**X-ray Crystallographic Structure of Thermophilic Rhodopsin**

**IMPLICATIONS FOR HIGH THERMAL STABILITY AND OPTOGENETIC FUNCTION**

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Thermophilic rhodopsin (TR) is a photoreceptor protein with an extremely high thermal stability and the first characterized light-driven proton pump derived from the extreme thermophile *Thermus thermophilus* JL-18. In this study, we confirmed its high thermal stability compared with other microbial rhodopsins and also report the potential availability of TR for optogenetics as a light-induced neural silencer. The x-ray crystal structure of TR revealed that its overall structure is quite similar to that of xantrhodopsin, including the presence of a putative binding site for a carotenoid antenna; but several distinct structural characteristics of TR, including a decreased surface charge and a larger number of hydrophobic residues and aromatic-aromatic interactions, were also clarified. Based on the crystal structure, the structural changes of TR upon thermal stimulation were investigated by molecular dynamics simulations. The simulations revealed the presence of a thermally induced structural substrate in which an increase of hydrophobic interactions in the extracellular domain, the movement of extracellular domains, the formation of a hydrogen bond, and the tilting of transmembrane helices were observed. From the computational and mutational analysis, we propose that an extracellular LPGG motif between helices F and G plays an important role in the thermal stability, acting as a “thermal sorter.” These findings will be valuable for understanding retinal proteins with regard to high protein stability and high optogenetic performance.

Light is one of the most essential factors for most organisms. On Earth, photoreceptor proteins have continuously evolved to use solar energy efficiently in organisms over the past 4.6 billion years. Rhodopsins, a type of photoreceptor protein, are known to exist in all domains of life (i.e. archaea, eubacteria, and euarya) (1), indicating their biological importance. Indeed, rhodopsins use light energy to exert a variety of biological functions, such as light-induced ion transportation and photosensing (2, 3). Despite such functional differences, rhodopsins are commonly composed of seven transmembrane α-helices (called helices A to G) with the retinal chromophore bound to a specific Lys residue in the seventh or G-helix via a protonated Schiff base linkage. Light absorption by rhodopsins triggers trans-cis or cis-trans isomerization of the retinal, and the photoisomerization induces structural changes of the transmembrane helices and soluble domains to exhibit their cognate biological functions. Rhodopsins are categorized into two types, type 1 for microbes and type 2 for animals (1).

Recently, a significant number of genes encoding type 1 rhodopsins have been identified from microbes living in a wide range of environments. Many of such newly discovered rhodopsins act as proton pumps in the cell membrane to produce adenosine triphosphate (ATP) upon photo-accumulation in collaboration with ATP-synthase (4, 5). Among the newly discovered rhodopsins, the eubacterial ones form the largest phylogenetic family. In 2012, a gene encoding a eubacterial proton pumping rhodopsin was identified in the genome of an extreme thermophile, *Thermus thermophilus* JL-18 strain, which was isolated in the Great Boiling Spring in the United States Great Basin at ~75 °C (6). Since that time, this protein, named thermophilic rhodopsin (TR), has been investigated to elucidate its molecular properties, including its light-driven proton pump activity, thermal stability, and photoconversion (7, 8). Those studies represent the first discovery and characterization of a rhodopsin from a thermophilic organism.

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1 The abbreviations used are: TR, thermophilic rhodopsin; MD, molecular dynamics; DDM, n-dodecyl-β-D-maltoside; LPC, lipidic cubic phase; BR, bacteriorhodopsin; AR3, archaerhodopsin-3; XR, xanthorhodopsin; SX, salinanxanthin; CCP, carbonyl cyanide 3-chlorophenylhydrazone; PDB, Protein Data Bank; r.m.s.d., root mean square deviation; RDF, radial distribution function.
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One of the physical characteristics of TR is its high thermal stability. TR maintains its visible color after incubation at 75 °C for more than 3 h even in the presence of a detergent (7). Because retinal is readily degraded above 50 °C (9), a robust protein structure is required to hold retinal inside the protein and retain its optimal function at the high temperature in the native habitat of *T. thermophilus* JL-18. Here, we confirmed the high thermal stability of TR compared with other microbial rhodopsins and characterized the robust neural silencing activity of TR as the first eubacterial rhodopsin available for optogenetics. Furthermore, we determined its x-ray crystal structure and elucidated possible mechanisms for its thermal stability together with the results of molecular dynamics (MD) simulations. These findings, which advance the understanding of ion transport mechanisms and the thermal stability of membrane proteins, should lead to the rational design of rhodopsin-based optogenetic tools.

