Functional Importance of the Interhelical Hydrogen Bond between Thr$^{204}$ and Tyr$^{174}$ of Sensory Rhodopsin II and Its Alteration during the Signaling Process$^*$

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Sensory rhodopsin II (SRII), a receptor for negative phototaxis in haloarchaea, transmits light signals through changes in protein-protein interaction with its transducer HtrII. Light-induced structural changes throughout the SRII-HtrII interface, which spans the periplasmic region, membrane-embedded domains, and cytoplasmic domains near the membrane, have been identified by several studies. Here we demonstrate by site-specific mutagenesis and analysis of phototaxis behavior that two residues in SRII near the membrane-embedded interface (Tyr$^{174}$ on helix F and Thr$^{204}$ on helix G) are essential for signaling by the SRII-HtrII complex. These residues, which are the first in SRII shown to be required for phototaxis function, provide biological significance to the previous observation that the hydrogen bond formation between them is strengthened upon the formation of the earliest SRII photointermediate (SRIIK) only when SRII is complexed with HtrII. Here we report frequency changes of the S-H stretch of a cysteine substituted for SRII Thr$^{204}$ in the signaling state intermediates of the SRII photocycle, as well as an influence of HtrII on the hydrogen bond strength, supporting a direct role of the hydrogen bond in SRII-HtrII signal relay chemistry. Our results suggest that the light signal is transmitted to HtrII from the energized interhelical hydrogen bond between Thr$^{204}$ and Tyr$^{174}$, which is located at both the retinal chromophore pocket and in helices F and G that form the membrane-embedded interaction surface to the signal-bearing second transmembrane helix of HtrII. The results argue for a critical process in signal relay occurring at this membrane interfacial region of the complex.

Sensory rhodopsin II (SRII, also known as phoborhodopsin) is a negative phototaxis receptor in haloarchaeal prokaryotes, including *Halobacterium salinarum* and *Natronomonas pharaonis* (1–5). The SRII photoreceptor subunit forms a 2:2 complex with its transducer subunit, HtrII, in membranes and transmits light signals through changes in protein-protein interaction. The photochemical reaction cycle (6) and atomic structure of SRII (7–9) are well characterized. SRII bound to an N-terminal fragment of HtrII have provided atomic details of the two proteins’ interaction surface in the periplasm and within the membrane (10), and interaction of the HtrII membrane-proximal domain with the cytoplasmic domain of the receptor has been demonstrated by fluorescent probe accessibility and Förster resonance energy transfer measurements (11), EPR of spin-labels (12), and *in vitro* binding of HtrII peptides to SRII (13). The signal relay mechanism from SRII to HtrII in the complex has become a focus of interest in part because of its importance to the general understanding of interaction between integral membrane proteins.

The results from several different methods show that light-induced structural changes occur all along the SRII-HtrII interface, which includes the region on the periplasmic side of the membrane, the membrane-embedded domain, and the cytoplasmic membrane-proximal domain: (i) FTIR light-dark difference spectra of the complex in proteoliposomes show both the periplasmic and membrane-embedded hydrogen-bonded regions undergo major structural changes (14)$^3$; (ii) EPR spectra of proteoliposomes indicate a rotatory motion of the HtrII second transmembrane helix (TM2) near the membrane/cytoplasm interface (15); a small (0.9 Å) displacement and rotation of this region of TM2 has also been reported in illuminated crystals of the complex (16); (iii) fluorescent probes in detergent-solubilized complex show that light-induced structural changes also occur in the cytoplasmic membrane proximal domain of the complex (11).

Determination of which SRII-HtrII interface changes are crucial for signal relay requires functional studies to complement the structural data. Site-specific mutagenesis provides a possible method to identify residues in both proteins crucial for this process, but there are no residue mutations in SRII that have been reported to eliminate phototaxis signaling by the receptor. Here we report that Thr$^{204}$ in helix G and Tyr$^{174}$ in...

