Reconstitution photoactive yellow protein from apoprotein and *p*-coumaric acid derivatives

Yasushi Imamoto, Takeshi Ito**, Mikio Kataoka, Fumio Tokunaga*

Department of Earth and Space Science, Faculty of Science, Osaka University, Toyonaka, Osaka 560, Japan

Received 4 September 1995

Abstract **We report reconstitution of photoactive yellow protein (PYP) from apoPYP and *p*-coumaric acid derivatives. The addition of *p*-coumaric acid to the apoPYP sample did not result in the recovery of PYP. In contrast, yellow products were obtained by the addition of *p*-coumaryl thiophenyl ester or *p*-coumaric anhydride to the apoPYP sample, the absorption spectra of which were indistinguishable from the spectrum of intact PYP. Our findings provide strong evidence that PYP has the *p*-coumaryl chromophore. This reconstitution technique opens the way for further biophysical studies of PYP using artificial chromophore analogs.**

Key words: Photoactive yellow protein; Photoreceptor protein; *p*-Coumaric acid; Chromophore; Reconstitution; *Ectothiorhodospira halophila*

1. Introduction

Photoactive yellow protein (PYP) is present in *Ectothiorhodospira halophila* as a soluble small chromoprotein [1]. It has a broad absorption band, the absorption maximum of which is at 446 nm [1]. It is considered a photoreceptor protein for the negative phototaxis of *E. halophila* [2]. PYP has a photoreaction cycle comprised of several intermediates, the spectral and kinetic properties of which are very similar to those of the retinal proteins in halobacteria [3–5]. Formation of the bathochromic photoproduct occurs upon light absorption, the photoproduct being converted to a near-UV intermediate in a microsecond time scale and reverting to the ground state in a subsecond time scale. These intermediates correspond to the K (batho) or L (lumi) and M (meta) intermediates of the retinal proteins. The relation between PYP and the retinal proteins, therefore, is a matter of interest, but the nature of the PYP protein moiety is very different from the moieties of the retinal proteins [6,7]. Retinal proteins are membrane proteins composed of seven transmembrane helices [8,9] and have molecular weights of 24,000–40,000. PYP is a soluble small protein of 14 kDa which crystal structure analysis to 1.4-Å resolution has shown to have the α/β-fold structure [7].

The PYP chromophore first was thought to be retinal because its photochemical properties are similar to those of retinal proteins [10]. The chromophore-binding site was tentatively assigned to the lysine residue at the 111 position [10]; but mass spectroscopy has shown that the molecular weight of the chromophore is 147, smaller than that of retinal [6]. Moreover, the chromophore binding site is a unique cysteine residue at position 69 [6]. These findings suggest that the chromophore is *not* retinal. Recent studies have shown that the PYP chromophore is *p*-coumaric acid (4-hydroxycinnamic acid) which binds to the cysteine residue via a thioester bond [11,12] (Fig. 1a,b).

Although studies based on NMR analysis of the chromophore released from digested PYP [11] or high-resolution crystallography of PYP [12] clearly have shown that PYP has the *p*-coumaryl chromophore, direct confirmation would depend on the reconstitution of PYP from its apoprotein and *p*-coumaric acid. Moreover, an effective reconstitution technique would provide an important means of furthering biochemical and biophysical studies of PYP using artificial chromophore analogs.

In spite of its importance, the reconstitution of PYP has not been achieved, probably because the thioester bond is difficult to obtain by the direct reaction of the carboxyl and thiol groups under physiological conditions. We, therefore, investigated formation of the thioester bond using *p*-coumaric acid derivatives and succeeded in reconstituting PYP which had an absorption spectrum indistinguishable from that of intact PYP.

