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Amino acid sequence surrounding the retinal-binding site in retinochrome of the squid, *Todarodes pacificus*

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Squid (*Todarodes pacificus*) retinochrome was reduced to *N*-retinyl protein with borane dimethylamine and cleaved by CNBr. The retinyl peptide was then isolated by chromatography while being monitored for absorbances at 215 and 330 nm, and the N-terminal amino acid sequence was determined to be Ser-Lys-Thr-Gly-X-Ala-Leu-Phe-Pro. This sequence was the same that we had observed at the 7th transmembrane domain of retinochrome whose structure was reported previously. During Edman degradation of the retinyl peptide, the yield of the PTH-lysine at the second cycle was lower than those of the other PTH-amino acids, proving that the lysine residue forms a Schiff's base with retinal (Lys-275 in retinochrome). The amino acid sequence surrounding the retinal-binding lysine in retinochrome greatly differed from those in a variety of known visual pigments. This fact would be associated with the difference in the photoisomerization of chromophore between retinochrome and rhodopsin. The protein structure of retinochrome is also compared with that of rhodopsin in *Todarodes*.

Rhodopsin; Retinochrome; Retinal-binding site; Photoisomerization; Visual cell; *Todarodes pacificus*

1. INTRODUCTION

Retinochrome is a photosensitive pigment that was originally extracted from the retina of the Japanese common squid, *Todarodes pacificus*. In the molluscs such as cephalopods and gastropods, the visual cell possesses retinochrome in the inner segments in addition to rhodopsin contained in the outer segments. As has been ascertained by our previous work, the intracellular transport of retinals and the exchange of retinal chromophores between the two photoisomerization systems of rhodopsin and retinochrome are conducted by the role of a retinal-binding protein (RALBP), yielding the rhodopsin–retinochrome conjugate system [1]. The two photopigments, retinochrome and rhodopsin, are very similar to each other in the absorption spectrum, but quite different in the stereoisomeric form of their retinal chromophores, which is all-*trans* in retinochrome but 11-*cis* in rhodopsin. Upon exposure to light, they are changed to metaretinochrome and metarhodopsin, whose chromophores are in the 11-*cis* and the all-*trans* forms, respectively. Particularly, retinochrome-protein can combine with various geometrical isomers of retinal and in visible light catalyze their isomerization to 11-*cis*, the isomer required by opsin to form rhodopsin. Aiming at understanding such a specific *trans*-to-*cis* photoisomerase activity, we previously reported the primary protein structure of *Todarodes* retinochrome deduced from the cDNA sequence [2]. The present work was carried out to determine the all-*trans*-retinal-binding site in the retinochrome molecule. We have shown it to be lysine located at the 7th transmembrane helix, and compared the amino acid sequence around it with those in vertebrate and invertebrate visual pigments. The structure of retinochrome is also described in comparison with that of *Todarodes* rhodopsin we reported recently [3].

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2. MATERIALS AND METHODS

2.1. *Reduction of purified retinochrome*

Retinochrome extract (ca. 0.4 mg retinochrome/ml) was prepared from the squid (*Todarodes pacificus*) retina by our routine method [4] using 12 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) in 67 mM phosphate buffer, pH 6.5. To 2.1 ml of the retinochrome extract was added 35 μl of 6 N HCl to give a pH 1.4 and then added 35 mg of borane dimethylamine with gentle stirring to reduce retinochrome [5]. Upon reduction for 10 min at room temperature, the absorption maximum shifted from 495 to 330 nm, showing the conversion of retinochrome into *N*-retinyl retinochrome.

2.2. *Preparation of cyanogen bromide peptides of retinyl retinochrome and purification of the retinyl peptide*

The retinyl protein was cleaved by cyanogen bromide (CNBr) (15 mg CNBr per mg retinochrome) in 0.1 N HCl under N_2 atmosphere at room temperature. After incubation for 12 h in the dark, the reaction mixture was diluted by addition of 4-fold volume of water and then the CNBr peptides were lyophilized. They were dissolved in 300 μl of 1% trifluoroacetic acid (TFA) and centrifuged at 15,000 × *g* for 10 min to remove any small amounts of insoluble material. The supernatant (100 ml) was applied to a column (Develosil ODS-7, 4.6 (diameter) × 25 cm, Toyosoda, Japan) pre-equilibrated with degassed 0.1% TFA. The resultant retinyl peptide was separated on high-pressure liquid chromatography (HPLC) using a gradient of 0% to 100% ace-

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tonitrile in 0.1% TFA at a flow rate of 1 ml/min, while being monitored for absorbances at 215 and 330 nm. Finally, only one peak of absorption at 330 nm was found through the chromatography (Fig. 1). This peak fraction was collected and lyophilized.

2.3. *Determination of N-terminal amino acid sequences of proteins*

The lyophilized retinyl peptide was subjected to amino acid sequence analysis by Edman degradation, using a gas-phase automatic peptide sequencer (model 470A; Applied Biosystems, USA).