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$^2$The abbreviations used are: SRII, sensory rhodopsin II from *N. pharaonis* (also known as NpSRII or pprB); HtrII, halobacterial transducer protein II from *N. pharaonis*; BR, bacteriorhodopsin; SRIIK, SRIIM, SRIIO, K, M, and O intermediates of SRII; FTIR, Fourier transform infrared; TM2, second transmembrane helix.

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helix F are crucial for receptor signal relay to the transducer subunit and present FTIR evidence for alteration of their hydrogen bond in the signaling states of the SRII photocycle. This first clue for the importance of these two residues was the uniquely large HtrII-dependent light-induced strengthening of the hydrogen bond between them evident from its spectral downshift (110 cm\(^{-1}\)) upon formation of the earliest photocycle intermediate (SRIIK) (17). The results reported here and the location of the Thr\(^{204}\)-Tyr\(^{174}\) interhelical hydrogen bond between the retinal chromophore pocket and the interaction surface of SRII with HtrII (Fig. 1) argue for a critical process for signal relay occurring within their membrane-embedded contact surfaces.

**EXPERIMENTAL PROCEDURES**

**Strain for Transformation—**

*H. salinarum* strain Pho81Wr\(^{-}\) lacking the four archaeal rhodopsins (BR, HR, SRI, and SRII) as well as the two transducer proteins (HtrI and HtrII) was used for transformation (18) according to the protocol described previously (19). To obtain a high expression level, the stronger bop promoter was used instead of the native promoter (19).

**Plasmid Construction—** The pYS001 plasmid was modified from plasmid pJS010 that encodes the wild-type SRII-HtrII fusion gene (20). The Ncol and NsiI fragments were ligated into Ncol and NsiI sites of pJS010. This plasmid encodes six histidines in the C terminus, and it was named pYS001. Plasmids of T204A, T204S, T204C, and Y174F without HtrII were constructed as previously described (17, 21). For the preparation of T204C-HtrII, T204A-HtrII, T204S-HtrII, and Y174F-HtrII expression plasmid, the 5’- and 3’-ends of the mutant genes were mutated by PCR to get Ncol and Spel restriction sites, respectively. The resulting Ncol-Spel fragment was ligated with the large fragment of NcoI/SpeI-treated pYS001 vector.

**Membrane Preparation—** Proteins were expressed in *H. salinarum* Pho81Wr\(^{-}\) cells. The preparation of membrane vesicles was performed using essentially the same method as previously described (22, 23). Briefly, membrane vesicles were prepared by sonication, and the membranes were finally pelleted by centrifugation at 45,000 rpm (Beckman, rotor type 70 Ti), for 30 min at

**FIGURE 1.**

*a,* residues in positions corresponding to 174 and 204 in SRII focused on in this study. *b,* details of the x-ray crystallographic structure of the SRII-HtrII complex in the dark state focusing on the hydrogen bond between Thr\(^{204}\) and Tyr\(^{174}\) in SRII, the length of which is 3.2 Å. This view is from the extracellular side roughly along the membrane normal. Microbial rhodopsin gene name abbreviations are as described in Ref. 46.
Role of Thr^{204}-Tyr^{174} Interaction in Phototaxis Signaling

Flash Photolysis Measurements and Phototaxis Analysis—Flash-induced absorption changes in the millisecond to seconds time domain were acquired on a digital oscilloscope (ClampX 8.2) following an Nd-YAG laser (Continuum, Surelight I; 532 nm, 6 ns, 40 mJ) in a laboratory-constructed flash photolysis system as described (24); 64–256 transients were collected for each measurement. The membranes were resuspended in 50 mM Tris-Cl, pH 7.0, containing 4 M NaCl. All experiments were performed at 20 °C. Phototaxis responses were measured as swimming reversal frequency changes to a 100-ms 500-nm photostimulus as described (25), except a new motion analysis system with software package Celltrak 1.2 Beta (Motion Analysis Corp., Santa Clara CA) was used to analyze motility behavior.

