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Azide accelerates the decay of M-intermediate of *pharaonis phoborhodopsin*

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Abstract

Natronobacterium pharaonis has retinal proteins, one of which is *pharaonis* phoborhodopsin, abbreviated as *p*pR (or called *pharaonis* sensory rhodopsin II, *p*sR-II). This pigment protein functions as a photoreceptor of the negative phototaxis of this bacterium. On photoexcitation *p*pR undergoes photocycling; the photoexcited state relaxes in the dark and returns to the original state via several intermediates. The photocycle of *p*pR resembles that of bR except in wavelengths and rate. The cycle of bR is completed in 10 ms while that of *p*pR takes seconds. The Arrhenius analysis of M-intermediate ($p\mathrm{pR}_{\mathrm{M}}$) decay which is rate-limiting revealed that the slow decay is due to the large negative activation entropy of *p*pR. The addition of azide increases the decay rate 300-fold (at pH 7); Arrhenius analysis revealed decreases in the activation energy (activation enthalpy) and a further decrease in the activation entropy.

Keywords: Flash-photolysis; Photochemistry; Arrhenius analysis; Sensory rhodopsin; *Natronobacterium pharaonis*

1. Introduction

Halobacterium salinarium (*halobium*) has four retinal proteins: bacteriorhodopsin (bR [1,2]); halorhodopsin (hR [3,4]); sensory rhodopsin (sR or sR-I [5,6]); and phoborhodopsin (pR or sensory rhodopsin II, sR-II [7–11]). The former two work as ion-pumps and the latter two as photoreceptors of this bacterium. pR (sR-II) is a photoreceptor of negative phototaxis whose action maximum is located at approx. 500 nm; the primary structure was determined recently [12]. The content of pR in bacterial membranes is very low and solubilisation with detergents inactivates pR. Although there are several reports on photochemical reactions [7–10,13–15], these facts prevent further study.

We succeeded in purification of a phoborho-

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dopsin-like protein from haloalkaliphilic bacterium, *Natronobacterium pharaonis* [16–21] and named this pigment *pharaonis* phoborhodopsin (*p*pR; it is also called *pharaonis* sensory rhodopsin II, *p*sR-II). When this bacterium is irradiated with approx. 500 nm light, it shows a light-intensity-dependent avoidance reaction [22,23], implying that the pigment works as a photoreceptor for negative phototaxis. This pigment protein was first detected by Bivin and Stoeckenius [24], and Engelhard et al. [15,25] also investigated its properties. The primary structure was published by Seidel et al. [26].

The *p*pR photocycle was shown to be as follows [16,18,19]: *p*pR (498) $\rightarrow$ *p*pR$_{K}$ (approx. 540) $\rightarrow$ *p*pR$_{KL}$ (512) $\rightarrow$ *p*pR$_{L}$ (488) $\rightarrow$ *p*pR$_{M}$ (390) $\rightarrow$ *p*pR$_{O}$ (550) $\rightarrow$ *p*pR (498), where numbers in parentheses represent the maximum wavelength in nanometres. This photocycle closely resembles that of bR except in the wavelengths and photocycling rate: the cycle of bR is completed in several microseconds while that of *p*pR takes seconds. A mutant of bR whose Asp (96) is replaced with Asn, D96N has a very slow photocycle and lacks the proton-pumping activity. Tittor et al. [27] found the effect of the weak acid of azide, which accelerated the M-decay of the D96N mutant; for example, the half-time of the decay was as fast as 1 ms, much faster than that of wild bR. This experiment was based on an observation by Hegemann et al. [28] who found that azide, HN_3/N_3^- can give/receive protons to/from the Schiff base of the retinal in hR. In retinal proteins, retinal binds to a lysine residue of the protein to form the Schiff base. In the dark, bR and *p*pR have a protonated Schiff base and M-intermediates have a deprotonated Schiff base. The decay of M-intermediate corresponds to the protonation process of this base.

In this communication, we present data showing that azide accelerates *p*pR$_{M}$-decay, implying that azide can provide protons to the deprotonated Schiff base of *p*pR$_{M}$. The effects of azide on sensory rhodopsins [sR-I or sR-II (pR)] have not been reported so far, and hence this is the first report on the interaction of azide with sensory rhodopsins.