Experimental Procedures

**Plasmid DNA Preparation**—The cDNA of the codon-optimized wild-type TR (7) was inserted into the pET21c expression vector (Novagen, Madison, WI). The cDNA of the codon-optimized TR mutant called TR-ΔLPGG, which was deleted Leu211-Pro212-Gly213-Gly214 sequence in the extracellular loop between the F- and G-helices from the wild-type TR, was purchased from GenScript Inc., Tokyo, Japan. The cDNA of TR-ΔLPGG was then inserted into the pET22b expression vector (Novagen). Ndel and Xhol restriction enzymes were used. The correctness of the constructed plasmid DNA was confirmed by dideoxy sequencing (Applied Biosystems, Forster City, CA).

**Preparation of TR for Crystallization**—TR was prepared essentially the same procedures as described previously (7). Codon-optimized TR was heterologously expressed in *Escherichia coli* BL21(DE3) cells. The protein expression was induced in the presence of 1 mM isopropyl β-D-1-thiogalactopyranoside (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and 10 μM all-trans-retinal (Sigma-Aldrich). TR was then purified from the crude fraction using a three-step column chromatographic method with the detergent n-dodecyl-β-D-maltoside (DDM): 1) Ni⁺⁺-nitritolitriacetic acid affinity chromatography using a HisTrap HP column (GE Healthcare), 2) anion-exchange chromatography using a HiTrap-Q HP column (GE Healthcare), and 3) size-exclusion chromatography using a Superdex-200 column (GE Healthcare).

The degree of purification was verified by SDS-PAGE and UV-visible absorption spectroscopy (data not shown). The purified TR showed an absorption maximum at 530 nm in DDM micelles without any other absorption band in the visible region. Based on data from SDS-PAGE and the absorption spectrum, the purity of TR was determined to be ∼95%.

The purified sample was concentrated, and the buffer was exchanged using Amicon Ultra centrifugal filter units (Merck Millipore). Thereafter, the sample was applied to a HisTrap FF column (GE Healthcare) that had been equilibrated using an Amicon Ultra filter (30,000 molecular weight cut-off, Merck Millipore). As a result, all samples for measurements of thermal stability contained the same medium composition (50 mM Tris-Cl (pH 7.7), 1M NaCl, ∼300 mM imidazole, and 0.05% (w/v) DDM). The purified TR-WT and TR-ΔLPGG were suspended in 50 mM Tris-HCl buffer (pH 7.0) containing 1M NaCl, 1M imidazole, and 0.05% DDM. As a result, all samples for measurements of thermal stability contained the same medium composition (50 mM Tris-Cl (pH 7.7), 1M NaCl, ∼300 mM imidazole, and 0.05% (w/v) DDM).

For the measurement of thermal denaturation by UV-visible spectroscopy, the concentration of each protein was adjusted to ∼1 μM. The measurement was carried out by a method similar to that reported previously (7). The optical density (OD) of each sample (≈0.1 OD) at the wavelength of the absorption maximum (530, 550, 548, and 552 nm for TR-WT and TR-ΔLPGG, BR, XR, and AR3, respectively) was plotted against time for the incubation. The data were analyzed by a single exponential decay function to estimate the denaturation rate constant. Three independent experiments were averaged.

**Optogenetic Control of Caenorhabditis elegans**—Transgenic *C. elegans* worms expressing AR3 or TR were generated by microinjection of DNA into the distal arms of gonads of N2 hermaphrodites as described previously (12–14). The plasmid *myo-3p::TR::gfp, aex-3p::AR3::gfp* or *aex-3p::TR::gfp* was injected with *pRF4* (rol-6d), a transgenic marker conferring the roller phenotype, to create these three lines. All-trans-retinal (Sigma-Aldrich) was taken up by feeding *C. elegans* warm on an agar plate in the presence of retinal as described previously (12, 13). Light-induced neural silencing was monitored by locomotion paralysis with green light (550 nm, 0.8 milliwell/mm² for...
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muscle silencing or 4.4 milliwatts/mm² for neural silencing) and was quantified by the change in body length as described previously (12, 13). In this system, the body was divided along its entire length into 11 segments by placing 12 dots at equal intervals. The sum of the length of each line connecting adjacent dots was defined as the body length of the worm. The average time for half of the body length change was calculated with standard error.

