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Na^+ Binding and pH-Dependent Transport in *Nonlabens dokdonensis* Rhodopsin 2*

Dear editor,

Light-driven Na^+ pumping rhodopsins (NaRs), the next generation optogenetic tools, are a family of seven-helical transmembrane proteins and covalently link to a retinal chromophore[1]. NaRs have a putative Na^+-conducting motif of Asn112-Asp116-Gln123 [1], homologous to the H^+-conducting motif of Asp85-Thr89-Asp96 in H^+ pump bacteriorhodopsin (BR) [2]. Powered by light, NaRs actively transport Na^+ or H^+ outward to generate electrochemical potential energy[1].

To elucidate the mechanism of Na^+ transport, many NaRs have been characterized [3–7], including *Gillisia limnaea* rhodopsin (GLR)[4] and *Krokinobacter* rhodopsin 2 (KR2)[3]. Balashov *et al*. proposed that a high affinity Na^+ binding site in the vicinity of Schiff base counterions may be formed transiently when GLR was illuminated [4]. Thus, Na^+ may be recruited into cytoplasmic ion selectivity region near Gln123 [8], subsequently transported to the extracellular binding site [4, 8], resulting in a net outward transport of Na^+. However, Inoue *et al*.[3] and Gushchin *et al*.[9] reported that Na^+ may bind to KR2 in the ground state. They also pointed out that the bound Na^+ was not what was being transported[3, 9]. Therefore, Na^+ binding site and the mechanism of Na^+ transport of NaRs remains elusive.

We focused on another NaR, *Nonlabens dokdonensis* rhodopsin 2 (NdR2)[10–11], and found that Na^+ may not bind to NdR2 in the ground state. Without illumination, the characteristic absorption maximum (528.0 nm) of NdR2 in Na^+ solution was identical to that in K^+ solution (Figure 1a). pH titration revealed two p*K*a of ~5.0 and ~6.3, respectively in Na^+ solution (Figure 1b), similar to those in K^+ solution (Figure 1c). Fluorescence emission spectra were identical in both Na^+ and K^+ solutions (Figure 1d). These results excluded Na^+ binding to the local regions in the vicinity of retinal, Schiff base counterions and seven tryptophan residues, respectively. Furthermore, no binding between NdR2 and Na^+ was observed by isothermal titration calorimetry (ITC) (Figure 1e). Thus, we propose that Na^+ may transiently bind to NdR2 during the photocycle instead of binding in the ground state.

Due to the competition between Na^+ and H^+[12–13], this transiently formed binding site may be perturbed when H^+ concentration increases. Indeed, the photocycle kinetics showed that at pH 5.7, both the rise(~1.1 ms) and decay(~6.1 ms) of characteristic O intermediate, which represents the transport of Na^+, were slower than those at neutral or basic pH(Figure 2a). The accumulation of O intermediate was also significantly suppressed at pH 5.7. In addition, the characteristic absorption maximum of NdR2 was red-shifted at pH 5.7 (Figure 2b), which directly indicated that Schiff base counterion region (probably Asp251 and Asp116), also putative Na^+ binding site[4, 8], became partially protonated. This was confirmed by the red-shifted spectrum of D116N mutant (Figure 2b) and in line with reported GLR mutants D251N/E [4]. Therefore, both the static and the kinetic spectra suggested that the transport of Na^+ may be altered at acidic pH. Moreover, as we monitored the pump activity of NdR2 in proteoliposomes[3, 5], a light-induced acidification of proteoliposome suspension was observed at pH 5.7 in Na^+ solution, *i.e*., the