2. Materials and methods

2.1. Preparation of the ppR sample

N. pharaonis (NCMB2191) was grown in a medium as described elsewhere [16–21]. The purification procedure was essentially the same as described previously [16], but the final column (octyl-Sepharose CL-4B) procedure was repeated twice, so that the spectrum of samples used did not show the presence of heme proteins at 410–420 nm (see Fig. 1 and compare with Fig. 1 from Hirayama et al. [16]). The shoulder of the spectrum is peculiar to *p*pR or to pR [29]. The pH of the sample was changed by dialysis twice against approx. 100-fold volume of buffer solutions which contained 1 M NaCl and 0.5% *n*-octyl-β-D-glucoside adjusted to the desired pH. The buffers (50 mM) used were: for pH 4–5, potassium hydrogen phthalate or citrate; for approx. pH 6, 3-(*N*-morpholino)propanesulfonic acid; for pH 7–8, phosphate, and for pH 9, 2-(cyclohexyl-amino)ethanesulfonic acid. The pH was adjusted to a predetermined value at each temperature.

2.2. ppR_M- and ppR_O-decay measurements by flash photolysis

The apparatus and procedure were essentially the same as described [19]. Short flashes were provided by a Xe-flash lamp (duration 100 μs) in combination with a 540-nm interference filter (540 ± 10 nm, KL54, Toshiba) and a 530-nm cut-off filter (Toshiba). The temperature inside the cuvette was monitored with a thermocouple (Chino).

2.3. ppR_M formation measurements by flash photolysis

A new apparatus was constructed for this. An actinic light source (532 nm, 7 ns) was the second harmonic of the fundamental beam of the Q-switched Nd-YAG laser (DCR-2; Quanta-Ray). The photocycling rate of *p*pR was changed con-

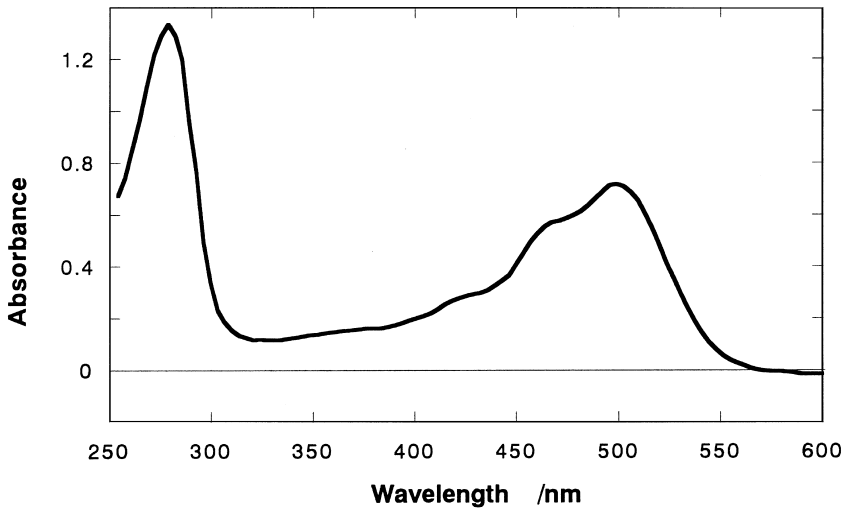

Fig. 1. Spectrum of *p*pR samples used. Purified samples were suspended in 4 M NaCl containing 0.5% *n*-octyl-β-D-glucoside and 50 mM Tris–HCl (pH 7.2). The spectrum does not show the presence of heme proteins whose absorption bands may appear at approx. 430–410 nm, but SDS-PAGE (stained with silver) showed a small amount of contamination whose molecular weight was approx. 97 kDa. Note that the shoulder is peculiar to pR or *p*pR.

siderably by variation of the azide concentration. Thus, the repetition frequency of the laser flash was adjusted according to the rate of photocycling. The source of monitoring light was a 150-W xenon arc lamp (C4251, Hamamatsu), the strength of which as it passed through the sample was detected by a photomultiplier (R2949, Hamamatsu). To select the measuring wavelength and exclude the scattered actinic flash from the sample, two monochromators were placed at the rear of the monitoring light source and in front of the photomultiplier. The output of the photomultiplier was amplified by a home-built I-V converter (~ 0.05-μs response time). The amplified signal was stored and averaged in a personal computer equipped with an A/D converter. The data was accumulated 100–200 times for each sample.

