Povsic, T. J., & Dervan, P. B. (1989) *J. Am. Chem. Soc. 111*, 8733–8735.
Praseuth, D., Perronault, L., Le Doan, T., Chassignol, M., Thuong, N., & Helene, C. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 1349–1353.
Radhakrishnan, I., Gao, X., de los Santos, C., Live, D., & Patel, D. J. (1991a) *Biochemistry 30*, 9022–9030.
Radhakrishnan, I., de los Santos, C., & Patel, D. J., (1991b) *J. Mol. Biol. 221*, 1403–1418.
Radhakrishnan, I., Patel, D. J., & Gao, X. (1991c) *J. Am. Chem. Soc. 113*, 8542–8544.
Rajagopal, P., & Feigon, J. (1989) *Biochemistry 28*, 7859–7870.
Sklenar, V., & Feigon, J. (1990) *Nature 345*, 6836–6838.
Strobel, S. A., & Dervan, P. B. (1990) *Science 249*, 73–75.
Strobel, S. A., & Dervan, P. B. (1991) *Nature 350*, 172–174.
Sun, J. S., Francois, J. C., Montenay-Gartesier, T., Saison-Behmoras, T., Roig, V., Thuong, N. T., & Helene, C. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 9198–9202.
Vuister, G. W., Boelens, R., & Kaptein, R. (1988) *J. Magn. Reson. 80*, 176–185.
Vuister, G. W., Boelens, R., Padilla, A., Kleywegt, G. J., & Kaptein, R. (1990) *Biochemistry 29*, 1829–1839.
Wells, R. D., Collier, D. A., Hanvey, J. C., Shimizu, M., & Wohlraub, F. (1988) *FASEB J. 2*, 2939–2949 (and references cited therein).
Wüthrich, K. (1986) *NMR of Proteins and Nucleic Acids*, Wiley, New York.
Xodo, L. E., Manzini, G., & Quadrifoglio, F. (1990) *Nucleic Acids Res. 18*, 3557–3564.
Zuiderweg, R. P., & Fesik, S. W. (1989) *Biochemistry 28*, 2387–2391.

Chromophore Configuration of *pharaonis* Phoborhodopsin and Its Isomerization on Photon Absorption†

Yasushi Imamoto,‡ Yoshinori Shichida,*,‡ Junichi Hirayama,§ Hiroaki Tomioka,§,∥ Naoki Kamo,§ and Tôru Yoshizawa⊥

Department of Biophysics, Faculty of Science, Kyoto University, Kyoto 606-01, Japan, Department of Biophysical Chemistry, Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo 060, Japan, and Department of Applied Physics and Chemistry, The University of Electro-Communications, Chofu, Tokyo 182, Japan

Received September 18, 1991; Revised Manuscript Received December 16, 1991

ABSTRACT: The configuration of the retinylidene chromophore in *pharaonis* phoborhodopsin (ppR) and its changes during the photoreaction cycle were investigated by means of a chromophore extraction method followed by HPLC analysis. The ppR has an all-trans chromophore, and unlike bacteriorhodopsin, it exhibits no dark isomerization of the chromophore. Irradiation of a ppR sample in the presence of 10 mM hydroxylamine, at which concentration a negligible amount of ppR was bleached, caused the formation of 90% 13-*cis*- and 10% *all-trans*-retinal oximes. Because the ppR sample under the continuous irradiation was a mixture containing original ppR, ppR_M, and a small amount of ppR_O, the above results showed that the chromophores of ppR_M and ppR_O are in a 13-cis form and an all-trans form, respectively. Therefore, the all-trans chromophore of ppR is isomerized to the 13-cis form on photon absorption, and it is thermally reisomerized to the all-trans form on the conversion process from ppR_M to ppR_O. The extracted retinal oximes from ppR and ppR_O were mainly the 15-syn form, while that from ppR_M was mainly the 15-anti form. This fact indicated that the attack of hydroxylamine on the chromophore is stereoselective owing to the unique structure of the chromophore binding site near the Schiff base region of the chromophore.

Negative phototactic response of a haloalkaliphilic bacterium, *Natronobacterium pharaonis*, is mediated by a retinal protein called *pharaonis* phoborhodopsin (ppR)[1] (Tomioka et al., 1990). It is very similar in its physiological functions (Takahashi et al., 1985; Tomioka et al., 1986) and spectroscopic properties (Bivin & Stoeckenius, 1986; Imamoto et al., 1991; Hirayama et al., 1992) to phoborhodopsin (pR) of *Halobacterium halobium*. The ppR has an absorption maximum at 498 nm, which is a 10 nm longer wavelength than that of pR, while both of the absorption spectra exhibit vibrational fine structures even at room temperature (Tomioka et al., 1990; Takahashi et al., 1990). Furthermore, our previous investigations of pR and ppR by low-temperature spectrophotometry revealed that they show very similar photochemical and subsequent thermal reactions to each other

† This work was supported in part by a Grant-in-Aid for Scientific research from the Japanese Ministry of Education, Culture and Science, by a Special Coordination Fund of the Science and Technology Agency of Japanese government, by a Grant from the Human Frontier Science Program, and by a Ciba-Geigy Foundation (Japan) for the Promotion of Science.