Crystalization, Data Collection, and Structure Determination—Purified TR was crystallized in lipidic cubic phase (LCP) at 23 °C as follows. The protein was diluted to ~34 mg/ml and mixed with monoolein (Nu-Chek Prep, Elysian, MN) at room temperature using a syringe mixing apparatus. The final protein:lipid ratio was 2:3 (w/w). After formation of a transparent LCP by overnight incubation at 23 °C, the mixture was dispensed on 96-well glass sandwich plates in a 50-nl drop and was overlaid with 800 nl of precipitant solution by a crystallization robot, Mosquito LCP (TTP Labtech Ltd., Melbourn, Hertfordshire, UK). TR crystals were grown in 100 mM sodium citrate (pH 3.6), 34% PEG 400 (v/v). Typical crystals matured to full size in 1 week. The crystals were harvested directly from the LCP using a MicroMount loop (MiTeGen LLC, Ithaca, NY) and were flash-cooled in liquid nitrogen.

TR crystals were screened at the BL-1A beamline at the Photon Factory and the BL32XU beamline at SPring-8 (15). Diffraction data were collected at BL-1A using a Pilatus 2M detector. The crystal was exposed to a 25-μm-wide, 10-μm-high beam for 0.5 s at 0.5° oscillation/frame with a 2-fold attenuation at a wavelength of 1.1 Å. Diffraction images were processed by XDS (16); the statistics are summarized in Table 1. General data manipulation was performed using the CCP4 suite of programs (17).

The structure was solved by molecular replacement using the structure of XR (18) (PDB ID: 3DDL) as a search model with the program Phaser (19). The correct molecular replacement solution contained four TR molecules packed in parallel in the asymmetric unit. Manual model building was performed in Coot (20) during iterative refinement. Early stage refinement was carried out with Refmac5 (21) using the jelly-body option. The later stages of refinement were performed using autoBUSTER (22) and Phenix (23) with TLS (translation-libration-screw), XYZ coordinates, and individual B-factor refinement. Structural analysis using Rampage (24) and the final refinement statistics are summarized in Table 1. Figures for the three-dimensional structures (Figs. 2, a, c, d, and e, and 5) were prepared using PyMOL (Schrödinger LLC), and figures for the electrostatic potential surfaces (Fig. 4, e and f) were prepared using CueMol2 with PDB2PQR (25) and APBS (26). The intra-molecular interactions of TR were analyzed using the PIC (Protein Interaction Calculator) Web server (27) using default parameters.

Molecular Dynamics Simulation—All MD simulations were performed with NAMD (versions 2.8 and 2.9) (28) under isothermal isobaric conditions using CHARMM22/CMAP protein force field (29, 30), CHARMM36 lipid force field (31), and a TIP3P water model. The previously determined force field of the retinal protonated Schiff base was employed for the chromophore (32, 33). Long-range electrostatic interactions were treated using the particle mesh Ewald method. The short-range interactions were cut off at 9 Å with a smoothing function ranging between 8 and 9 Å. The initial TR structure was taken from the x-ray crystallographic structure reported in the present study. Water molecules were placed by the DOWSER program (34) to fill internal cavities in TR. Glu106 was protonated according to the pKa value predicted by the PROPKA program (35). A monomer protein of TR was embedded into a lipid bilayer solvated in a water solution containing 150 mM NaCl added with an Autoionize plugin for VMD. The simulation system consisted of 206 POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine) lipids, 20,202 waters, 58 Cl⁻ ions, and 58 Na⁺ ions. The simulation box size was ~94 × 77 × 121 Å so that the closest distance between the proteins in the periodic images was sufficiently large (>50 Å), indicating that the numbers of lipid and water molecules in the simulation box was sufficient. The entire system was equilibrated at 300 K under 1 bar for 50 ns followed by the production runs. MD simulations were performed at two different temperatures, i.e. at low temperature (300 K) and at high temperature (348 K) starting with different initial coordinates and velocities taken from the trajectory at 60 ns in the production simulation at 300 K. The conformational changes characterized by the Cα distance matrices observed in the two independent MD simulations at 348 K (data not shown) were essentially the same, confirming the reproducibility and consistency of the thermally induced conformational changes.