3. Results

3.1. The slow decay of ppR_M is due to small activation entropy

The decay of $p\mathrm{pR_M}$ can be measured at 350 nm [19]. The decay constants were calculated with a single exponential equation and determined under varying temperatures. Data were analysed in terms of activation energy (E_a) or enthalpy ($\Delta H^{\neq}$) and activation entropy ($\Delta S^{\neq}$) which are defined as

$$k = \frac{k_B T}{h}\exp\left(\frac{\Delta S^{\neq}}{R}\right)\exp\left(-\frac{\Delta H^{\neq}}{RT}\right) \quad (1)$$

where k_B, T, h and R stand for Boltzmann constant, absolute temperature, Planck constant and gas constant, respectively. Since the relationship between E_a and $\Delta H^{\neq}$ is

$$\Delta H^{\neq} = E_a - RT, \quad (2)$$

Eq. (1) can be recast to

$$k = \frac{k_B T}{h}\exp\left(1 + \frac{\Delta S^{\neq}}{R}\right)\exp\left(-\frac{E_a}{RT}\right) \quad (3)$$

This equation means the so-called frequency factor is expressed by $(k_B T/\mathrm{h})\exp[1 + (\Delta S^{\neq}/R)]$.

The value of E_a of $p\mathrm{pR_M}$-decay at pH 8.0 was 79 kJ/mol which is comparable to that of the M-decay of wild-type bR, 71–79 kJ/mol [27] and 69–89 kJ/mol at pH 9 [30]. The $\Delta S^{\neq}$ value, on the other hand, was -10 J/(mol·K) which is in

sharp contrast to the value of $+50$ J/(mol·K) for the wild-type bR [27]. Although the ppR$_M$-decay became rapid in acidic media, E_a (or $\Delta H^{\neq}$) decreased and $\Delta S^{\neq}$ also decreased further to -50 or -70 J/(mol·K). The reason for the very slow decay of ppR$_M$ is therefore concluded to be the largely negative $\Delta S^{\neq}$ (small frequency factor), which might reflect a situation where the proton transfer to the deprotonated Schiff base is carried out by a selective pathway rather than by random access to this base. Our previous experiment using retinal analog [21] suggested tightness around the Schiff base, which is consistent with the present finding.

3.2. Effect of azide on ppR_M-decay

As shown in Fig. 2, the azide anion accelerated the ppR$_M$-decay appreciably. For example, the kinetic constant of the decay in the absence of azide was 0.65/s at pH 7.0, while addition of 100 mM of azide increased the rate to as large as 174/s, which is even faster than the M-decay of wild-type bR. This figure also shows that the effect was pH-dependent. The decay kinetic constants are plotted against azide concentration under varying pH in Fig. 2D. As shown, the azide effect is prominent when the pH becomes small,