* Correspondence should be addressed to this author.
‡ Kyoto University.
§ Hokkaido University.
∥ Present address: Laboratory for Bioelectronic Materials, Frontier Research Program, Riken Institute, Wako 351-01, Japan.
⊥ The University of Electro-Communications.

[1] Abbreviations: ppR, *pharaonis* phoborhodopsin; pR, phoborhodopsin; bR, bacteriorhodopsin; hR, halorhodopsin; sR, sensory rhodopsin; HPLC, high-performance liquid chromatography; Ts, *all-trans*-15-*syn*-retinal oxime; Ta, *all-trans*-15-*anti*-retinal oxime; 13s, 13-*cis*-15-*syn*-retinal oxime; 13a, 13-*cis*-15-*anti*-retinal oxime.

0006-2960/92/0431-2523$03.00/0

(Shichida et al., 1988a; Imamoto et al., 1991; Hirayama et al., 1992). The scheme of the photocycle of ppR reported so far is as follows: ppR (ppR_{500}) $\xrightarrow{h\nu}$ ppR_K (P_{540}) $\rightarrow$ ppR_M (P_{390}) $\rightarrow$ ppR_O (P_{560}) $\rightarrow$ ppR (Bivin & Stoeckenius, 1986; Tomioka et al., 1990; Hirayama et al., 1992). The noticeable properties of pR and ppR in their photocycle are that they have no L intermediate and that ppR_K and the corresponding intermediate of pR are thermally more stable than those of the other retinal proteins (i.e., bR and rhodopsin) (Imamoto et al., 1991; Hirayama et al., 1992). These characteristics of pR and ppR indicate that their chromophore/protein interactions are quite different from those of the other retinal proteins investigated so far, and their chromophore configurations should be determined first of all to reveal the molecular mechanism of photon absorption by pR and ppR.

Chromophores of bR and hR in the dark-adapted states are in the thermal equilibrium between *all-trans*- and 13-*cis*-retinals, while the all-trans chromophore is dominant in each of the light-adapted states (Maeda et al., 1977; Kamo et al., 1985). On absorption of a photon, all-trans chromophores of bR and hR are isomerized to 13-cis forms (Braiman & Mathies, 1982; Ogurusu et al., 1981). On the other hand, the chromophore of sR is only in an all-trans form, even in the dark-adapted state. However, its chromophore is also photoisomerized to the 13-cis form (Tsuda et al., 1985). In the pR system, it was suggested from the study using the locked 13-*trans*-retinal analogue that isomerization of the chromophore of pR from all-trans to 13-cis is essential for the formation of its intermediate and for the generation of physiological activity (Yan et al., 1990). However, no direct experimental data focusing on the dark/light adaptation and the photoreaction of the chromophore of pR were reported because an instability of pR to the detergents as well as a low quantity of pR in the cell prevents its purification and hampers the chromophore extraction experiments (Takahashi et al., 1988).

Unlike pR, ppR can be extracted without denaturation by a few detergents and purified using column chromatography (Tomioka et al., 1990; Hirayama et al., 1992). Using a purified ppR sample, we were able to elucidate the chromophore configuration of ppR and its photointermediate by means of chromophore extraction experiments followed by HPLC analysis.

Materials and Methods

Sample Preparation. A purified ppR sample was prepared from the *Natronobacterium pharaonis* (NCMB2191) (Hirayama et al., 1992) (buffer condition: 25 mM Tris-HCl, 4 M NaCl, and 0.5% octyl glucoside, pH 7.2). The retinylidene chromophores of ppR and its intermediates reacted with hydroxylamine, whose concentration in the sample was 10 or 100 mM, to form the retinal oximes before or after denaturation of the protein moiety of ppR by methanol and dichloromethane, followed by extraction with hexane (Shichida et al., 1988b). The denaturation and the extraction were carried out under ice-chilled conditions.

HPLC Analysis. The configurations of isomers of retinal oximes in the extracted mixture were analyzed by HPLC (LC-7A system, Shimadzu) equipped with a silica column (6 × 150 mm, YMC-A0123, Yamamura). The solvent was composed of 98.8% (v/v) benzene, 1.0% (v/v) diethyl ether, and 0.2% (v/v) 2-propanol, and the flow rate was 1.5 mL/min. The HPLC patterns were obtained by monitoring the absorbances at 360 nm. Using this system, 15-*syn*- and 15-*anti*-retinal oximes were able to be separated (Figure 1) (Tsukida et al., 1985). Each peak in the HPLC pattern was assigned by comparison of its retention time with those of the authentic

