Sodium-pumping rhodopsins (NaRs) are membrane transporters that utilize light energy to pump Na⁺ across the cellular membrane. Within the NaRs, the retinal Schiff base chromophore absorbs light, and a photochemically induced transient state, referred to as the “O intermediate”, performs both the uptake and release of Na⁺. However, the structure of the O intermediate remains unclear. Here, we use time-resolved cryo-Raman spectroscopy under preresonance conditions to study the structure of the retinal chromophore in the O intermediate of an NaR from the bacterium Indibacter alkaliphilus. We observed two O intermediates, termed O1 and O2, having distinct chromophore structures. We show O1 displays a distorted 13-cis chromophore, while O2 contains a distorted all-trans structure. This finding indicates that the uptake and release of Na⁺ are achieved not by a single O intermediate but by two sequential O intermediates that are toggled via isomerization of the retinal chromophore. These results provide crucial structural insight into the unidirectional Na⁺ transport mediated by the chromophore-binding pocket of NaRs.

The movements of substances into or out of cells are achieved via transmembrane proteins, known as membrane transporters. The polar molecules and charged ions, for instance, are prevented from entering the hydrophobic interior of cellular membranes. For these substances, the only way to traverse the cellular membrane is via a route provided by the membrane transporters. This transportation involves the uptake of a substrate from one side of the membrane and its release to the other side. These two steps are believed to require a distinct structural change of the protein to switch the accessibilities of the substrate from one side and the other (1). However, this structural change of the transporting process has not been observed for most proteins.

Discovered in 2013, sodium-pumping rhodopsins (NaRs) are unique photoreceptive membrane transporters that function as light-driven Na⁺ pumps (2). NaRs are rhodopsin-like photoreceptors that have seven transmembrane helices (A, B, ..., and G) as shown in Fig. 1A, where the retinal molecule is bound to the lysine residue on the helix G via a Schiff base linkage. This retinal Schiff base (RSB) chromophore is protonated and has the all-trans form before absorption of a photon (Fig. 1B). Then, photoabsorption induces the isomerization of the chromophore into the 13-cis form, and the subsequent chemical changes lead to Na⁺ transport from the cytoplasm to the extracellular side. This photoreaction is a cyclic process known as a photocycle and it produces multiple intermediate states typically expressed as NaR + hν → K → L/M → O → NaR (2–4). The K intermediate is the first state formed with a distorted 13-cis form (5). The next state, the L intermediate, is generated by structural relaxation of the distorted chromophore (6). This L intermediate is in equilibrium with the M intermediate, where the Schiff base moiety of the chromophore is deprotonated. Then, the recovery to the dark state is mediated by the O intermediate, which finally transports Na⁺ across the membrane.

Of these multiple photointermediates, the O intermediate is recognized to play the most crucial role in membrane transport of Na⁺ (7). So far, most studies assumed a single state for the O intermediate. In an early transient absorption study, the redshifted absorption band was observed in the millisecond region, and this band was attributed to the O intermediate (2). Then, FTIR, Raman, and NMR studies of NaRs were conducted and reported the single O-intermediate spectrum (8–11). However, recent studies came to find the evidences of multiple O intermediates. The X-ray crystallographic studies of an NaR from Krokinobacter eikastus (KR2), which were independently carried out by Skopintsev et al. (12) and Kovalev et al. (13), reported the different structures of the O intermediate. The former study by Skopintsev et al. also measured the time-resolved UV-visible and IR absorption spectra to report two O intermediates from a global fit analysis. We recently measured the time-resolved absorption spectra of a NaR from Indibacter alkaliphilus (lnNaR) and revealed the clear presence of two O intermediates, O1 and O2, exhibiting distinct spectra and kinetics (14). The photocycle of lnNaR is shown in Fig. 1C, where O1 (lifetime: 2.4 ms) has an absorption maximum (λmax) at 596 nm, while the subsequently formed O2 (lifetime: 7.6 ms) has a λmax at
Retinal reisomerization switches Na$^+$ uptake/release by NaR