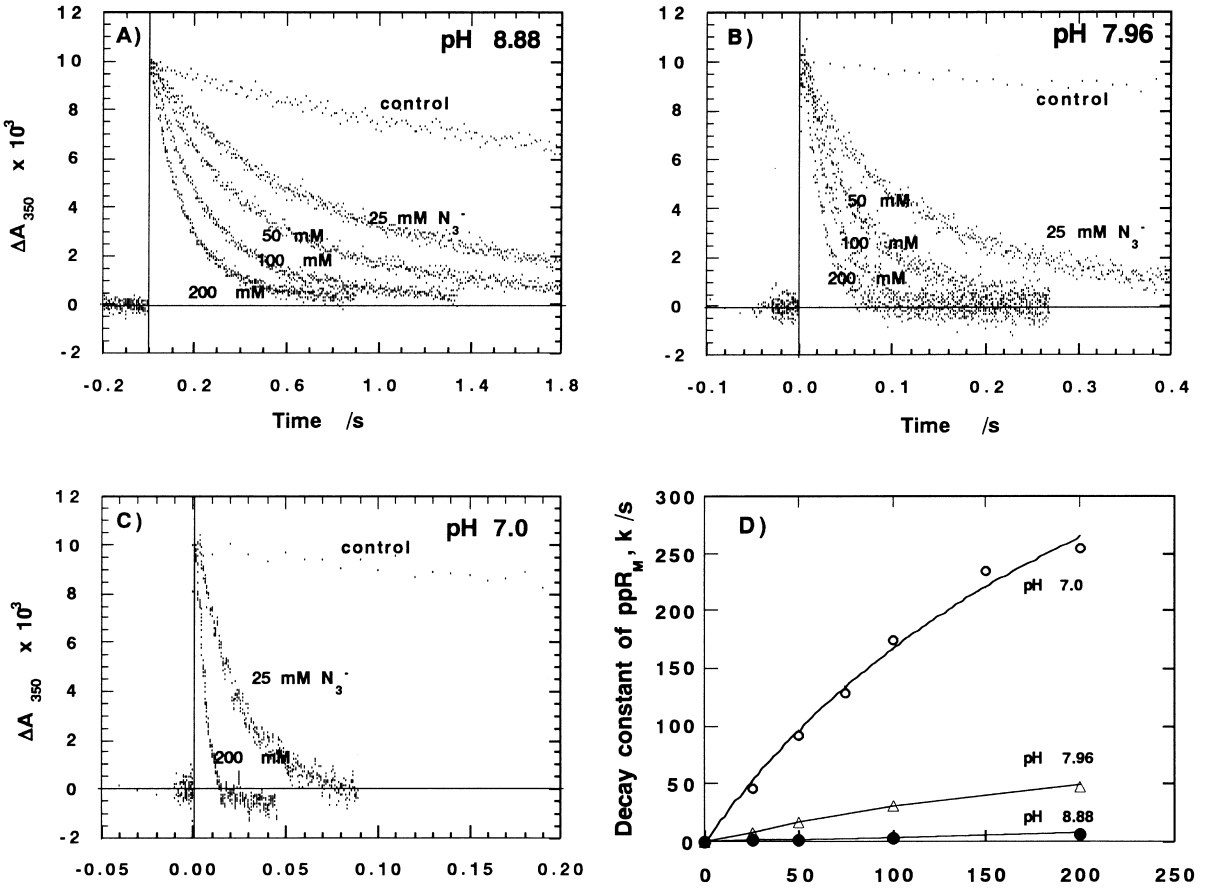

Fig. 2. Azide accelerates ppR$_M$ decay in a pH-dependent manner. Charts from A to C show the flash-photolysis data at pH 8.88, 7.96 and 7.0, respectively. The azide concentrations are noted. The decay of ppR$_M$ was monitored at 350 nm. Temperature was 25°C. These time-dependent absorbance changes were analyzed with a single exponential equation to calculate the rate constant. The rate constants were plotted against azide concentration added in D, showing clearly that with a decrease in pH, the effect became prominent.

Table 1
Effect of various anions of weak acid on ppR$_M$-decay

Anion	ppR[a]			D96N of bR[b]
	pK_a	K_m/mM	k_{max}/s^{-1}	K_m/mM
Azide	4.5	607	1160	3–4
Cyanate	3.7	41.8	6.02	0.9
Nitrite	3.3	42.8	3.36	10
Acetate	4.8	No effect	No effect	139
Thiocyanate	0.85	1250	2.0	No effect

The meanings of K_m and k_{max} are given in the text.
[a] ppR was suspended in 1 M NaCl, 0.5% n-octyl-β-D-glucoside buffered with 50 mM phosphate (pH 7.0 and 25°C). The decay constant in the absence of anions, k_0 (rate constants in the absence of azide) is 0.65/s. The decay of ppR$_M$ was monitored by the absorbance at 350 nm.
[b] Data were taken from Tittor et al. [27]. The D96N mutant bR was suspended in 100 mM NaCl, 10 mM MOPS, pH 6.4.*

suggesting that the acceleration effect may be due to the associated form of HN_3.

3.3. Anion specificity

Anions of weak acids other than azide were examined. The increment in the decay constant, $k-k_0$ was analysed by the Michaelis–Menten-like equation with respect to added anion concentration, and the effect of anions was expressed in terms of K_m and k_{max}:

$$k-k_0=\frac{k_{max}[\text{anion}]}{K_m+[\text{anion}]}$$

where k_0 is the decay constant in the absence of anion, and k_{max} and K_m have their usual meanings.

