545 cm\textsuperscript{-1} and the carboxyl stretching mode at 1764 cm\textsuperscript{-1} exhibit an exponential decay (data not shown) with a time decay constant similar to that previously reported (38). Because there is no noticeable delay between the decay of these receptor vibrations and the 1694 cm\textsuperscript{-1} band assigned to Asn\textsuperscript{74} from the transducer, we conclude that the recovery of the Tyr\textsuperscript{199}/Asn\textsuperscript{74} hydrogen bonding occurs at a similar rate as SRII returns to the unphotolyzed state.

DISCUSSION

In an earlier study, we compared the structural changes occurring in the transducer-free receptor SRII and a SRII-HtrII fusion complex using time-resolved FTIR difference spectroscopy (27). Several spectral differences were found between the two systems including bands characteristic of asparagine and tyrosine vibrations. In the present study, we focused on the interaction between Tyr\textsuperscript{199} and Asn\textsuperscript{74} that is observed in the crystal structure of SRII-HtrII in its dark state (12). These two residues form a hydrogen bond that functions as an interhelical hydrogen bond perturbation.
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Fig. 4. The decrease of difference signal for wild-type SRII-HtrII spectra recorded in the 50–250 ms time range. Several time slices were averaged to produce each trace.

pin between helix G of SRII (Tyr199) and TM2 of HtrII (Asn74) embedded near the middle of the membrane interior (12). As such, changes in the vibrational spectra of Tyr199 and Asn74 represent a native probe of possible movements that involve these two helices in the middle of the membrane.

To study the structural changes that specifically involve these two residues we utilized site-directed mutagenesis in combination with time-resolved FTIR. The SRII-HtrII fusion construct used here included the full-length receptor and the first 120 amino acids of the transducer, which constitute the transmembrane and membrane-proximal domains of HtrII. The use of a truncated transducer allowed us to focus on the mechanism of signal transduction from SRII to HtrII while excluding downstream events associated with signal propagation within the transducer.

The results of this work show that the major spectral differences observed between the truncated SRII-HtrII complex and free receptor (27) can be ascribed to structural changes of the receptor Tyr199 and transducer Asn74 groups. Considering the sensitivity of infrared spectroscopy to detect very small alterations in the hydrogen bonding of polar groups (39–41), it is likely that perturbations of these residues represent one of the most significant changes in the hydrogen-bonding structure of the receptor-transducer complex. These results, however, do not exclude the possibility of additional structural changes in the complex. One example is the displacements of transducer helices TM1 and TM2 in response to the receptor conformational changes. These movements are expected to give rise to difference bands in the amide I and amide II spectral regions. In fact, several amide I bands in the receptor spectra are altered in the presence of transducer (27). The detailed analysis of vibrations in this region is a subject of a future study.

Our finding that the replacement of either Tyr199 or Asn74 alters light-induced negative bands arising from both of these groups establishes that they interact in proteoliposomes in the SRII-HtrII dark state, in agreement with the crystallographic structure (Fig. 5) (12). The frequencies of negative bands arising from the side chain vibrations of Tyr199 and Asn74 are also consistent with the presence of a hydrogen bond between them. The x-ray diffraction data does not resolve unambiguously whether the hydroxyl group of Tyr199 is hydrogen-bonded to the oxygen or amide nitrogen of Asn74 side chain (12, 34). The frequency of the Asn74 peak (1694 cm\(^{-1}\)) detected in this study is considerably higher compared with the asparagine model compounds (1677 cm\(^{-1}\)) indicating that the side chain carbonyl group is only weakly hydrogen-bonded and therefore the interaction with the Tyr199 hydroxyl group is more likely through the amide nitrogen.

The FTIR results provide insight regarding the environment of Tyr199 and Asn74 in the light-activated state. The frequency downshift from 1694 to 1670 cm\(^{-1}\) for Asn74 points to the formation of a considerably stronger hydrogen bond in the photointermediate. This shift is larger than normally associated with environmental changes of hydrogen-bonded asparagine groups. For example, relatively small differences of 6 and 8 cm\(^{-1}\) were observed between the initial and M states for Asn105 in SRII (25) and Asn230 in proteorhodopsin (26), respectively. On the other hand, the data for HtrII are consistent with formation of a strong hydrogen bond involving the C=O group of Asn. A large upshift of the Tyr199 vibrational mode initially near 1265 cm\(^{-1}\) to above 1270 cm\(^{-1}\) also indicates significant changes in the hydrogen bonding interaction. In particular, a shift of similar amplitude was observed for this mode in the case of Tyrosine D in photosystem II upon the replacement of nearby His100 with a Gln (32). Therefore, both Tyr199 and Asn74 undergo large structural alterations during the photoactivation as a result of the movements of helices F and TM2 (17).

The observed FTIR signals in the dark and intermediate states most likely reflect disruption of the Tyr199-Asn74 interaction and formation of new stronger hydrogen bonds. However, we do not exclude the possibility that these residues still interact in the M intermediate. Such interaction may be indirect, for example through a network of hydrogen-bonded groups. Such a weak interaction is in agreement with the observation that the replacement of Tyr199 with Phe has smaller effect on the transducer affinity for the receptor in the M intermediate compared with the dark state (42).

In conclusion, we demonstrated that FTIR difference spectroscopy in combination with site-directed mutagenesis can be successfully used to probe specific conformational changes that arise from interaction between two membrane proteins. In the case of the SRII-HtrII complex reported here, changes in the transmembrane domain occur during the early steps in trans-
ducer activation. The extension of this work including mutations in the cytoplasmic and extracellular protein domains should be able to provide a more comprehensive picture of the SRII-HtrII interaction and ultimately lead to the identification of the specific protein residues responsible for the transfer of the phototaxis signal from the receptor to the transducer. In addition to the peak assignment, the site-directed mutagenesis can be also used to correlate protein structural changes with the cell phototaxis response assessed from functional studies. An example of such an approach is demonstrated in the case of the receptor D75N mutant. The difference spectra of the mutant complex revealed the presence of a Tyr^{199}-Asn^{74} interaction in the dark state and its perturbation upon photoactivation despite the absence of the Schiff base counterion. The similar conformational changes between the wild-type and mutant complex in the transmembrane region suggested by these results are in agreement with the ability of D75N SRII to activate the transducer.

Comparison of the kinetic spectra recorded during the last photocycle transition reveals that the transducer return to the initial state, measured by disappearance of the Asn^{74} signal, occurs concomitantly with the decay of the phototivated receptor. This result differs from the observation of Wegener et al. (17) of slower kinetics for the recovery of paramagnetic label attached to the transducer residue Val^{78}. The difference may be due to several factors including different measurement conditions, probing different positions in the transducer and possible additional structural changes resulting from the introduced label in the case of EPR.

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