Figure 1. Structure and photocycle of NaR. (A), crystal structure (PDB entry: 6REW). (B), chemical structure of the retinal Schiff base chromophore. (C), photocycle of lNaR from ref. (14). The time constants shown with the photointermediates are obtained at 298 K in Tris–HCl buffer (pH 8) with 50 mM NaCl. NaR, sodium-pumping rhodopsin; lNaR, NaR from *Indibacter alkaliphilus.*

Figure 2. Photoreaction of lNaR at 268 K and room temperature. (A), time-resolved absorption spectra of lNaR at 268 K. The time resolution is 1 s. See Experimental procedures for details. (B), time-resolved absorption spectra of lNaR (with 50 mM NaCl) measured in our previous nanosecond flash photolysis study (14) at room temperature. The spectra are plotted at selected time points—namely, 2.0 ms (red), 7.1 ms (blue), 30 ms (purple), and 135 ms (cyan)—and O1, O2, N, and NaR make the major contributions to these four spectra, respectively (14). NaR, sodium-pumping rhodopsin; lNaR, NaR from *Indibacter alkaliphilus.*

Results

We first describe the photoreaction of *l*NaR at 268 K based on the time-resolved absorption data. Fig. 2A shows the time-resolved absorption spectra at 268 K. In this measurement, the *l*NaR sample was irradiated by green light (at 532 nm) for 1 s and the light-induced absorbance changes were measured at the delay times ($\Delta T$s) up to 240 s with the time resolution of 1 s. The spectrum at $\Delta T = 0$ s was measured at the photostationary state under continuous irradiation. As seen from the figure, the green light irradiation produced the redshifted absorption component, which decayed in a few hundred seconds. The spectra also exhibited the small shifts with $\Delta T$. The time-resolved spectra showed an $\sim 10$-nm blueshift from $\Delta T = 0$ to 1 s and it then slightly redshifted afterward. Previously, we carried out the nanosecond flash photolysis of *l*NaR at room temperature (14) and observed the redshifted intermediates in the millisecond region as shown in Fig. 2B. The detailed kinetic analysis revealed that the millisecond process includes the four redshifted intermediates referred to as O1, O2, N, and NaR’ (Fig. 1C). In Fig. 2B, the spectral change from 2 to 7 ms is mainly caused by the O1 → O2 process. The spectral change after 7 ms is then attributed to the subsequent process of O2 → N → NaR’. The former spectral change at room temperature shows close similarity with that from 0 to 1 s at 268 K, and the latter spectral change corresponds to that after 1 s at 268 K. Thus, the time-resolved absorption data at 268 K confirms the production of the O intermediates and the subsequent transient states with their prolonged lifetimes.

Fig. 3 shows the time-resolved Raman spectra at 268 K in comparison to the spectrum for the unphotolyzed state. These
spectra were measured by 785-nm excitation and obtained in the same way as we did for the time-resolved absorption measurement; the IaNaR sample was photoirradiated at 532 nm for 1 s and the Raman spectra were measured from ΔT = 0 s to 240 s with the time resolution of 1 s. The photostationary state was measured for ΔT = 0 s. In the figure, the vibrational modes of the RSB chromophore were selectively observed due to the preresonance enhancement. Before a green-light irradiation, the unphotolyzed state exhibited the intense C=C stretch (1533 cm⁻¹), C-C stretch (1168 and 1200 cm⁻¹), and CH₃ rocking (1006 cm⁻¹)(17, 18). In addition, the unphotolyzed state shows the moderately strong bands assigned to the C=N stretch (1643 cm⁻¹), CH in-plane bending (1270–1350 cm⁻¹), and hydrogen out-of-plane (HOOP) modes (826, 879, 970 cm⁻¹)(17, 18). The double peaked pattern of C-C stretching modes is characteristic of the all-trans form of the chromophore. This observed spectrum of the unphotolyzed state agrees with those reported previously (5, 6, 9, 19).