Results obtained at pH 7.0, 25°C are listed in Table 1. Azide was the most effective with the maximum value of decay constant being as fast as 1160/s, which should be compared to the value in the absence of azide, 0.645/s. This table also lists the K_m values for the D96N mutant taken from [27]. Similar results on the anion series in halorhodopsin were also obtained by Lanyi [31]. Comparison of K_m for ppR and D96N reveals that very high concentrations of anions were needed for ppR. This requirement for higher concentration might be due to the fact that the interaction between anions and deprotonated Schiff base occurs through a relatively selective mode. For both D96N and ppR, K_m of cyanate was smaller than azide, but k_{max} was not large (although the values of D96N are not described in [27]).

3.4. Arrhenius analysis of ppR_M-decay

Fig. 3 shows the typical Arrhenius plots of the decay constant of ppR$_M$ at varying pH, where azide concentration is 500 mM. The decay rate is seen to be increased by a factor of one to six units in the ln (natural logarithmic) scale. Interestingly, the activation energy in acidic media is almost zero (see Fig. 4). This small activation energy suggests the proton transfer process is composed of at least two elementary processes.

Fig. 4 shows E_a (A) and $\Delta S^{\neq}$ (B) of ppR$_M$-decay which was calculated at varying pH in the absence (closed circles) and presence (open circles) of 500 mM azide. Data in the presence of azide show an inflection point at approx. 6 or 6.5; in the absence of azide, the inflection point appeared at approximately the same pH (see Fig. 6 and Fig. 8 from Miyazaki et al. [19] and also the 'closed circle' data in this figure). Data points for both E_a and $\Delta S^{\neq}$ are almost parallel in the presence and absence of azide, although the difference is somewhat larger in acidic medium.

3.5. Effect of ppR_M-rise and ppR_O-decay

The effect on the ppR$_M$ formation was investigated and the results listed in Table 2 show there is essentially no effect. This is in contrast to that of wild-type bR M-formation, which is increased approximately twofold by the addition of azide [32].

The ppR$_O$-decay constants in the absence and presence of 100 mM of azide were 0.46 and 2.3/s, respectively, at pH 7.0, and 0.32 and 1.96/s, respectively, at pH 9.0. It is noted that the 300-fold increase in the ppR$_M$-decay was observed. For wild-type bR, on the other hand, azide was effective on the O-decay in acidic media (pH < 6); the decay rates increased by 20–30% [33]. In alkaline media, azide showed no effect. For E204Q mu-