Binding Analysis and FTIR Measurements—SRII and HtrII-(1–159) proteins were prepared as described previously (26, 27). A truncated HtrII protein expressed from position 1 to position 159, which has been shown to exhibit the same interaction with SRII as the full-length protein (28, 29), was used for measurements other than phototaxis behavior and flash photolysis. Briefly, the proteins with a hexahistidine tag at the C terminus were expressed in Escherichia coli cells, solubilized with 1.0% N-dodecyl-β-D-maltoside, and purified with a nickel-nitrilotriacetic acid column as described previously (26, 27).

Confirmation of HtrII binding to SRII was based on the inhibition of heat denaturation of SRII by HtrII (30). Purified wild-type or mutant SRII were incubated without and with HtrII protein in a 1SRII:2HtrII molar ratio in a UV-visible spectrophotometer. The temperature was maintained at 81 °C for 10 min, where the maximum of the SRII visible band (30). During incubation, the suspensions became turbid, presumably because of denatured protein aggregation. Therefore, before spectrum measurements the samples were centrifuged at 15,000 x g briefly for 1 min to remove the aggregate.

For FTIR measurements, purified SRII and HtrII proteins were mixed in a 1:1 molar ratio and incubated for 1 h at 4 °C. Two samples, SRII and the SRII-HtrII mixture, were then reconstituted into l-α-phosphatidylcholine (PC) liposomes by removal with Bio-Beads (SM22; Bio-Rad, Hercules, CA), where the molar ratio of added PC was 50 times that of the SRII protein (26, 31). Low temperature FTIR spectroscopy was performed as described previously (32, 33). The samples were washed three times with a buffer at pH 7.0 (2 mM phosphate) for measurement of M intermediate spectra, or pH 5.0 (2 mM citrate, 5 mM NaCl) for measurement of O intermediate spectra. 90 μl of the sample was dried on a BaF_{2} window with a diameter of 18 mm. After hydration by H_{2}O, the sample was placed in a cell that was mounted in an Oxford DN-1704 cryostat placed in the Bio-Rad FTS-60 spectrometer. All samples were hydrated with H_{2}O.

The SRII_{M} minus SRII difference spectra were measured at 230 K and pH 7.0 as follows. To convert SRII to SRII_{M}, the sample was irradiated for 2 min with >480 nm of light (YV-50; Toshiba, Shizuoka, Japan); subsequent illumination with UV light (UG-5; Melles Griot, Irvine, CA) for 90 s reconverted SRII_{M} into SRII (32). The difference spectrum was calculated from the spectra constructed with 128 interferograms collected before and after the illumination. Twenty-four spectra obtained in this way were averaged for the SRII_{M} minus SRII spectrum. For the measurement of the O intermediate of SRII, we used an acidic film (pH 5.0). To accumulate SRII_{O}, the sample was irradiated for 2 min with light at 260 K through a band pass filter whose transmittance spectrum (<510 nm) is shown in a previous article (33). After the illumination, the film was kept in the dark for 2 min, where SRII_{O} returns to the original state of SRII almost completely. The difference spectrum was calculated from the spectra constructed with 128 interferograms collected after and before the illumination (SRII_{O} minus SRII) and before and after releasing in the dark (SRII minus SRII_{O}). Twenty-four difference spectra obtained in this way were averaged.