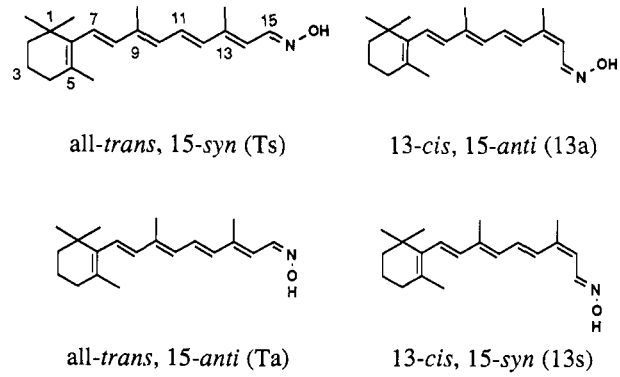

FIGURE 1: Structures of retinal oximes. Top left: *all-trans*-15-*syn*-retinal oxime (Ts). Bottom left: *all-trans*-15-*anti*-retinal oxime (Ta). Top right: 13-*cis*-15-*anti*-retinal oxime (13a). Bottom right: 13-*cis*-15-*syn*-retinal oxime (13s). It should be noted that the hydroxyl group in the syn (or anti) form of retinal oxime is located in the direction opposite to that of the carbon atoms of the lysine residue in the syn (or anti) form of retinylidene Schiff base. The retinal oximes are drawn in the 6s-cis conformations, while C_6–C_7 conformations of the retinylidene Schiff base in the protein have not been revealed.

retinal oximes. HPLC patterns were stored by a control computer (Chromatopack CR-5A, Shimadzu) and transferred to a personal computer (PC-9801RA, NEC) for analysis.

The molar compositions of the retinal isomers extracted from ppR samples were calculated from the areas of the peaks in the HPLC patterns. The extinction coefficients of Ts, Ta, 13s, and 13a at 360 nm in benzene were previously reported as 52 200, 51 600, 47 800, and 52 100, respectively (Tsukida et al., 1985; Trehan et al., 1990).

Spectrophotometry. Absorption spectra and time courses of absorbance changes of the samples were recorded by a recording spectrophotometer (MPS-2000, Shimadzu) which was interfaced with a personal computer (PC-9801RA, NEC) for storing and analyzing the spectral and absorbance data. A sample cell holder connected with a thermostatic circulator was installed in the sample compartment of the spectrophotometer to keep the sample at a constant temperature (20 or 0 °C). The sample was irradiated with a green light (501 nm) or a yellow light (>480 nm) from a 1-kW projector lamp (Sanko) which had passed through an interference filter (Nihonshinku, half-bandwidth = 2 nm) or a glass cutoff filter (Y-50, Toshiba). A 5-cm water layer was placed in front of the projector lamp to remove heat radiations.

Results

Configuration of the Chromophore Contained in the Dark-Adapted ppR. The purified ppR sample was kept in the dark for more than 2 days at 4 °C and served as a dark-adapted ppR sample. Its absorption spectrum is shown in Figure 2a (curve 1). No carotenoids (bacterioruberin) were detected, while a small amount of heme protein was contaminated (absorption maximum, about 420 nm). If ppR exhibits dark/light adaptation, dark-adapted ppR should be in a mixture. Therefore, it was examined from the following two aspects: its bleaching kinetics and its chromophore configuration.

Since ppR is unstable against hydroxylamine (Bivin & Stoeckenius, 1986), bleaching kinetics of the dark-adapted ppR was monitored in the presence of 100 mM hydroxylamine at 20 °C (Figure 2). The spectral changes of the sample in the course of the bleaching are shown in Figure 2a. The decreases of absorbances at the peak (500 nm, circles) and the shoulder (460 nm, triangles) in this course were plotted in the semilogarithmic scale (Figure 2b), indicating that they showed the parallel linear lines. This fact strongly suggests that the

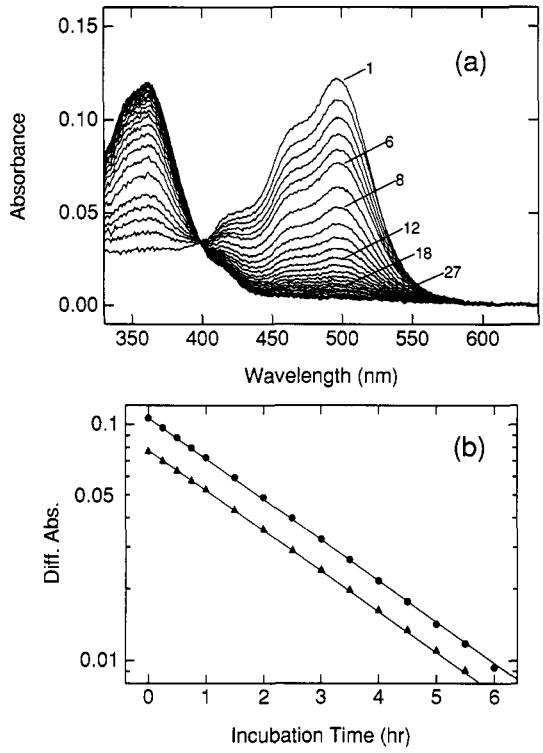

FIGURE 2: The course of bleaching of ppR by hydroxylamine in the dark. (a) The absorption spectrum of a dark-adapted ppR sample was recorded at 20 °C (curve 1). After addition of a one-tenth volume of 1 M hydroxylamine (curve 2), the sample was incubated at 20 °C until the ppR in the sample was completely bleached (15 h). The spectra were recorded at intervals of 15 min (curves 3–6), 30 min (curves 6–24), and 1 h (curves 24–27), respectively. (b) The bleaching kinetics of ppR in the sample. The difference absorbances at the peak (500 nm, circles) and the shoulder (460 nm, triangles) on the bleaching process of ppR shown in (a) were plotted in the semilogarithmic scales versus the incubation times.