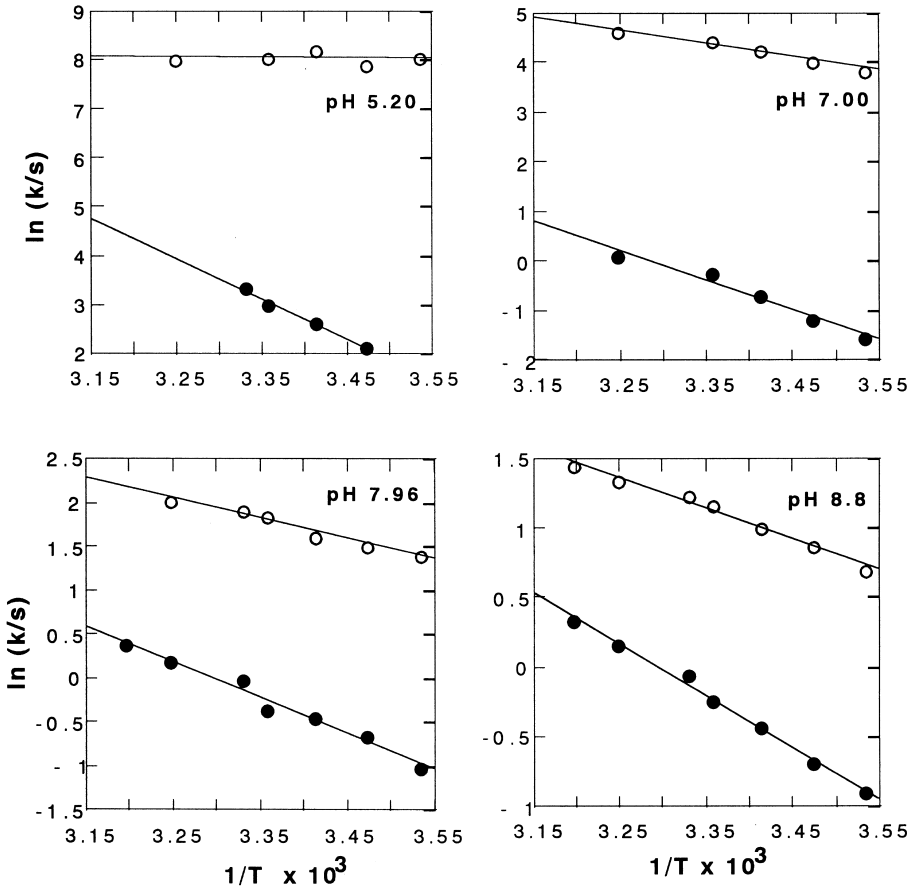

Fig. 3. Arrhenius plots of the rate constants of ppR$_M$-decay at typical pH. Note that the ordinate is a natural log scale. ● represents the rate constant k (in seconds) in the absence of azide; and ○ represents k in the presence of 500 mM NaN_3.

tant bR, azide increased the O-decay rates by 30-90% [33]. These are interpreted as follows: proton is released from E204. When the proton translocation between D85 and E204 works normally under the condition of the carboxyl group of E204 being dissociated, no azide effect is observed, and under the condition of the carboxyl group being incapable of the proton transport, azide shows its effect on EC (extracellular channel). Azide took effects on ppR$_O$-decay in spite of the much smaller effect than that of ppR$_M$. The increase rates of the ppR$_O$-decay, however, were larger than those of wild-type bR or E204Q mutant. This might mean the lack of the amino acid residue in ppR corresponding to E204 of bR, although alignment analysis of the amino acid sequences suggests that the corresponding residue in ppR is Asp (data not shown). Tittor et al. [34] also obtained results showing that azide was effective on EC as well as CP (cytoplasmic channel).

4. Discussion

Coutre et al. [32] investigated the azide-induced acceleration of the mutant bR$_M$-decay using time-resolution FTIR (Fourier-transform infrared spectroscopy); they showed that azide locates near D85 and proposed the influence of azide on the arrangement of the intramolecular hydrogen-bonded network of water. Although we do not know whether this is the case for ppR or not, a

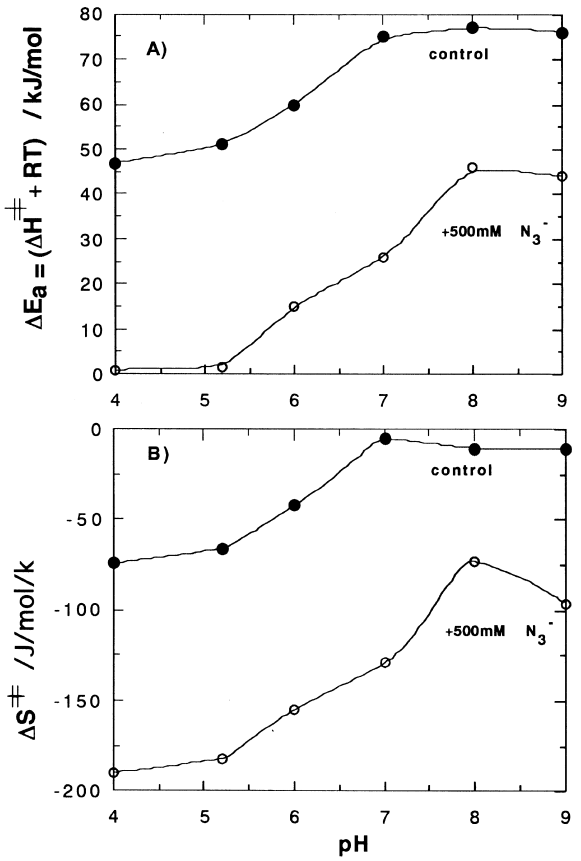

Fig. 4. Activation energy (A) and activation entropy (B) calculated from Fig. 3 were plotted against pH in the media. Notations are the same as those in Fig. 3.

shuttle mechanism may be involved since the slope of a plot of log k(ppR$_M$-decay constant) against pH in the medium was 0.98 from pH 9–5 (in the presence of 100 mM N_3^-, data not shown). For the D96N bR mutant, the same pH dependence was reported [30,35]. Here, the shuttle mechanism is that the protonated azide gives the proton to the deprotonated Schiff base and the azide anion diffuses to the membrane surface to return the protonated form, which diffuses back again to the Schiff base.