RESULTS

Phototaxis Behavior of the Mutants—We assessed motility behavior of cells containing wild-type SRII-HtrII, T204C-HtrII, T204A-HtrII, T204S-HtrII, and Y174F-HtrII fusion complexes exposed to a 100-ms 500-nm light stimulus (Fig. 2). The SRII-HtrII complexes mediated a phototaxis response similar to that of the wild type, whereas T204A-HtrII, T204S-HtrII, and Y174F-HtrII complexes did not mediate detectable responses (Fig. 2). Although the Ser residue of T204S like Thr has an hydroxyl group, it did not mediate phototaxis responses. The previous FTIR study of this mutant showed that the O-H stretching vibration of the hydroxyl group of Thr^{204} exhibits a frequency downshift of 110 cm^{-1} upon retinal photoisomerization in the SRII-HtrII complex, whereas that of Ser^{204} exhibits a much smaller frequency downshift of 24 cm^{-1} (17). Thus, this result suggests that the downshift of Ser^{204} is insufficient to activate HtrII and supports that the hydrogen bond between the Thr^{204} hydroxyl and Tyr^{174} is important for the signaling.

Confirmation of the Complex Formation—To confirm complex formation between HtrII and SRII mutants, we used the substantial protection against heat denaturation of SRII by HtrII as a semi-quantitative assessment of binding (30). HtrII significantly inhibits denaturation of wild type, T204C, T204A, T204S, and Y174F of SRII (Fig. 3). The Y199A mutant does not mediate detectable responses (Fig. 2). Although the Ser residue of T204S like Thr has an hydroxyl group, it did not mediate phototaxis responses. The previous FTIR study of this mutant showed that the O-H stretching vibration of the hydroxyl group of Thr^{204} exhibits a frequency downshift of 110 cm^{-1} upon retinal photoisomerization in the SRII-HtrII complex, whereas that of Ser^{204} exhibits a much smaller frequency downshift of 24 cm^{-1} (17). Thus, this result suggests that the downshift of Ser^{204} is insufficient to activate HtrII and supports that the hydrogen bond between the Thr^{204} hydroxyl and Tyr^{174} is important for the signaling.
and O intermediates, respectively. The photocycle kinetics of T204C-HtrII, T204A-HtrII, and T204S-HtrII complexes were similar to that of wild-type SRII-HtrII complex (Fig. 4). Therefore, they are all expressed and form photoactive pigments in the *H. salinarum* membranes. In the T204C mutant, the absorbance change at 560 nm (O intermediate) is small because of a fast O-decay, consistent with previous reports that the O-decay of a quadruple mutant of SRII including T204C is much faster than that of wild-type SRII (21, 36). On the other hand, mutation of Tyr174 to Phe greatly altered the photocycle kinetics. The Y174F mutant exhibits a fast M-decay and slow O-decay (Fig. 4). Also in the case of BR, mutation of Tyr185 (corresponding to Tyr174 in SRII) strongly stabilizes the O-state (37). Therefore, Tyr residues at this position, which are within van der Waals contact distance of the retinal chromophore, appear to be important for the normal photocycle kinetics of these retinal proteins.

Infrared Spectral Changes of SRII upon the Formation of the M and O Intermediates in T204C in the S-H Stretching Frequency Region—S-H stretching vibrations of cysteine residues are well isolated from other vibrations (38) and useful for monitoring their hydrogen bonding strength. Because wild-type SRII and HtrII-(1–159) both lack cysteine residues, no signal in the S-H stretching region (2580–2525 cm⁻¹) was observed in the wild-type SRII-HtrII complex. The T204C mutation in SRII

![Figure 2. Phototaxis responses measured as swimming reversal frequency changes to a 100-ms 500-nm photostimulus (arrow).](image)