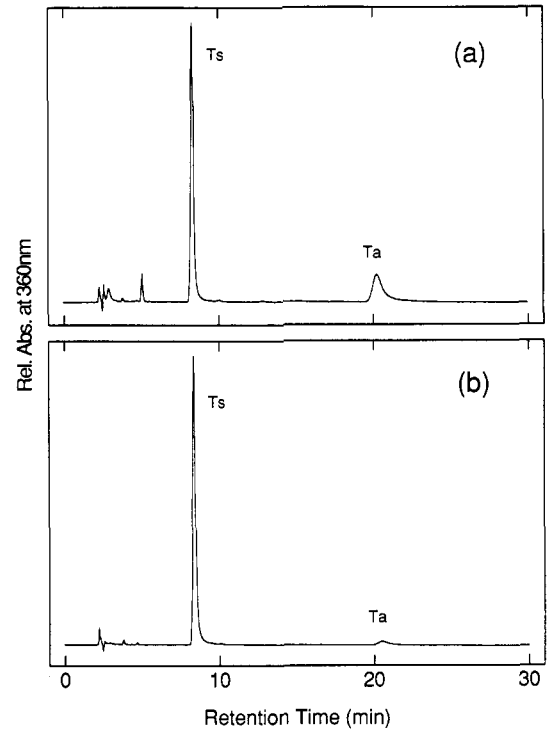

FIGURE 3: The chromophore configuration of dark-adapted ppR. (a) The dark-adapted sample (corresponds to curve 1 in Figure 2a) was denatured by methanol and dichloromethane, followed by addition of 100 mM hydroxylamine to form the retinal oximes. Then, the retinal oximes in the sample were extracted by hexane and analyzed by HPLC with the detection beam set at 360 nm. (b) The ppR sample completely bleached by hydroxylamine (curve 27 in Figure 2a) was supplemented with methanol and dichloromethane, followed by extraction of retinal oximes by hexane. The extracted retinal oximes were analyzed by HPLC with the detection beam set at 360 nm.

dark-adapted ppR was composed of only one species which has a fine structure in its absorption spectrum.

Then, the analysis of chromophore configuration was carried out. The retinylidene chromophores were extracted from the samples before and after bleaching by hydroxylamine. The ppR in the unbleached sample (corresponds to curve 1 in Figure 2a) was denatured by the addition of methanol and dichloromethane, followed by addition of hydroxylamine (final concentration of 100 mM) and extraction with hexane. On the other hand, from the sample completely bleached by hydroxylamine (curve 27 in Figure 2a), the retinal oximes were extracted by hexane after denaturation of the protein moiety by methanol and dichloromethane.

The retinal oximes extracted from the samples described above were analyzed by HPLC (Figure 3). In both cases, only *all-trans*-retinal was detected. However, the ratio of Ts to Ta extracted from the hydroxylamine-bleached sample (Figure 3b) was larger than that from chemically denatured ppR (Figure 3a).

Configuration of the Chromophores in the Photointermediates of ppR. The ppR sample was successively irradiated with green light (501 nm) at 20 °C in the presence of 10 mM hydroxylamine (Figure 4a), resulting in bleaching of ppR in the sample to the retinal oximes and the protein moiety. Since ppR undergoes its photoreaction cycle, the continuous light irradiation of the sample produces a steady state composed of ppR and its photointermediates. However, the separate experiments on the stability of ppR against hydroxylamine in the dark showed that the half-decay time of ppR in the presence of 10 mM hydroxylamine at 20 °C was about 10 h (data not shown), while the half-decay time of ppR under irradiation was about 10 min. These facts indicate that the photoproducts of ppR were selectively bleached by hydroxylamine. Therefore, the retinal oximes formed in this process can be regarded as the chromophores of the photoproducts of ppR. The chromophores were extracted by hexane after the denaturation of the protein moiety and analyzed by HPLC. In the HPLC pattern, the main component was 13a, but 13s, Ts, and Ta were also detected (Figure 4b).

To clarify the origins of the extracted chromophores, we tested which intermediate of ppR existed under continuous irradiation (Figure 4c). Until now, the formations of ppR_M and ppR_O were reported on the millisecond time scale, on which hydroxylamine seems to be able to attack the Schiff base linkages of the intermediates. The sample was irradiated with 501-nm light at 20 °C for 30 s in the absence of hydroxylamine, followed by monitoring the absorbance changes at 500, 390, and 560 nm, which reflect the recovery of ppR and the decays of ppR_M and ppR_O, respectively. The extents of difference absorbances at 0 s (immediately after the irradiation) are attributed to the contents of ppR and its intermediates present in the sample under the irradiation. The results showed that the sample contained mainly ppR_M together with a small amount of ppR_O, but ppR_K was not detected on this time scale. Therefore, 13-*cis*- and *all-trans*-retinal oximes extracted from the irradiated sample seem to be the chromophores of ppR_M and ppR_O, respectively. To pursue these identifications, the irradiation condition was changed.