Fig. 4 shows that for both cases in the absence and the presence of N_3^-, E_a and $\Delta S^{\neq}$ are sigmoidal, and that the middle pH was approx. 6.5. Usual interpretation may be the presence of proton-donating amino acid group(s) which gives the

Table 2
Effect of azide on the rate constant of ppR$_M$ formation

Azide/ mM	Rate constant of ppR$_M$ formation ($k \times 10^{-3}$)/s^{-1}	
	pH 5.0	pH 9.0
0	70	61
20	69	73
40	72	78
60	78	66
80	76	57

Notes. Media contained 1 M NaCl, 0.5% n-octyl-β-D-glucoside buffered with the following: for pH 5.0, 50 mM potassium hydrogen phthalate and for pH 9.0, 50 mM 2-(cyclohexylamino)ethanesulfonic acid. Temperature was 25°C.

proton to the Schiff base. This may not be consistent with the above shuttle mechanism; then we can infer that among these pH ranges the conformational change might happen triggered by association/dissociation of a certain amino acid residue and that this conformation change alters the Arrhenius parameters. Another is that amino acid residue(s) may change due to the medium pH change which leads to the change in the surface potential, and this potential change may alter the effective proton concentration near the membrane surface.

Arrhenius analysis of bR mutants (D96N, D96G) revealed $\Delta H^{\neq}$ of 29.6 kJ/mol and $\Delta S^{\neq}$ of -60 J/(mol·K) for the D96N mutant, and $\Delta H^{\neq}$ of 15.6 kJ/mol and $\Delta S^{\neq}$ of -106 J/(mol·K) for the D96G mutant [36]. It is noted that these mutants show very slow decay of M-intermediate, similar to ppR$_M$. Addition of 100 mM azide to D96N mutant accelerated the M-decay and values of $\Delta H^{\neq}$ and $\Delta S^{\neq}$ were reported to be 48 kJ/mol and -26 J/(mol·K), respectively [27]. Different values were reported [30]: for D96N bR mutant, E_a and $\Delta S^{\neq}$ were 33.2 kJ/mol and -178 J/(mol·K), respectively, and addition of 5 mM N_3^- changed them to 24.3 kJ/mol and -160 J/(mol·K) (5 mM N_3^-, 50 mM phosphate at pH 9.0). For the bR mutant, azide increased $\Delta S^{\neq}$ (although the absolute values are not equal reported by Tittor et al. [27] and Cao et al. [30]).

The present paper shows that azide increases ppR$_M$-decay approx. 300-fold (at pH 7.0). Arrhe-

nius analysis of data in the presence of azide showed a decrease in both $\Delta H^{\neq}$ and $\Delta S^{\neq}$. Under an acidic condition, $\Delta H^{\neq}$ was almost zero, and $\Delta S^{\neq}$ decreased to as small as -190 J/(mol·K). The finding that the small values of $\Delta S^{\neq}$ for ppR$_M$-decay and further decrease in $\Delta S^{\neq}$ by addition of azide (see Fig. 4B) is interesting. On the other hand, for bR azide increases $\Delta S^{\neq}$. The molecular interpretation of this $\Delta S^{\neq}$ is a further problem; this may be related to the implication from our previous retinal–analog experiment [20,21] that the retinal binding pocket in ppR may be tight, so the proton or HN_3 might approach the Schiff base via a selected pathway.

There is a question of whether the proton transfer in ppR induces generation of the membrane potential (proton transport across the membrane) in the presence or absence of azide. There is possibility that at least, in the presence of azide, ppR might generate the membrane potential on illumination: azide increases ppR$_M$-decay, which is indicative of the proton transfer in CP (cytoplasmic channel), and the formation of ppR$_O$ suggest the proton transfer in EC (extracellular channel). It is noted that sR (sR-I) pumps proton when Htr-I is removed [37,38].

As far as we know, there are no reports concerned with the effect of weak-base anions on sensory rhodopsins other than ppR (i.e. sR or pR), and consideration on their effects in connection with the primary structures might be interesting.

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