Then, after the irradiation by green light, the Raman spectra of photointermediates appeared. As shown in the figure, the photointermediate showed the C-C stretches with three peaks at 1173, ~1200, and 1216 cm⁻¹. The C=C stretch of the photointermediate was significantly downshifted relative to that of the unphotolyzed state (e.g., 1524 cm⁻¹ at ΔT = 1 s versus 1533 cm⁻¹ at unphotolyzed state). As ΔT increased, these spectral features showed the changes that appear to consist of two phases. The first phase corresponds to the spectral change at ΔT = 0 to 1 s, where the C=C stretch shifted by +4 cm⁻¹ and the intensity pattern in the C-C stretching region (1150–1250 cm⁻¹) slightly changed. The second phase is after ΔT = 1 s, as a large intensity decrease was observed for the HOOP modes and the C-C stretch at 1173 cm⁻¹.

In Fig. 4, we provide an analysis of the Raman spectra in the first phase, that is, ΔT = 0 and 1 s (traces a and b). On the basis of the time-resolved absorption data (Fig. 2), this time region corresponds to the O1 → O2 process. The spectra at 0 and 1 s are characterized by the downshifted C=C stretch, the triple-peaked C-C stretches, and the intense HOOP modes. First, the C=C stretching frequency (νC=C) is known to correlate with the maximum absorption wavelength (λ maxi); +1 cm⁻¹ shift of νC=C generally corresponds to ~4 nm shift of λ maxi (20–23). Thus, the downshifted C=C stretch is consistent with the redshift of the absorption band at 0 and 1 s. Next, the intensity pattern of the C-C stretch is sensitive to the geometry of the chromophore. This observed spectrum of the unphotolyzed state agrees with those reported previously (5, 6, 9, 19).

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of the polyene chain (17, 18, 24). The C-C stretching bands have three peaks at 1173, ~1200, and 1216 cm\(^{-1}\). For purposes of comparison, we measured the O intermediate of bacteriorhodopsin (BR) and plotted its spectrum in the figure (trace c). As seen, the O intermediate of BR shows the C-C stretches whose intensity patterns are very similar to the time-resolved Raman spectrum of \(\text{IaNaR}\), especially at \(\Delta T = 1\) s. The O intermediate of BR has the all-trans chromophore (25). This indicates that the all-trans chromophore makes a major contribution in the time-resolved spectrum at 1 s. Moreover, the intense HOOP modes were observed at 794 and 954 cm\(^{-1}\). These modes have been assigned to the HOOP vibrations of C14H and HC11=C12H (17, 18), the intensities of which are these modes have been assigned to the HOOP vibrations of C14H and HC11=C12H (17, 18), the intensities of which are

Table 1 lists the vibrational frequencies and \(\lambda_{\text{max}}\) values characteristic of O1 and O2. In the previous time-resolved absorption study of \(\text{IaNaR}\), we estimated that the \(\lambda_{\text{max}}\) values of O1 and O2 were 596 and 570 nm, respectively, at room temperature (14). The 26 nm blueshift of \(\lambda_{\text{max}}\) is consistent with the 7 cm\(^{-1}\) upshift of \(\nu_{\text{C=C}}\) from O1 to O2, considering the general correlation between \(\nu_{\text{C=C}}\) and \(\lambda_{\text{max}}\) (Supporting Information, Fig. S3). We also carried out the experiment at lower temperatures (250 and 260 K) and confirmed that the intermediate, which we assigned to O1 at 268 K, was actually produced from the L-like precursor (Supporting Information, Fig. S4).

Then, we examine the time-resolved Raman spectra in the second phase (\(\Delta T = 1–240\) s) in Fig. 5. As described above, the
Retinal reisomerization switches Na⁺ uptake/release by NaR

Discussion

From the time-resolved Raman spectra of IaNaR at 268 K, the multiple redshifted intermediates (O1, O2, and N), which have distinct chromophore structures, are revealed. The vibrational spectra of these photointermediates showed that O1 has the distorted 13-cis chromophore, O2 has the distorted all-trans chromophore, and N has the relaxed all-trans form. This result also points out that the structures of the O intermediates reported to date should be reconsidered because a single O intermediate was assumed in most of previous studies of NaRs. The early light-induced FTIR measurement of an NaR from Gillisia limnaea revealed the O intermediate with all-trans chromophore under photostationary state (10), and the spectrum was close to that of the O intermediate of Gillisia limnaea (vide supra). Recent QM/MM (quantum mechanical/molecular mechanical) calculations showed that the Na⁺ binding to these sites causes the significant redshift of the absorption band of the O intermediates (29). We consider that either O1 or O2 was almost selectively observed in these studies. Normally, it would be natural to assume that Na⁺ moves to an extracellular site from O1 to O2 transition (29, 30). Therefore, in terms of the Na⁺-binding position, the one structure (PDB ID: 6XYT) may correspond to O1 and the other (PDB ID: 6TK2) to O2. If such is the case, however, the puzzling point is that the chromophore structures are inconsistent with our observation. As both groups discussed the coexistence of all-trans and 13-cis forms (12, 13), the chromophore structures in these crystallographic studies need to be carefully interpreted.