Since the amount of ppR_O in the sample decreased on cooling the sample to lower temperatures (unpublished data), the sample was irradiated with green light (501 nm) at 0 °C

Table I: Molar Ratios of Retinal Oxime Isomers Extracted from the Samples

	all-trans (%)			13-cis (%)		
	syn	anti	syn + anti	syn	anti	syn + anti
denatured[a] in the dark (original sample)	72.4	27.6	100.0	0.0	0.0	0.0
bleached[b] by NH_2OH in the dark	95.5	4.5	100.0	0.0	0.0	0.0
photobleached[c] by 501-nm light, 20 °C	9.6	1.2	10.8	14.7	74.5	89.2
photobleached[c] by 501-nm light, 0 °C	6.8	1.1	7.9	14.3	77.8	92.1
photobleached[c] by >480-nm light, 20 °C	7.9	1.0	8.9	11.7	79.4	91.1
denatured[a] under 501-nm light, 0 °C	10.8	1.9	12.7	66.1	21.2	87.3

[a] The term denatured means that the protein moiety of the sample was denatured by methanol and dichloromethane prior to addition of the hydroxylamine. [b] The term bleached means that the chromophore in the native chromophore binding site reacted with hydroxylamine. [c] The sample was photobleached in the presence of 10 mM hydroxylamine.

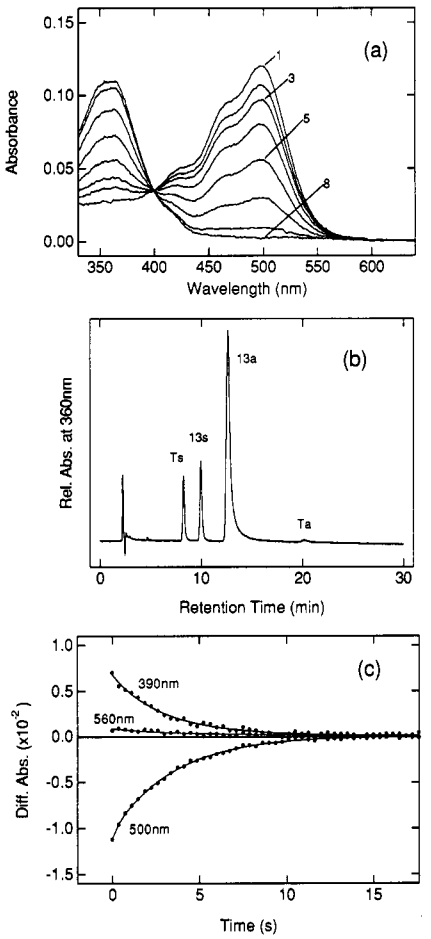

FIGURE 4: The chromophore configuration(s) of the photointermediate(s) of ppR. (a) The photobleaching process of ppR in the presence of hydroxylamine. The ppR sample was supplemented with 10 mM hydroxylamine, and the spectrum was recorded at 20 °C (curve 1). The sample was then irradiated with green light (501 nm) for a total of 2, 4, 8, 16, 32, 64, and 128 min at 20 °C (curves 2–8, respectively). (b) The HPLC pattern of the chromophores extracted from the ppR sample after complete bleaching with 501-nm light. The detection beam of the HPLC pattern was set at 360 nm. (c) The kinetics of decay of the intermediates produced by the continuous irradiation of the ppR sample with 501-nm light. The ppR sample was irradiated with green light (501 nm) at 20 °C for 30 s, and immediately after irradiation, the absorbance changes at 560, 500, and 390 nm were monitored on the several seconds time scale.

in the presence of 10 mM hydroxylamine. It should be noted that the half-decay time was almost identical with that at 20 °C (10 min). After the complete bleaching, the chromophore was extracted and analyzed by HPLC. The amounts of *all-trans*-retinal oximes were decreased (Table I).

Next, the sample was irradiated with a yellow light (>480 nm) at 20 °C in the presence of 10 mM hydroxylamine. Since ppR_O has an absorption spectrum more red-shifted than those of ppR and ppR_M, it absorbs the yellow light more preferentially than ppR and ppR_M. In this case, the half-bleaching time of ppR was about 6 min. Chromophore extraction experiments indicated that a less amount of *all-trans*-retinal was present in this sample than in that irradiated with 501-nm light (Table I).

It should be noted that in all the experiments described above 13-cis chromophores were extractable mainly in a 15-anti form of the retinal oximes, while the all-trans chromophores were mainly in a 15-syn form. Therefore, it is very interesting to examine whether the stereoselective reaction of the chromophore with hydroxylamine is caused by the unique binding site of the protein moiety or the sole property of the 13-cis retinylidene Schiff base. Thus, the ppR sample was irradiated with 501-nm light at 0 °C for 30 s in the absence of hydroxylamine. Then, a mixture of methanol, dichloromethane, and hydroxylamine (final concentration of 100 mM) was added to the sample, followed by vigorous mixing of the sample under the irradiation. In this manipulation, the protein moiety of ppR_M is expected to be rapidly denatured by methanol and dichloromethane and then the 13-cis chromophore is expected to react with hydroxylamine. The extracted 13-*cis*-retinal oxime was mainly the 15-syn form (Table I), suggesting that the stereoselective reaction of the 13-cis chromophore is mainly due to the unique binding site of the chromophore in ppR_M.