Measurement of Proton Pump Activity—The proton pump activity was measured by the light-induced pH changes of E. coli suspension as described previously (7). Briefly, TR-expressed E. coli BL21(DE3) cells were washed three times in 100 mM NaCl and then suspended in the same solution. The pH change was monitored by using a LAQUA F-72 pH meter equipped with a micro ToupH pH electrode (HORIBA, Ltd., Kyoto, Japan). The cell suspension was kept in the dark and then illuminated for 3 min with a Xe light source, MAX-302 (Asahi Spectra Co., Ltd., Tokyo, Japan), equipped with a 520 ± 10-nm band-pass filter for TR-WT and the TR-ΔLPGG mutant. When necessary, a proton-selective ionophore, carboxylic cyanide 3-chlorophenylhydrazone (CCCp, Sigma-ALdrich), was used.

Results and Discussion

Thermal Stability of TR

The proton-pumping rhodopsins are divided into distinct archaeal, eubacterial, and eukaryotic groups (Fig. 1a). Fig. 1b shows the time-dependent decrease in visible absorption, which is considered thermal denaturation and therefore was used to measure the thermal stability of the rhodopsins. As reported previously, TR maintains its visible color even when it is incubated at physiological temperature for T. thermophilus (75 °C) for several hours (Fig. 1b) (7). To compare its thermal stability with other proton-pumping rhodopsins, we performed similar experiments with BR from the archaeon H. salinarum, archaerhodopsin-3 from the archaeon Halorubrum sodomense,
and xanthorhodopsin from the eubacterium *S. ruber* (Fig. 1, *a* and *b*). Of note, we succeeded in expressing carotenoid-free XR using a cell-free protein expression system (11). As shown in Fig. 1b, BR, AR3, and XR lost their visible color within 20 min at 75 °C. The denaturation rates were estimated by fitting to a single exponential function. These experiments reveal that the thermal stability of TR (denaturation rate constant, $8.0 \times 10^{-3}$ [min$^{-1}$]) is much higher (20–100-fold) than those of the others (denaturation rate constants, $1.6 \times 10^{-1}$ [min$^{-1}$] for XR, $5.2 \times 10^{-1}$ [min$^{-1}$] for BR, and $6.1 \times 10^{-1}$ [min$^{-1}$] for AR3) (Fig. 1c), although the functionally important residues located in the proton transport pathway are conserved among them (Fig. 2).

**Applicability of TR as an Optogenetic Tool**

In 2010, Chow *et al.* reported that proton-pumping rhodopsins can be applied to optogenetics technology as light-dependent neural silencers (36). They found the archaeal AR3 to be the most powerful among the seven proton pumps tested (36). Here, to investigate the availability of TR as a silencer of excitable cells, we first expressed TR as a fusion construct with green fluorescent protein (GFP) in body wall muscle cells of *C. elegans*, which drive locomotion of worms, under the control of a promoter of the myosin heavy chain gene *myo-3* (12–14). Then the locomotion of the worms was measured with and without green light (550 nm), which overlaps the absorption spectra of both TR (530 nm) and AR3 (552 nm). Green light illumination of worms expressing TR-GFP led to locomotion paralysis, and the body length was increased significantly during 1 s of illumination, as shown in Fig. 1e. Upon switching off the light, the body length immediately returned to normal. The increased body length indicates that the paralysis was elicited by the relaxation of the body wall muscles, consistent with the presumed silencing ability of TR.