The chromophore structure of O1 and O2 can provide crucial insight about the Na⁺ pumping mediated by the O intermediates. Our time-resolved absorption and membrane potential measurements revealed that O1 is responsible for Na⁺ uptake, O2 is responsible for Na⁺ release, and the O1 → O2 transition was irreversible (7, 14). The present Raman study indicates that the reisomerization of the RSB chromophore acts as the irreversible switch between the Na⁺ uptake and release states. During the Na⁺ pumping through O1 and O2, an Na⁺-binding site inside the protein needs to be accessible from the cytoplasmic side only in the uptake process (L/M → O1), and the binding site then must be open to the extracellular side in the release process (O2 → N). In Fig. 6, we propose a molecular model of this Na⁺ transport for IaNaR. In this model, Na⁺ passes in close proximity to the neutral deprotonated chromophore in the M/L state, and it binds to the D115 (D116 in KR2) in O1. For this binding site, we referred to the crystal structure (PDB ID: 6XYT) solved by Kovalev et al. (13) (vide supra). The protonated 13-cis chromophore in O1 is considered to flip the positively charged Schiff base moiety opposite to D115. Thus, the formation of the electrostatic pair of Na⁺ and D115 is temporarily allowed. In O1, however, the protonated Schiff base cannot be stabilized due to the lack of efficient electrostatic interaction with the counter ion. This situation may drive the isomerization of the chromophore to interact with D115 and produce O2. In forming O2, the all-trans chromophore points the Schiff base moiety toward D115, which destabilizes the Na⁺ binding to D115 and causes Na⁺ to move to the D250 site (D251 in KR2). We referred to the crystal structure (PDB ID: 6TK2) solved by Skopintsev et al. (12) (vide supra) for the interaction between Na⁺ and D250. When Na⁺ binds to D250, the ionic hydrogen bond (or salt bridge) of D250-R108 would be transiently broken. This would make the binding site open to the extracellular side. Then, Na⁺ is finally pumped to the outside of the membrane in the O2 → N step. In the N intermediate, we assume that the R108-D250 salt bridge is reformed to prevent the backflow of Na⁺. This protein structural change may be
associated with the relaxation of the chromophore conformation.

**Conclusion**

We used time-resolved Raman spectroscopy to study the structures of O intermediates of IaNaR at 268 K. The present time-resolved Raman experiment under the preresonance condition allowed us to observe high quality vibrational spectra of three intermediates. These intermediates have distinct chromophore structures, and they were attributed to O1, O2, and N intermediates. O1 has the distorted 13-cis chromophore while O2 has the distorted all-trans form. The subsequent N has the relaxed all-trans chromophore. This study indicated that the thermal reisomerization of the chromophore occurs during the O1 → O2 process, where O1 is responsible for Na⁺-uptake and O2 is responsible for Na⁺-release. The irreversibility of the O1 → O2 step, which is key for the unidirectional Na⁺ pump, is also realized by the reisomerization of the RSB chromophore. The isomerization of the RSB chromophore is considered to drastically change the electrostatic interactions in the active site to translocate Na⁺. The O2 → N process is associated with the chromophore structural relaxation. This structural relaxation of the chromophore should involve the protein conformational change to prevent the backflow of Na⁺.