Discussion

Chromophore Configuration of ppR and Its Intermediates. The present investigations demonstrated that the bleaching kinetics of dark-adapted ppR by hydroxylamine at both the peak (500 nm) and the shoulder (460 nm) showed first-order kinetics with the same time constant (Figure 2b) and that chromophore extracted from ppR in the dark-adapted state was only in an all-trans form (Figure 3). These facts clearly show that like sR (Tsuda et al., 1985) ppR has only *all-trans*-retinal as its chromophore, even in the dark-adapted state, and exhibits no dark isomerization of the chromophore. The latter is in contrast to bR and hR, whose chromophores undergo the thermal equilibria between the all-trans and 13-cis forms (Maeda et al., 1977; Kamo et al., 1985). Considering the facts that sR and ppR act as photoreceptor proteins for positive and negative phototaxis (Spudich & Bogomolni, 1988; Tomioka et al., 1990), while bR and hR act as those of proton and chloride pumps (Oesterhelt & Stoeckenius, 1974; Lanyi, 1986), the absence or presence of the thermal equilibrium between all-trans and 13-cis chromophores is one of the distinguishable properties between photosensor proteins and ion pumps in the halophilic bacteria.

When the retinylidene chromophores were extracted after bleaching of the ppR by irradiation in the presence of hydroxylamine, about 90% 13-*cis*- and 10% *all-trans*-retinal were detected. Since ppR_M and ppR_O exist besides ppR under the

irradiation, the extracted 13-cis and all-trans chromophores would originate from ppR_M or ppR_O. As shown in Figure 4c, the difference absorbances at 500 (ppR), 390 (ppR_M), and 560 nm (ppR_O) under irradiation at 20 °C with 501-nm light were −0.0112, 0.0068, and 0.00086, respectively. The ratio of difference absorbance at 390 nm to that at 560 nm was about 8, which is comparable to the ratio of the amount of 13-*cis*- to that of *all-trans*-retinal (about 9) extracted from the sample. Although the extinction coefficients of ppR_M and ppR_O have not been determined yet, it is reasonable to assume that they are not so different from each other. Therefore, the chromophores of ppR_M and ppR_O should be in the 13-cis and all-trans forms, respectively. These identifications were supported by the fact that the amount of *all-trans*-retinal oxime was decreased when the sample was irradiated with >480-nm light or irradiated at 0 °C, where a less amount of ppR_O was formed. The results obtained by the irradiation of >480-nm light also deny the possibility that *all-trans*-retinal oxime originated from the photoproduct formed from ppR_O because if so, the amount of *all-trans*-retinal oxime should be increased by the >480-nm light irradiation by which ppR_O is photoconverted more preferentially than by the 501-nm light irradiation. It should be noted that the chromophore of the O intermediate of bR is also in an all-trans form (Smith et al., 1983).

From these results, we can summarize the photochemical events of the chromophore of ppR as follows. The *all-trans*-retinal, the chromophore of ppR, is isomerized to the 13-cis form on a photon absorption. While we do not have direct data, it is reasonable to assume that the chromophore of ppR_K is 13-*cis*-retinal because its thermal product, ppR_M, has a 13-*cis*-retinal. The conversion from ppR_K to ppR_M would be the release of the proton attached to the Schiff base linkage, judging from the absorption maximum of ppR_M (390 nm). The event on the formation of ppR_O is the thermal reisomerization of the chromophore from the 13-cis form to the all-trans form, and the proton would attach again. Since the configurational changes of the chromophore of ppR during its photocycle are very similar to those of bR, the peculiarity of ppR in its absorption spectrum and the absence of the L intermediate are caused by the specificity of the protein moiety.

Geometry of the C=N Configuration of the Retinal Oximes Extracted from ppR and ppR_M. Another important observation is that the ratio of 15-syn to 15-anti forms of *all-trans*-retinal oximes extracted from ppR is dependent on the extraction conditions. When ppR was denatured by chemical reagents, followed by addition of hydroxylamine (Figure 3a), the ratio of 15-syn to 15-anti forms was about 3 (Table I), while it was about 20 when ppR was bleached by hydroxylamine in the dark (Figure 3b). In the former manipulations, the structure of the protein moiety was first destroyed by the chemical reagents (methanol and dichloromethane) and then the chromophores reacted with hydroxylamine to form retinal oximes. On the other hand, in the latter case, the retinylidene chromophores bound to the protein moiety directly reacted with hydroxylamine to form retinal oximes, and then they were extracted by hexane. The results described above probably indicate the presence of a confined chromophore binding site in ppR. That is, the chromophore is partially protected by the protein moiety which effectively directs the approach of hydroxylamine to the imine group of the chromophore.