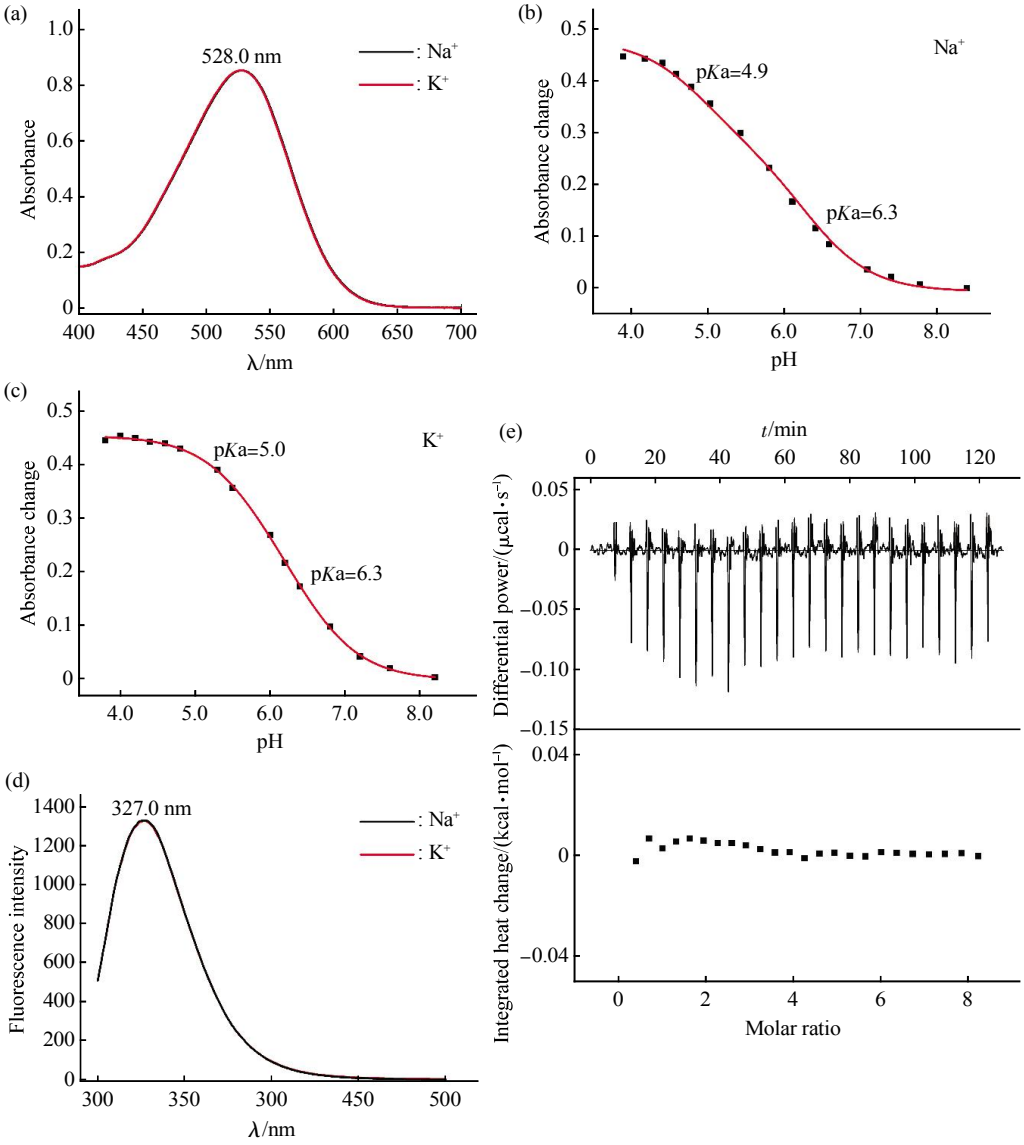

Fig. 1 NdR2-Na^+ binding assay

(a) Absorption spectra of NdR2 in 0.2 mol/L Na^+ (black) and K^+ (red) solutions, 0.1% DDM, pH 7.5. (b, c) Absorbance change of NdR2 at 590 nm in Na^+ (b) or K^+ (c) solution. Given the facts that Asp116 and Asp251 are putative counterions, titration data was fitted by Henderson-Hasselbalch equation (red) with two p*K*a components[3-4]. (d) Fluorescence emission spectra of NdR2. (e) Binding isotherm for the interaction of NdR2 with Na^+.

polarity of the pH change was opposite to that at pH 7.0 (Figure 2c). Typically, an active transport of H^+ outward is expected to be involved, because H^+ pump activity increases as the H^+ concentration increase due to the competitive transport of Na^+ and H^+ [12–13]. However, the rise and decay of O intermediate at pH 5.7 were less than ～10 ms (Figure 2a), which was close to the characteristic kinetics of Na^+ transport, but was significantly faster than the characteristic kinetics of H^+ transport (～hundreds of ms) [3]. Thus, the reversed pH change suggested that the transport of Na^+ might be inverted at pH 5.7 (Figure 2d), similar to the reversed transport of H^+ in proteorhodopsin[14–15].

Here, we hypothesize a pH-dependent bi-directional Na^+ transport model (Figure 2d) for NdR2. Because the transiently formed extracellular binding site has a high affinity for Na^+ at neutral pH[4, 8], Na^+ that has been taken up by cytoplasmic ion selectivity region in the vicinity of Gln123 [8], is subsequently transported to this site and released to the

extracellular surface (Figure 2d, left). However, due to electric repulsion, Na^+ affinity of this binding site may significantly decrease at acidic pH, as Schiff base counterions of Asp251 and Asp116 become partially protonated. Thus, upon illumination, Na^+ may be captured by this weakened binding site from extracellular side, subsequently recruited by the cytoplasmic Gln123 region whose affinity for Na^+ remains unaltered, and finally released to the cytoplasmic surface (Figure 2d, right). At present, due to the lack of a well-established functional assay to directly monitor Na^+, Na^+ transport mechanism at acidic pH remains obscure. This hypothesis needs to be validated in the future work.