2. Materials and methods

2.1. Purification of PYP

E. halophila (BN 9629) was provided by Dr. K. Shimada (Tokyo Metropolitan University). Cells cultured in 5–10 l culture medium [1] for 7–10 days under sunlight were harvested by centrifugation ($9000 \times g$, 20 min), then stored at −80°C until use. PYP was isolated from *E. halophila* by the slightly modified method reported previously [1]. Briefly, the cells were suspended in Tris buffer (10 mM Tris-HCl, pH 7.4) supplemented with a few mg of DNase I then disrupted by osmotic shock by being dialyzed against distilled water at 4°C. The desalted lysate was centrifuged ($48,000 \times g$, 60 min) and the supernatant collected. Ammonium sulfate powder was added to 50% saturation, after which the mixture was stirred for 30 min at 4°C, then centrifuged ($48,000 \times g$, 20 min). The supernatant was dialyzed against Tris buffer, then applied to a DEAE-Sepharose CL6B column (Pharmacia) equilibrated with Tris buffer. After washing the column with Tris buffer, PYP was eluted with a linear gradient of NaCl (100–200 mM) in the same buffer. The yellow fractions eluted with ~150 mM NaCl were collected, DNase I was added and the fractions dialyzed overnight at 4°C against Tris buffer containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$ after which DEAE-Sepharose CL6B column chromatography was repeated. Fractions that contained PYP were collected and concentrated with an ultrafiltration membrane (Amicon, Centriprep-3). The concentrate was applied to a gel-filtration column (Pharmacia, PD-10) equilibrated with Tris buffer and PYP eluted with the same buffer. This PYP was adsorbed on a small DEAE-Sepharose column equilibrated with Tris buffer, then eluted with the same buffer containing 200 mM NaCl. The optical purity index (Abs_{275}/Abs_{446}) was ~0.5.

2.2. Preparation of apoPYP

Purified PYP was bleached in the dark for 16 h at 20°C in the

*Corresponding author. Department of Earth and Space Science, Faculty of Science, Osaka University, Toyonaka, Osaka 560, Japan. Fax: (81) (6) 850 5542.

**Present address:* Earthquake Research Institute, University of Tokyo, Bunkyo-ku, Tokyo 113, Japan.

SSDI 0014-5793(95)01096-3

presence of 100 mM hydroxylamine and 1 mM dithiothreitol (DTT). To remove the hydroxylamine and DTT, the bleached PYP (tentatively called apoPYP) sample was applied to a gel-filtration column (Pharmacia, PD-10) equilibrated with Tris buffer and the column flushed with same buffer.

2.3. Synthesis of p-coumaryl thiophenyl ester

p-Coumaric acid (Sigma) and thiophenol (Wako) first were dissolved in *N,N'*-dimethylformamide (DMF), then mixed on ice with dicyclohexylcarbodiimide (DCC) dissolved in DMF. The reaction mixture was stirred for several hours and kept at 4°C overnight and, after the addition of distilled water, centrifuged. *p*-Coumaryl thiophenyl ester was extracted from the reaction mixture with diethylether. The extract was treated with charcoal, evaporated under a flow of N_2 gas and the residue dissolved in DMF (Fig. 1c).

2.4. Synthesis of p-coumaric anhydride

The *p*-coumaric acid dissolved in DMF was mixed with DCC dissolved in DMF on ice. After being stirred for several hours, the reaction mixture was kept at 4°C overnight, then centrifuged. The supernatant was diluted 4-fold with DMF and used in the following experiments (Fig. 1d).

2.5. Reconstitution and purification of PYP

Each *p*-coumaric acid derivative dissolved in DMF was added to an apoPYP sample and incubated at 4°C. There was a 2–3-fold molar excess based on the assumption that the respective extinction coefficients of *p*-coumaryl thiophenyl ester and *p*-coumaric anhydride were 20,000 and 40,000. After incubation at 4°C overnight, the sample was applied to a small DEAE-Sepharose column equilibrated with Tris buffer. The column was washed with 50 mM NaCl in Tris buffer and the reconstituted PYP eluted with 200 mM NaCl in Tris buffer.

2.6. Spectroscopy

Absorption spectra were recorded with a Hitachi 3210 recording spectrophotometer interfaced with a PC (NEC 9801 NS/R) that stored and analysed the spectral data. Temperature-controlled water was circulated to keep the sample cell at a constant temperature (4 or 20°C).

3. Results

The apoprotein of PYP (apoPYP) was prepared in the active form and had the ability to bind the *p*-coumaryl chromophore and form PYP. PYP was bleached with hydroxylamine in the dark (Fig. 2). Incubation of PYP in the presence of 100 mM hydroxylamine caused an absorbance decrease at 446 nm, indicative of the bleaching of PYP (Fig. 2a). Absorbance decreases at 446 nm on the semilogarithmic scale were plotted against incubation time (Fig. 2b), the plot being linear. The bleaching time constant ($1/e$) was estimated to be 154 min. Because the thioester bond is cleaved into a thiol group and hydroxamic acid by hydroxylamine, as shown in the coenzyme