![Figure 3. Confirmation of complex formation of SRII and HtrII.](image)
does not inhibit its photosensory function as established above. Therefore, we introduced a cysteine residue at the position of Thr\(^{204}\) in SRII to monitor hydrogen bond changes at the 204 position in signaling states of the receptor. The S-H stretch of Cys\(^{204}\) strengthens upon formation of the K-intermediate like the O-H stretch of Thr\(^{204}\), and the T204C mutant is functional. Therefore, the behavior of the Cys\(^{204}\) is likely to mimic that of Thr\(^{204}\) in the wild-type SRII. It should be noted that fingerprint vibrations (1250–1100 cm\(^{-1}\)) show that the M and O intermediates are normally produced in T204C (data not shown). The difference infrared spectra of SRIIM minus SRII and SRIIO minus SRII measured with the T204C-HtrII complex and T204C in the absence of HtrII are shown in Fig. 5. In the SRIIM minus SRII spectrum, the S-H vibration bands appeared at 2560 (−)/2570 (+) cm\(^{-1}\) in T204C-HtrII complex and 2555 (−)/2564 (+) cm\(^{-1}\) in the SRII T204C mutant in the absence of HtrII, respectively. These results suggest that the hydrogen bond assigned to Thr\(^{204}\) and Tyr\(^{174}\) in the dark state is replaced by weaker hydrogen bonding in the M state. The negative and positive bands, corresponding to SRII and SRIIM, respectively, were upshifted (5 cm\(^{-1}\)) by the complex formation of SRII with HtrII, implying that the binding of HtrII influences the environment around Thr\(^{204}\) not only in the unphotolysed state but also in the M state. On the other hand, in the SRIIO spectrum, the S–H vibration bands appeared at 2560 (−)/2570 (+) cm\(^{-1}\) in T204C-HtrII complex and 2555 (−)/2564 (+) cm\(^{-1}\) in the SRII T204C mutant in the absence of HtrII, respectively. These results suggest that the hydrogen bond assigned to Thr\(^{204}\) and Tyr\(^{174}\) in the dark state is replaced by weaker hydrogen bonding in the M state. The negative and positive bands, corresponding to SRII and SRIIM, respectively, were upshifted (5 cm\(^{-1}\)) by the complex formation of SRII with HtrII, implying that the binding of HtrII influences the environment around Thr\(^{204}\) not only in the unphotolysed state but also in the M state. On the other hand, in the SRIIO spectrum, the S–H vibration bands appeared at 2560 (−)/2570 (+) cm\(^{-1}\) in T204C-HtrII complex and 2555 (−)/2564 (+) cm\(^{-1}\) in the SRII T204C mutant in the absence of HtrII, respectively.
Role of Thr²⁰⁴-Tyr¹⁷⁴ Interaction in Phototaxis Signaling

FIGURE 6. A, SRIIM, minus SRII and SRII, minus SRII IR difference spectra of T79C alone (dotted lines) and T79C-HtrII (solid lines) in the 2600–2500 cm⁻¹ region. B, SRIIM minus SRII and SRII, minus SRII IR difference spectra of D193C (dotted lines) alone and D193C-HtrII (solid lines) in the 2600–2500 cm⁻¹ region.

side chain of Arg⁷² differs from that of other haloarchaeal rhodopsins (8, 9). Are the HtrII-dependent structural changes, as seen for T204C, characteristic for the 204 position? We tested whether the hydrogen bonds between Thr⁷⁹-Asp⁷⁵ and Asp¹⁹³-Arg⁷² are altered by association with HtrII. Fingerprint vibrations (1250–1100 cm⁻¹) are altered by association with HtrII. In addition, no difference is observed in the spectra of the D193C mutant. In contrast, a negative peak at 2537 cm⁻¹ is observed involving helices F and G in SRII and TM2 in HtrII. Observation of active intermediates.

DISCUSSION

The results above demonstrate that Thr²⁰⁴ and Tyr¹⁷⁴ in SRII are each critical residues for phototaxis signaling. Further, the FTIR data argue that the hydrogen bond between them, which is altered during the photocycle, is crucial to development of an active signaling state in the SRII-HtrII complex. Binding of HtrII influences the Thr²⁰⁴-Tyr¹⁷⁴ hydrogen bond in SRII and also alters hydrogen bonding attributed to these residues in SRIIM and SRIIO. Although Thr²⁰⁴ has been known as an important residue for color tuning and photocycle kinetics of SRII (21, 36, 39), the results here demonstrate a more critical importance of Thr²⁰⁴, namely for production of a phototaxis signal.