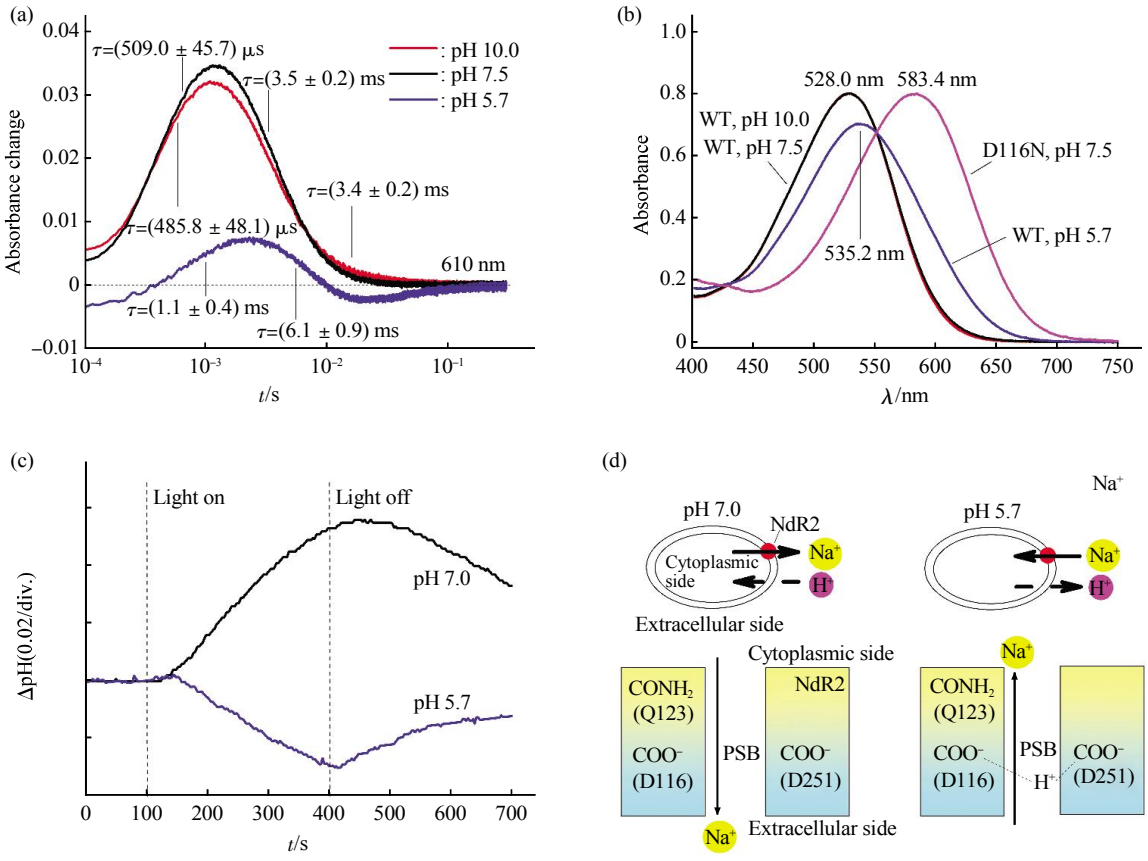

Fig. 2　Light-driven Na^+ transport in NdR2

(a) Photocycle kinetics of NdR2 in 0.1 mol/L Na_2SO_4, 0.1% DDM at 610 nm. The rise and decay time constants are indicated. (b) Absorption spectra of NdR2 and D116N. (c) Light-induced pH changes of NdR2 proteoliposome (d) Schematic representation (top) of Na^+ and H^+ movements as in (c) and tentative scheme (bottom) for Na^+ transport in NdR2 at pH 7.0 (left) and pH 5.7 (right). Solid and dash arrows refer to active and passive ion movement, respectively. PSB, protonated Schiff base.

Our findings suggest a Na^+ binding during the photocycle of NdR2 and a pH dependent transport of Na^+, which might shed some light on the mechanism of Na^+ transport in NaRs. Since the reversed Na^+ transport drives passive flow of H^+ outward, NdR2 may play an important physiological role in maintaining intracellular neutral pH in acidic environments. In addition, NaRs have been reported to inhibit neuron spikes[8] due to the transport of Na^+. Thus, NdR2 may also be applied in the optogenetics and the development of novel biosensors and photoelectric devices.

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