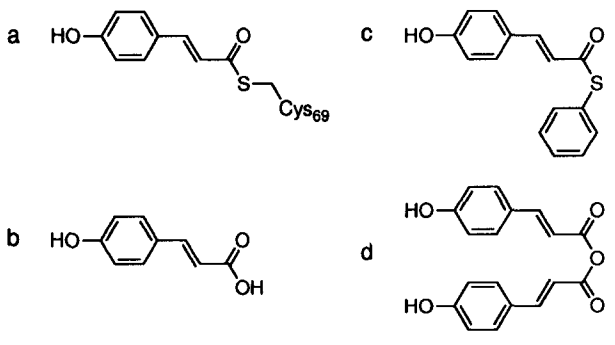

Fig. 1. Structures of the *p*-coumaryl chromophore of PYP (a), *p*-coumaric acid (b), *p*-coumaryl thiophenyl ester (c) and *p*-coumaric anhydride (d).

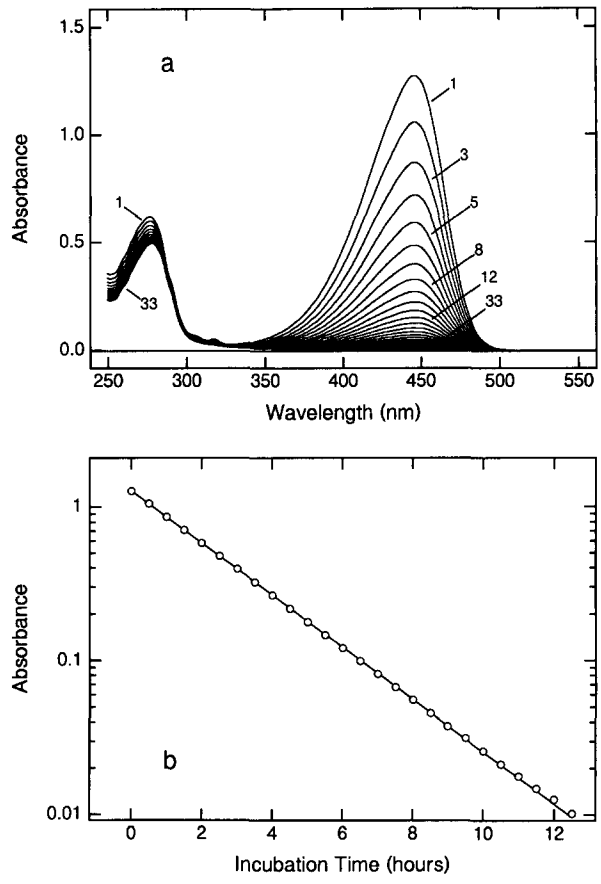

Fig. 2. Bleaching of PYP with hydroxylamine. (a) The PYP sample was supplemented with hydroxylamine and DTT at the final respective concentrations of 100 and 1 mM (curve 1) after which the sample was incubated at 20°C in the dark. Absorption spectra were recorded at intervals of 30 min (curves 2–33). (b) The absorbance decreases at 446 nm on the bleaching of PYP were plotted on a semilogarithmic scale against incubation time (circles). The estimated bleaching time constant ($1/e$) was 154 min.

A system, *p*-coumaric acid (the chromophore of PYP) was expected to be released and the free thiol of cysteine formed. Quantitative analysis was made of the thiol group exposed on the bleaching of PYP with hydroxylamine using DTNB [5,5'-dithiobis(2-nitrobenzoic acid)] in Tris buffer without DTT. Results showed that ~0.9 of a thiol group was formed from one PYP molecule (data not shown).

The hydroxylamine and DTT in the apoPYP sample were removed by gel-filtration column chromatography. An addition of *p*-coumaric acid to the apoPYP sample did not regenerate PYP which suggests that the thioesterification between *p*-coumaric acid and cysteine does not proceed directly (see below). Taking into account a method for the acylation of coenzyme A [13], we made two different chemical modifications of *p*-coumaric acid.