We next expressed GFP-tagged TR and AR3 in neurons by using a pan-neuronal promoter. As shown by the *green-colored* cells in Fig. 1d, GFP signals were clearly observed in the soma and the axon of many neurons in the head, mid body, and tail for both TR and AR3, indicating their successful expression. Similar to the worms carrying the transgene *myo-3p::TR*, green light illumination led to locomotion paralysis with ~5% increased body length in worms carrying either TR or AR3 (Fig. 1e). These changes are likely to be mediated by the silencing of motor neurons, which govern muscular contraction, as the paralysis was associated with an increase in the body length. The body length immediately returned to normal upon switching off the light. The time for half of the body length change, indicating the apparent neural silencing activities of the rhodopsins, was then calculated, and their averages are shown in Fig. 1f with *error bars*. Because the wavelength of light used here (550 nm) corresponds well to the absorption maximum of AR3 (552 nm) rather than TR (530 nm), we concluded that TR receives the light with a lower efficiency than AR3. These results indicate that TR is an efficient and powerful neural silencer, comparable to AR3. Although a light-driven chloride-pumping rhodopsin, halorhodopsin, is also used as a neural silencer in the optogenetics technique (37), the silencing activity of halorhodopsin is comparably smaller than that of AR3.

Some eubacterial proton pumps can bind and use carotenoids as a secondary chromophore for their photoreactive functions (38, 39). The amino acid residues necessary for carotenoid binding are also conserved in TR, as described in the following section. Therefore, in addition to the availability of
TR itself, the TR-carotenoid complex will be available for optogenetics studies in the near future. A recent report has demonstrated the availability of animal rhodopsin and melanopsin for the optogenetics of *C. elegans* worm (40). The technique is advantageous for direct monitoring of the cellular behavior caused by the coupling reaction between these proteins and their cognate G proteins. On the other hand, microbial rhodopsins including TR can directly depolarize or hyperpolarize the host neurons without involving other cellular responses. From the point of basic molecular properties, microbial rhodopsins are advantageous for duplicate measurements because these molecules can continue to hold the retinal chromophore after one photoreaction. Although the use of microbial and animal rhodopsins in optogenetics has both advantages and disadvantages, the usefulness of TR shown here would give the researchers valuable information. To understand and utilize those properties, we determined the three-dimensional structure of TR at an atomic resolution as described below.

**Structure Determination of TR and Comparison with XR and BR**

TR was crystalized into LCP. The structure was solved by molecular replacement using XR structure (18) as a searching model and was determined at 2.8 Å resolution (PDB ID: 5AZD). In the crystal, the asymmetric unit contains four TR molecules (denoted as chains A–D) that are almost identical in structure (data not shown). Therefore, we used the data from chain A in all figures (Figs. 3–5) and in the following discussion; the structural statistics of TR are summarized in Table 1. Similar to the known structures of other microbial rhodopsins, the overall structure of TR is composed of seven transmembrane helices, with the all-trans-retinal chromophore covalently bound to Lys233 at the center of the G-helix via a protonated Schiff base linkage (Fig. 3a) (1). Note that about 50% of Asp95, one of the putative counterions for the protonated Schiff base, is protonated at pH 3.6 for the crystallization, because the pKₐ for Asp95 was determined to be 3.4 in our previous study (7).

As shown in Fig. 3b, the structure of TR was compared with the archaeal proton pump XR (18) by superimposition of those structures. The structure of TR was similar to that of XR (r.m.s.d. 1.11 Å), as expected from the high sequence identity and similarity between TR and XR (57% and 85%, respectively). It has been reported that XR binds a carotenoid molecule called SX as a secondary chromophore in the crystal structure because XR was purified from the native habitat having a carotenoid synthesis system (38). In the XR-SX complex, light energy transfer from SX to the retinal in XR occurs (38).
The residues responsible for SX binding, including a critical Gly residue (Gly152 in TR) (18, 39), are completely conserved in TR, and SX can be fitted into the structure of TR with an appropriate energy-transferable distance between SX and retinal (Fig. 3c) (18). Thus, in addition to the characteristics described above, TR is assumed to have a putative carotenoid-binding cavity in the E-helix (Fig. 3c). Because SX has a broad and blue-shifted absorption spectrum with a large molecular extinction coefficient, the TR-SX complex would be useful for optogenetics as a molecule that absorbs a wide range of the visible region with high efficiency. Notably, it has been reported that the native *T. thermophilus* has a carotenoid synthesis system and a kind of carotenoid named thermozeaxanthin, which shares a similar structure with SX, as a major component (42). Thus, it is assumed that TR has a potential carotenoid binding ability. Further studies to confirm the carotenoid binding are now under way.