**Experimental procedures**

The Raman spectra were measured using a continuous wave laser (08-NLD(M), Cobolt) operating at 785 nm. The laser power at the sample was 350 mW. The back scattered light from the sample was collected and dispersed by a 30 cm polychromator with a 600 groove/mm grating (Acton SP-2300; Princeton Instruments). The spectrum was then measured by a charge-coupled device detector (Pixis 256E; Princeton Instruments). Using this setup, the preresonance Raman spectra of IaNaR were obtained with a resolution of 8.5 cm⁻¹. The time-resolved Raman measurement of IaNaR was carried out at 268 K. At this temperature, the photocycle of IaNaR was slowed enough that the lifetimes of photointermediates were extended for a duration on the order of seconds. To perform the time-resolved measurement, we irradiated the sample with a 532 nm light-emitting diode (LED) (0.38 mW; thorlabs) for 1 s, and the Raman spectra were collected with an exposure time of 1 s at the selected time delays (ΔT’s). The time resolution was therefore 1 s. The photostationary state under continuous irradiation was measured for ΔT = 0. The Raman spectra of photointermediates were obtained by subtracting the unphotolyzed component from the spectra after light irradiation (Supporting Information, Figs. S1 and S2). We also checked the LED-irradiation power dependence of the Raman spectrum and confirmed that there was no photoproduct due to a multiphoton process (Supporting Information, Figs. S1 and S2). For a comparison, we prepared a BR sample using a 10 mM citrate buffer solution (pH 4.0) and measured the O intermediate under a photostationary state at room temperature (31).

The time-resolved absorption measurement was also performed at 268 K. We used a white LED (Edison) as the probe light. After the sample was irradiated by the 532-nm LED for 1 s, the probe light transmitted through the sample was dispersed by a 30 cm polychromator (with a 300 groove/mm grating), and the spectrum was measured by the charge-coupled device detector with an exposure time of 1 s at the selected ΔT’s. The photostationary state was measured for ΔT = 0. The light-induced absorbance change was calculated from the spectra of transmitted lights before and after the photoirradiation, and the time-resolved absorption spectra were obtained.

The IaNaR sample was prepared as reported previously (6). Briefly, IaNaR, having a C-terminal histidine tag, was expressed in Escherichia coli strain BL21 by using the pKA001 vector which contains IaNaR gene (GenBank accession number: EQZ93469) under the lacUV5 promoter. The cells were disrupted by sonication and the membrane fragments were collected by ultracentrifugation. After solubilization with the detergent n-dodecyl β-D-maltopyranoside, IaNaR was purified using a nickel–nitritoltriacetic acid agarose column. The purified sample was reconstituted in Egg-PC liposome at a protein:

**Figure 6. Molecular model of Na⁺ translocation mediated by O intermediates in IaNaR.** CP, cytoplasm; EC, extracellular side; and RSB, retinal Schiff base. The amino acid sequence numbers in IaNaR are used. IaNaR, NaR from Indibacter alkaliphilus.
lipid molar ratio of 1:50 and stored in a 50 mM Tris buffer solution (pH 8.0 with 0.4 M NaCl). We used a film-like sample for the time-resolved Raman and absorption measurements at 268 K. After rinsing the NaR sample with 2 mM Tris buffer solution (pH 8.0 with 3 mM NaCl), ~1 μl drop of the suspending solution was dried on a glass plate and this drying was repeated about 7 times to concentrate the sample on the plate. Then, a water drop (~1 μl) was added on the film-like sample to humidify it. The sample was sealed with an O ring and another glass plate and set in the liquid-nitrogen-cooled cryostat (Optstat DN2; Oxford Instruments) for the spectroscopic measurements.

Data availability

All the data supporting the findings of this study are available within the article and the supporting information.

Supporting information—This article contains supporting information (6, 17, 18, 25, 28, 32–37).

Author contributions—T. F., T. K., and M. U. methodology; T. F., K. K., and T. K. investigation; T. F., K. K., and T. K. formal analysis; T. F., T. K., and M. U. writing—original draft.

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Conflicts of interest—The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: BR, bacteriorhodopsin; HOOP, hydrogen out-of-plane; NaR, NaR from Indibacter alkaliphilus; LED, light emitting diode; NaR, sodium-pumping rhodopsin; RSB, retinal Schiff base.

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Retinal reisomerization switches Na+ uptake/release by NaR