First, the *p*-coumaric acid was linked with thiophenol via a thioester bond with DCC as the dehydration reagent. The *p*-coumaryl thiophenyl ester formed was added to the apoPYP sample and the whole incubated at 4°C (Fig. 3a). An absorbance increase occurred at 446 nm with a concomitant decrease at ~350 nm, indicative that PYP was regenerated as the result of transthioesterification from the thiophenyl ester to the thiol

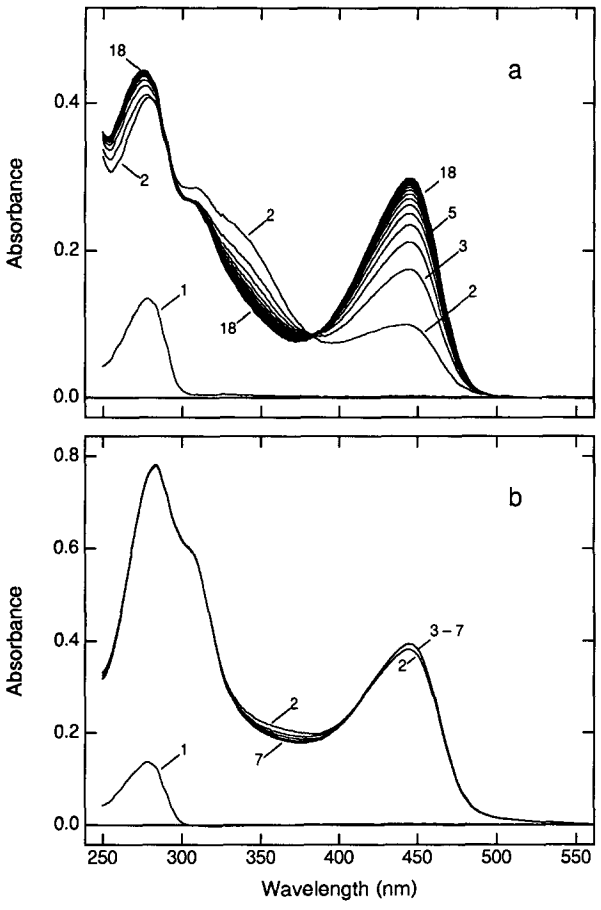

Fig. 3. Reconstitution of PYP from apoPYP and *p*-coumaric acid derivatives. (a) The apoPYP sample (curve 1) was supplemented with *p*-coumaryl thiophenyl ester dissolved in DMF (curve 2), then incubated at 4°C in the dark. Absorption spectra were recorded at intervals of 10 min (curves 3–18). (b) The apoPYP sample (curve 1) was supplemented with *p*-coumaric anhydride dissolved in DMF (curve 2), then incubated at 4°C in the dark. Absorption spectra were recorded at intervals of 10 min (curves 3–7).

of cysteine. The absorbance increase at 446 nm was plotted against incubation time (Fig. 4a). After removal of excess *p*-coumaryl thiophenyl ester by small DEAE-Sepharose column chromatography, the absorption spectrum of the regenerated PYP was compared with that of intact PYP (Fig. 4b). In the visible region, their absorption spectra were indistinguishable. The slight increase in the absorbance ratio at 280–446 nm would be due to oxidization of the thiol group of the cysteine and/or to the denaturation of apoPYP during the manipulations.

Next, the apoPYP was incubated with the *p*-coumaric anhydride formed from *p*-coumaric acid with DCC as the dehydration reagent (Fig. 3b). Just after the addition of *p*-coumaric anhydride, the sample turned yellow and the absorbance increase at 446 nm was saturated within 10 min (Fig. 4a). Excess *p*-coumaric anhydride was removed by small DEAE-Sepharose column chromatography and the absorption spectrum of the product compared with that of intact PYP. Their spectra also were indistinguishable (Fig. 4b).

When *p*-coumaric acid dissolved in DMF was added to the apoPYP sample, there was no absorbance increase at 446 nm even on incubation at 20°C (Fig. 4a). After 14 h, the *p*-coumaryl thiophenyl ester was added to the mixture. As a result, ~80% of the PYP was regenerated, evidence that most of the apoPYP maintained its ability to bind its chromophore (data not shown). We concluded that no thioester bond was formed by the direct reaction of the carboxyl group of *p*-coumaric acid and the thiol group of cysteine under the experimental conditions used.

4. Discussion

Our findings clearly show that PYP is bleached by cleavage of the thioester bond by hydroxylamine and that PYP can be regenerated by the use of *p*-coumaric acid derivatives. The absorption spectra of the reconstituted PYPs are indistinguishable from the spectrum of intact PYP, evidence that the structure of the reconstituted PYP is identical to that of the intact PYP. These observations are direct proof that PYP has the *p*-coumaryl chromophore as reported previously [11,12].