In contrast, the retinal oximes formed by the reaction of ppR_M with hydroxylamine were mainly 15-anti form. However, the phenomena were similar to those of ppR in that the ratios of 15-syn to 15-anti forms are dependent on the extraction conditions. The 13-cis chromophore of ppR_M was largely extracted as the 15-*anti*-retinal oxime when it reacted with hydroxylamine under the conditions where the protein moiety was undenatured, while 15-syn form was dominantly extracted when it reacted with hydroxylamine after the denaturation of the protein moiety (Table I). These facts indicate that the selectivity of C=N geometry in 13-*cis*-retinal oxime extracted from ppR_M is also caused by the structure of its protein moiety near the Schiff base.

From these observations, we can discuss the cause of the selectivity of the C=N geometry of the retinal oximes extracted from ppR and ppR_M. When a free *all-trans*- or 13-*cis*-retinal reacts with hydroxylamine, the ratio of 15-*syn*- to 15-*anti*-retinal oximes is 5–3:1. The preference formations of the 15-syn forms are likely to be caused by the steric interaction between the hydroxyl group (OH) and the hydrogen attached to the retinal at 14 position (Figure 1). Therefore, the geometry of C=N configuration of the retinal oxime extracted from the protein could be also affected by the steric and/or electrostatic interaction with some amino acids near the C=N of the chromophore. Although many amino acid residues could be selected for the candidate(s) to affect the stereoselective formation of the retinal oxime, we propose the counterion, the negatively charged residue present near the Schiff base linkage of the chromophore in ppR, as one of the most plausible candidates for the following reasons.

The *all-trans*- and 13-*cis*-retinal oximes extracted from ppR and ppR_M under nondenaturation conditions of the protein moieties are mainly in the 15-syn and 15-anti forms, respectively, both of which have hydroxyl groups oriented toward the methyl groups attached to the side chain of the retinal oxime at the 9 and 13 positions (see Figure 1). These facts suggest that a putative amino acid residue(s) affecting the geometry of the C=N configuration of the retinal oxime should be oriented in the direction opposite to that of the hydroxyl group of retinal oxime. Since the Schiff base linkage of ppR as well as that of ppR_M is expected to be in a 15-anti form, because all the retinal proteins investigated so far have 15-anti forms (except for 13-*cis*-bR; Smith et al., 1987), the counterion of ppR should be present in the same direction as that of the putative amino acid residue(s) (Figure 1). On the other hand, the chromophore of ppR_M is thought to be in an unprotonated form, and therefore no counterion should be present in ppR_M. However, it is reasonable to assume that like bR (Henderson et al., 1990) the counterion of ppR should be protonated by taking the proton from the Schiff base during the course of the formation of ppR_M but its position relative to the Schiff base linkage should not largely move. From these ideas, it is most likely that the stereoselective formation of the retinal oxime is caused by the presence of the counterion or its protonated form near the retinal chromophore.

Registry No. *All-trans*-retinal, 116-31-4; 13-*cis*-retinal, 472-86-6; hydroxylamine, 7803-49-8.

References

Bivin, D. B., & Stoeckenius, W. (1986) *J. Gen. Microbiol. 132*, 2167–2177.

Braiman, M., & Mathies, R. A. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 403–407.

Henderson, R., Baldwin, J. M., Ceska, T. A., Zemlin, F., Beckmann, E., & Downing, K. H. (1990) *J. Mol. Biol. 213*, 899–929.

Hirayama, J., Imamoto, Y., Shichida, Y., Kamo, N., Tomioka, H., & Yoshizawa, T. (1992) *Biochemistry* (in press).

Imamoto, Y., Shichida, Y., Yoshizawa, T., Tomioka, H., Takahashi, T., Fujikawa, K., Kamo, N., & Kobatake, Y.