Regarding the proton pumping function of TR, Fig. 3d shows an expanded view of the putative proton transport pathway in TR. As with the other proton-pumping rhodopsins, in TR there are two putative counterions, Asp95 and Asp229 (7), for the protonated Schiff base nitrogen of Lys233. When aspartate works as a primary proton acceptor, a point mutation of that residue (Gly152 in TR) (18, 39), are completely conserved in TR, and SX can be fitted into the structure of TR with an appropriate energy-transferable distance between SX and retinal (Fig. 3c) (18). Thus, in addition to the characteristics described above, TR is assumed to have a putative carotenoid-binding cavity in the E-helix (Fig. 3c). Because SX has a broad and blue-shifted absorption spectrum with a large molecular extinction coefficient, the TR-SX complex would be useful for optogenetics as a molecule that absorbs a wide range of the visible region with high efficiency. Notably, it has been reported that the native *T. thermophilus* has a carotenoid synthesis system and a kind of carotenoid named thermozeaxanthin, which shares a similar structure with SX, as a major component (42). Thus, it is assumed that TR has a potential carotenoid binding ability. Further studies to confirm the carotenoid binding are now under way.

During the reaction, His11 and Arg92 are thought to be involved in the maintenance of both the pKₐ values of the charged residues and the proper structural changes of the peptide backbone analogous to the eubacterial proton pumps including XR (18, 43–46). Unfortunately, we cannot discuss the structural details of TR at the atomic level by precisely comparing them with XR or other microbial rhodopsins because of the present modest resolution (2.8 Å). However, the proton-translocating
mechanism of TR is basically the same as those of conventional proton-pumping rhodopsins (8). The improvement in the crystallization of TR to achieve a much better resolution is currently in progress.

**Thermal Stabilization Mechanism Suggested by the Structural Comparison**

As shown in Fig. 1, b and c, TR showed a 20-fold higher thermal stability than XR. There are several potential reasons why TR is more stable than XR, which are discussed here in the context of their structural comparison. Fig. 4a shows the superimposed structures of TR and XR. The identical residues, shown in white, are especially localized to the inside of the helices, which are composed of the proton transport pathway. In contrast, non-identical residues, shown by color, are found mainly on the exposed surfaces of the proteins. From the comparison, it was revealed that TR has 172 hydrophobic residues involving in 297 hydrophobic interactions, the number of which is greater than that of XR, as shown in Fig. 4b. It is noteworthy that seven non-identical aromatic residues (Phe9, Trp13, Tyr29, Phe66, Phe160, Tyr161, and Phe241) of TR form aromatic-aromatic interactions, whereas only two non-identical residues (Phe20 and Tyr248) of XR contribute to such interactions (Fig. 4, c and d). On the other hand, TR has a lesser number of polar amino acids than XR (66 and 87, respectively), indicating that the number of polar interactions in TR is also less than in XR (Fig. 4b). These findings suggest that the high thermal stability of TR is achieved by the hydrophobic and aromatic-aromatic interactions within the protein and with the membrane lipid molecules surrounding TR under the native environment.

**Atomic Mechanisms of the Thermal Stability**

To examine the structural and dynamic behaviors of TR at high temperature, we performed MD simulations by using TR structure in the technically well established lipid bilayer system at 348 K (75 °C) and compared them with those at an ambient temperature (300 K) (27 °C) for 1-μs duration. An increase in the temperature from 300 to 348 K induced a distinct conformational transition to a characteristic conformational substate where the r.m.s.d. values of the Cα atoms of the protein in the trajectories with respect to the x-ray structure were significantly altered (Fig. 5a). Although the r.m.s.d. at 300 K does not vary greatly from ~1.4 Å over the entire simulation time, the r.m.s.d. values of the two independent MD trajectories at 348 K exhibit drastic increases from ~1.4 to 2.2 Å within 200 ns after heating from 300 K (data not shown), indicating that TR undergoes a thermally induced conformational transition upon heating to 348 K. The thermally induced conformational changes are spatially extensive and involve at least three concerted local structural changes, which could contribute to stabilizing the conformational substate at that high temperature (Fig. 5b) as follows.