Our results presented here demonstrate that the Thr²⁰⁴-Tyr¹⁷⁴ hydrogen bond in SRII is essential for either SRII activation or signal relay or both. Recently we observed that substitution of a Thr for Ala²¹⁵ in the proton pump BR, which is expected to introduce the hydrogen bond corresponding to Thr²⁰⁴-Tyr¹⁷⁴ into the pump, is sufficient to enable a small but detectable response of HtrII to BR photoactivation (40). Two additional mutations that align BR and HtrII in a similar manner as SRII and HtrII enhance this small response to a robust response comparable with that of the SRII-HtrII complex (40). Our interpretation of these results is that the hydrogen bond is essential for signal relay, because it confers signal relay activity to BR. The bond may also be essential for receptor activation.

What chemical role does the Thr²⁰⁴-Tyr¹⁷⁴ hydrogen bond play in signaling? Thr²⁰⁴ and Tyr¹⁷⁴ are very close to the retinylidene Schiff base (4.3 and 5.1 Å, respectively) (Fig. 1), whose hydrogen bond with the water hydrating its counter ion is disrupted upon trans-cis photoisomerization of the retinal chromophore. Previously, the hydrogen bonding interaction between Thr²⁰⁴ and Tyr¹⁷⁴ was found to be greatly strengthened (according to the frequency change of 110 cm⁻¹) after the retinal isomerization in an HtrII-dependent manner (17). It is important to note that frequency changes are not large in the K intermediate in general, because protein structural changes are very limited at this early stage of the photocycle. For example, frequency shifts are 18 cm⁻¹ for Thr¹⁷, 13 cm⁻¹ for Thr¹²¹, and 60 cm⁻¹ for Thr⁸⁹ in bacteriorhodopsin (41, 42). Thus, the large spectral shift of 110 cm⁻¹ stands out as an unusual storage of energy in this bond. The O-H frequency differences at the 204 position are 110 cm⁻¹ (wild type, i.e. Thr²⁰⁴, Tyr¹⁷⁴), 24 cm⁻¹ (T204S), and 28 cm⁻¹ (Y174F) as reported previously (17). The lower values for T204S and Y174F correlate with the lack of signaling function by these mutants. In the case of the Y174F mutant, Thr²⁰⁴ may form a hydrogen bond with the peptide carbonyl C = O of Leu²⁰⁰. In addition, an enhanced strong peak appeared at 2244 cm⁻¹ for the K intermediate of SRII possessing a C₁₄-D-labeled retinal (43). It is likely that Thr²⁰⁴ is the counterpart of the C₁₄-D group, and enhanced absorption probably originates from the local steric constraint at the C₁₄-D position after the C₁₃=C₁₄ double bond rotation. In this interpretation, steric hindrance between Thr²⁰⁴ and C₁₄-D is caused by photoisomerization, and relaxation of the hindrance that we reported as the restored O-H stretch of Thr²⁰⁴ in the M state (44) may induce the structural changes of the F and G helices in SRII and thereby in the HtrII TM2 helix that is tightly wedged between them. Thus, the steric hindrance between Thr²⁰⁴ and the retinal C₁₄-D and the interhelical hydrogen bonding alteration between them may be crucial for conformational changes observed involving helices F and G in SRII and TM2 in HtrII. Wegener et al. (45) reported that the F-helix SRII moves toward HtrII following light activation of SRII and TM2 of HtrII rotates (15). The x-ray structure of SRIIM does not show a movement of the F helix and rather indicates G helix motion (16); however, the crystal lattice forces clearly greatly inhibit and may distort the resulting motion. Yang et al. (11) reported that the distance
between the E-F loop of SRII and the HAMP domain extension of TM2 of HtrII is altered by light activation. Finally, Bergo et al. (14) observe substantial structural changes in hydrogen bonding between helices F and G and TM2. A causative link between chromophore photoisomerization and these later events may be the early structural change that resolves the steric hindrance between Thr^204 and the retinal C_{14}-H.

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