PYP was bleached with 100 mM hydroxylamine in the dark. During this bleaching, no absorbance increase due to the released chromophore was detected (Fig. 2a). We suspect that the λ_{max} of *p*-coumaryl hydroxamic acid is shorter than 250 nm or that its extinction coefficient is very small. Note that the absorbance at ~280 nm decreased and the spectral fine structures at 318 and 306 nm disappeared on the bleaching of PYP. These findings strongly suggest that PYP has a second absorption band at ~280 nm which corresponds to the *b*-bands of retinal proteins that appear at ~350 nm. The difference in the locations of the *b*-bands must be due to the difference in the lengths of the conjugated double bond system between *p*-coumaric acid and the retinal.

The Schiff base bond of the retinylidene chromophore is easily formed under physiological conditions and incubation of opsin with the retinal that has the appropriate configuration results in the formation of pigment [14–17]. The thioester bond is difficult to obtain under the mild conditions at which protein is not denatured. We, therefore, reconstituted PYP from its apoprotein using *p*-coumaryl thiophenyl ester or *p*-coumaric anhydride; but these reactions would differ markedly from those in the biosynthetic route of PYP in *E. halophila*. It is reasonable to assume that a specific enzyme is required for the thioesterification of the *p*-coumaryl group and apoPYP in vivo. Although reconstitution with such an enzyme would be efficient, enzyme isolation would be difficult as the amount isolated would be small because of the small amount of PYP in the cell [1].

We succeeded in regenerating PYP by the use of two *p*-coumaric acid derivatives. In general, on reconstitution of the chromoprotein from the apoprotein and lyophilic chromophore at least two processes must be considered; the binding of the chromophore to the hydrophobic region of the apoprotein and the formation of a covalent bond with the specific amino acid residue. For example, on the regeneration of rhodopsin, the retinal chromophore first enters the hydrophobic region, then a Schiff base bond is formed [18]. In the case of PYP, reconstitution would proceed in two steps similar to those for rhodopsin. Regeneration with *p*-coumaric anhydride was much faster than that with *p*-coumaryl thiophenyl ester. A comparison of the *p*-coumaryl thiophenyl ester with *p*-coumaric anhydride shows that the molecular weight of the former is

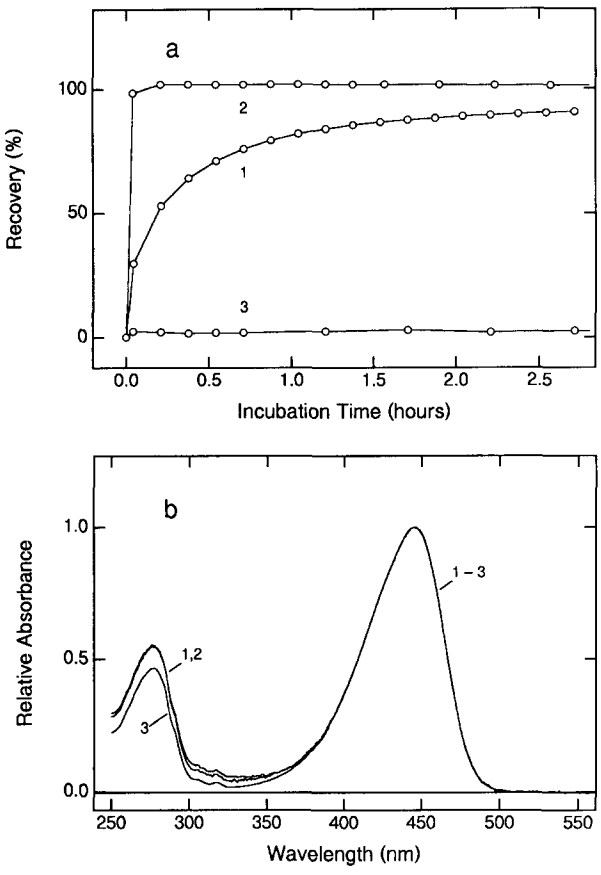

Fig. 4. (a) Comparison of the PYP regeneration rates. *p*-Coumaryl thiophenyl ester (curve 1), *p*-coumaric anhydride (curve 2) or *p*-coumaric acid (curve 3) dissolved in DMF was added to the apoPYP sample. The absorbance increases at 446 nm were plotted against incubation time. The incubation temperature was 4°C (curves 1 and 2) or 20°C (curve 3). (b) Comparison of the absorption spectra of the reconstituted PYP from *p*-coumaryl thiophenyl ester (curve 1) and *p*-coumaric anhydride (curve 2) with that of intact PYP (curve 3). Spectra were normalized at 446 nm.

smaller and its hydrophobicity is higher; therefore, binding to the hydrophobic region should be faster for the *p*-coumaryl thiophenyl ester than for *p*-coumaric anhydride which differs from the observed regeneration rates. The difference in the regeneration rates of these two derivatives is attributable to the formation of a thioester bond, a rate-limiting factor for the regeneration of PYP.