**LPGG Sequence in the FG Loop**—The structural changes were found in the extracellular side of the F-helix and in the extracellular loop between the F- and G-helices (called the FG loop (Fig. 5, c and d)). The loop includes a Leu211-Pro212-Lys213-Gly214-Gly215 (LPGG) sequence, which is absent in BR and XR (Fig. 2). At 300 K, the F- and G-helices are nearly parallel and distant from each other. Accordingly, the FG loop forms in an extended conformation to the bulk water environment (Fig. 5, c and d), which can be monitored by the distance between the Cα atoms of Leu211 and Gly214 at both ends of the FG loop connecting the F- and G-helices (Fig. 5, c and g) and by the radial distribution function (RDF) of water molecules from the Cα atom of Leu211 located at the beginning of the FG loop (Fig. 5i). Upon heating to 348 K, on the other hand, the extracellular side of the F-helix moves toward the G-helix, and the FG loop intrudes into the hydrophobic space between the F- and G-helices (Fig. 5, c and d) establishing hydrophobic packing among them, accompanied by the exclusion of water molecules (Fig. 5i). The hydrophobic interaction in the extracellular region is considered to be formed by a gain of translational and rotational entropy and thus is more enhanced at a higher temperature.

**Hydrophobic Core around Retinal**—The movement of the F-helix toward the G-helix leads to the formation of a hydrophobic core around the retinal (Fig. 5e), accompanying the movement of the C-helix (Fig. 5d) and the exclusion of water molecules from this region, which can be measured by the RDF of water molecules from the Cn2 atom of Phe246 in the C-helix, located proximal to the retinal (Fig. 5f). The formation of the hydrophobic core is considered to stabilize the conformational
substate at the higher temperature. To clarify the structural changes of TR upon thermal stimulation experimentally, spin-label EPR experiments and fluorescence measurements of Trp residues around the retinal chromophore are useful.

**Hydrogen Bond**—In addition, a hydrogen bond formation between the C- and D-helices at high temperature was observed. As a result of the displacement of the C-helix, the main-chain carbonyl group of Leu211, located around the center of the C-helix, which lacks a hydrogen bond partner at low temperature because of a kink in the C-helix introduced by Pro101, approaches the hydroxyl group of Ser129 in the D-helix (Fig. 5h) and forms an inter-helix hydrogen-bond with it (Fig. 5f).

As described above, TR has a large number of hydrophobic residues and characteristic aromatic residues involved in the aromatic-aromatic interaction (Fig. 4, b and c). Such unique structural properties, revealed by the x-ray structure and the structural transitions that enhance the hydrophobic interaction upon the thermal stimulation suggested by MD simulations, are a possible mechanism for the thermal stabilization of TR at high temperature. Note that no sequence similarity of the FG loops of the possible mechanisms of high structural stability, we then prepared the mutant TR, which was the deleted Leu211-Pro212-Gly213-Gly214 sequence in the FG loop from the wild-type TR (named TR−LPGG (Figs. 5c and 6a)). The TR−LPGG mutant was functionally expressed in E. coli cells, showed outward proton pumping activity, and exhibited an absorption maximum at 530 nm in the presence of DDM, almost the same as the wild-type TR (Fig. 6b and c). We then measured the thermal stability of the ΔLPGG and wild-type TR according to the same procedure reported previously (7). Fig. 6d and e, shows the time-dependent decrease in absorption at 530 nm at 75°C. The TR−ΔLPGG mutant was almost denatured in 210 min, whereas more than 60% of the wild-type TR remained. From the denaturation kinetics (Fig. 6f), the data of which were analyzed by the single exponential function, we determined the denaturation rate constants as (2.54 ± 0.10) × 10−3 min−1 for the wild-type TR and (1.06 ± 0.03) × 10−2 min−1 for the TR−ΔLPGG mutant, respectively (Fig. 6g).

These data indicate that the deletion of the LPGG sequence in the FG loop affected the thermal stability of TR. We presume that the deletion of LPGG suppressed the conformational transition of the FG loop and the consequent formation of the tight packing caused by hydrophobic interactions at high tempera-
we determined the crystal structure of TR at 2.8 Å resolution. This gave us information about the structure with a putative secondary chromophore-binding cavity at atomic resolution. Combined with the MD simulations, we assume the importance of the hydrophobic interactions in TR as contributing to its high thermal stability. Thus these findings will be valuable for understanding and utilizing retinal proteins in respect to high protein stability and high optogenetic performance.

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