(1991) *Biochemistry 30*, 7416-7424.
Kamo, N., Hazemoto, N., Kobatake, Y., & Mukohata, Y. (1985) *Arch. Biochem. Biophys. 238*, 90-96.
Lanyi, J. K. (1986) *Annu. Rev. Biophys. Biophys. Chem. 15*, 11-28.
Maeda, A., Iwasa, T., & Yoshizawa, T. (1977) *J. Biochem. 82*, 1599-1604.
Oesterhelt, D., & Stoeckenius, W. (1974) *Methods Enzymol. 31*, 667-678.
Ogurusu, T., Maeda, A., Sasaki, N., & Yoshizawa, T. (1981) *J. Biochem. 90*, 1267-1273.
Shichida, Y., Imamoto, Y., Yoshizawa, T., Takahashi, T., Tomioka, H., Kamo, N., & Kobatake, Y. (1988a) *FEBS Lett. 236*, 333-336.
Shichida, Y., Nakamura, K., Yoshizawa, T., Trehan, A., Denny, M., & Liu, R. S. H. (1988b) *Biochemistry 27*, 6495-6499.
Spudich, J. L., & Bogomolni, R. A. (1988) *Annu. Rev. Biophys. Biophys. Chem. 17*, 193-215.
Smith, S. O., Pardoen, J. A., Mulder, P. P. J., Curry, B., Lugtenburg, J., & Mathies, R. A. (1983) *Biochemistry 22*, 6141-6148.
Smith, S. O., Pardoen, J. A., Lugtenburg, J., & Mathies, R. A. (1987) *J. Phys. Chem. 91*, 804-819.
Takahashi, T., Tomioka, H., Kamo, N., & Kobatake, Y. (1985) *FEMS Microbiol. Lett. 28*, 161-164.
Takahashi, T., Tomioka, H., Nakamori, Y., Tsujimoto, K., Kamo, N., & Kobatake, Y. (1988) in *Molecular Physiology of Retinal Proteins* (Hara, T., Ed.) pp 149-154, Yamada Science Foundation, Osaka, Japan.
Takahashi, T., Yan, B., Mazur, P., Nakanishi, K., & Spudich, J. L. (1990) *Biochemistry 29*, 8467-8474.
Tomioka, H., Takahashi, T., Kamo, N., & Kobatake, Y. (1986) *Biochem. Biophys. Res. Commun. 139*, 389-395.
Tomioka, H., Otomo, J., Hirayama, J., Kamo, N., & Sasabe, H. (1990) *IV International Conference on Retinal Proteins*, Santa Cruz.
Trehan, A., Liu, R. S. H., Shichida, Y., Imamoto, Y., Nakamura, K., & Yoshizawa, T. (1990) *Bioorg. Chem. 18*, 30-40.
Tsuda, M., Nelson, B., Chang, C.-H., Govindjee, R., & Ebrey, T. G. (1985) *Biophys. J. 47*, 721-724.
Tsukida, K., Ito, M., Tanaka, T., & Yagi, I. (1985) *J. Chromatogr. 331*, 265-272.
Yan, B., Takahashi, T., Johnson, R., Derguini, F., Nakanishi, K., & Spudich, J. L. (1990) *Biophys. J. 57*, 807-814.

Chemical Modification of Prostaglandin H Synthase with Diethyl Pyrocarbonate†

Xuhan Zhang, Ah-Lim Tsai, and Richard J. Kulmacz*
Division of Hematology and Oncology, University of Texas Health Science Center at Houston, Houston, Texas 77225
Received August 1, 1991; Revised Manuscript Received November 22, 1991

ABSTRACT: The role of histidine in catalysis by prostaglandin H synthase has been investigated using chemical modification with diethyl pyrocarbonate (DEPC), an agent that has been found to rather selectively derivatize histidine residues in proteins under mild conditions. Incubation of the synthase apoprotein with DEPC at pH 7.2 resulted in a progressive loss of the capacity for both cyclooxygenase and peroxidase catalytic activities. The kinetics of inactivation of the cyclooxygenase activity were dependent on the concentration of DEPC; a second-order rate constant of 680 M^{-1} min^{-1} was estimated for reaction of the apoenzyme at pH 7.2 and 0 °C. The kinetics of inactivation of the cyclooxygenase by DEPC exhibited a sigmoidal dependence on the pH, indicating that deprotonation of a group with a pK_a of 6.3 was required for inactivation. The presence of the heme prosthetic group slowed, but did not prevent, inactivation by DEPC. The stoichiometry of histidine modification of apoenzyme during inactivation determined from absorbance increases at 242 nm agreed well with the overall stoichiometry of derivatized residues determined with [^{14}C]DEPC, indicating that modification by DEPC was quite selective for histidine residues on the synthase. Although modification of several histidine residues by DEPC was observed, only one of the histidine residues was essential for cyclooxygenase activity. Modification of the holoenzyme with DEPC altered the EPR signal of the hydroperoxide-induced tyrosyl free radical from the wide doublet (35 G, peak-to-trough) found with the native synthase to a narrower singlet (28 G, peak-to-trough) quite like that found in the indomethacin–synthase complex. Reaction of the indomethacin–synthase complex with DEPC was found to increase the cyclooxygenase velocity by 9 times its initial value, to about one-third of the uninhibited value, without displacement of the indomethacin; the peroxidase was significantly inactivated under the same conditions. Histidyl residues in the synthase are thus likely to have important roles not only in cyclooxygenase and peroxidase catalysis but also in the interaction of the synthase with indomethacin.

The cyclooxygenase activity of prostaglandin H (PGH)[1] synthase represents the first committed step in the biosynthesis of prostaglandins, prostacyclin, and thromboxane (Samuelsson et al., 1978). The pure synthase also exhibits a heme-dependent peroxidase activity that has been proposed to play a crucial role in the initiation of the cyclooxygenase reaction (Kulmacz et al., 1985; Dietz et al., 1988; Kulmacz, 1986). Spectroscopic observations have indicated that histidine res-

† This work was supported in part by National Institutes of Health Grant GM 30509.
* To whom correspondence should be addressed at the Division of Hematology and Oncology, University of Texas Health Science Center, P.O. Box 20708, Houston, TX 77225.

[1] Abbreviations: PGH, prostaglandin H; DEPC, diethyl pyrocarbonate; EtOOH, ethyl hydroperoxide; EPR, electron paramagnetic resonance.

0006-2960/92/0431-2528$03.00/0