Rhodopsin analogs obtained with chemically synthesized retinal analogs are widely used to clarify the structures and functions of retinal proteins [19]. The PYP reconstitution technique reported here opens the way for further biophysical studies of PYP using chemically modified chromophores.

Acknowledgements: We thank Dr. K. Shimada (Tokyo Metropolitan University) for the kind gift of *E. halophila* strain BN 9629. This work was supported in part by grant-in-aids from the Japanese Ministry of Education, Culture and Science and by SUNBOR.

References

[1] Meyer, T.E. (1985) Biochim. Biophys. Acta 806, 175–183.
[2] Sprenger, W.W., Hoff, W.D., Armitage, J.P. and Hellingwerf, K.J. (1993) J. Bacteriol. 175, 3096–3104.
[3] Hoff, W.D., Kwa, S.L.S., van Grondelle, R. and Hellingwerf, K.J. (1992) Photochem. Photobiol. 56, 529–539.
[4] Hoff, W.D., van Stokkum, I.H.M., van Ramesdonk, H.J., van Brederode, M.E., Brouwer, A.M., Fitch, J.C., Meyer, T.E., van Grondelle, R. and Hellingwerf, K.J. (1994) Biophys. J. 67, 1691–1705.
[5] Meyer, T.E., Yakali, E., Cusanovich, M.A. and Tollin, G. (1987) Biochemistry 26, 418–423.
[6] van Beeumen, J., Devreese, B., van Bun, S., Hoff, W.D., Hellingwerf, K.J., Meyer, T.E., McRee, D.E. and Cusanovich, M.A. (1993) Protein Sci. 2, 1114–1125.
[7] Bolgstahl, G.E.O., Williams, D.R. and Getzoff, E.D. (1995) Biochemistry 34, 6278–6287.
[8] Henderson, R., Baldwin, J.M., Ceska, T.A., Zemlin, F., Beckmann, E. and Downing, K.H. (1990) J. Mol. Biol. 213, 899–929.
[9] Schertler, G.F.X., Villa, C. and Henderson, R. (1993) Nature (London) 362, 770–772.
[10] McRee, D.E., Tainer, J.A., Meyer, T.E., van Beeumen, J., Cusanovich, M.A. and Getzoff, E.D. (1989) Proc. Natl. Acad. Sci. USA 86, 6533–6537.
[11] Hoff, W.D., Düx, P., Hård, K., Devreese, B., Nugteren-Roodzant, I.M., Crielaard, W., Boelens, R., Kaptein, R., van Beeumen, J. and Hellingwerf, K.J. (1994) Biochemistry 33, 13959–13962.
[12] Baca, M., Borgstahl, G.E.O., Boissinot, M., Burke, P.M., Williams, D.R., Slater, K.A. and Getzoff, E.D. (1994) Biochemistry 33, 14369–14377.
[13] Nishizuka, Y. and Wakabayashi, K. (1975) in: Vitamins and Coenzymes (Japanese Biochemical Society, Ed.) Methods in Biochemistry, Vol. 13, pp. 315–324, Tokyo-Kagaku-Dojin, Tokyo, Japan. [In Japanese.]
[14] Hubbard, R. and Wald, G. (1952) J. Gen. Physiol. 36, 269–307.
[15] Oesterhelt, D. and Schumann, L. (1974) FEBS Lett. 44, 262–265.
[16] Maeda, A., Iwasa, T. and Yoshizawa, T. (1977) J. Biochem. 82, 1599–1604.
[17] Shichida, Y., Imai, H., Imamoto, Y., Fukada, Y. and Yoshizawa, T. (1994) Biochemistry 33, 9040–9044.
[18] Matsumoto, H. and Yoshizawa, T. (1975) Nature (London) 258, 523–526.
[19] Liu, R.S.H. and Shichida, Y. (1991) in: Photochemistry in Organized and Constrained Media (Ramamurthy. V., Ed.) pp. 817–840, VCH Publishers, New York, NY.