All the above procedures were carried out under dim red light.

3. RESULTS AND DISCUSSION

The N-terminal amino acid sequence of the isolated retinyl peptide of *Todarodes* retinochrome was determined to be Ser-Lys-Thr-Gly-X-Ala-Leu-Phe-Pro. This sequence was consistent with the amino acid residues from 274 to 282 that we had observed in the 7th membrane spanning domain of retinochrome [2]. Table I shows the amount of PTH-amino acid from each cycle of the stepwise Edman degradation. It is usual for Edman degradation to gain a relatively high yield of PTH-lysine. However, the actual yield of the PTH-lysine at the second cycle was lower than those of the other PTH-amino acids. This indicated that the retinal should be combined with the lysine of the peptide to form a retinal Schiff's base linkage. Consequently, the all-*trans*-retinal-binding site in retinochrome was determined to be Lys-275 in the 7th transmembrane helix, as illustrated later in Fig. 3.

The amino acid sequences surrounding the retinal-binding lysine in various photopigments is presented in Fig. 2. They are very homologous among visual pigments, rhodopsins and cone pigments. However, the sequence in retinochrome is greatly different from those in the visual pigments. It is only the proline at the 7th position following the lysine that is conserved throughout the photopigments including retinochrome. The phenylalanine at the 2nd position preceding the lysine, the asparagine at the 6th and the tyrosine at the 10th following the lysine are conserved in all the visual pigments. In retinochrome, however, the phenylalanine, asparagine and tyrosine are substituted by methionine, phenylalanine and isoleucine, respectively. The histidine at the 5th position from the lysine is a characteristic found only in cephalopod rhodopsins (tyrosine in insect and vertebrate visual pigments) [3,6–8]. This histidine is also replaced by leucine in retinochrome. Distinct differences with regard to the amino acid sequence be-

Table I

Amino acid sequence of the isolated retinyl peptide determined by Edman degradation, using a gas phase automatic sequencer

Cycle no.	PTH-amino acid identified	Amount recovered (pmol)
1	Ser	13.3
2	Lys	3.9
3	Thr	8.6
4	Gly	47.7
5*	–	–
6	Ala	8.0
7	Leu	6.9
8	Phe	5.7

*Amino acid at the 5th cycle was predicted to be cysteine from the primary sequence of retinochrome. PTH-cysteine is difficult to be detected with our present sequencer.

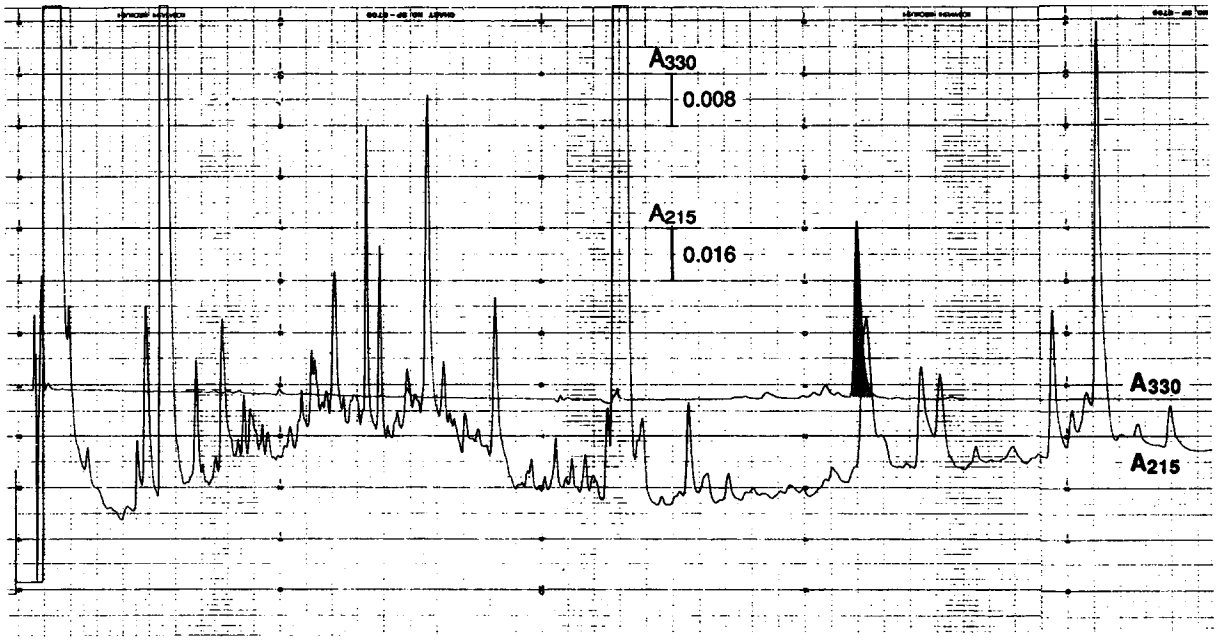

Fig. 1. Isolation of the retinyl peptide of squid retinochrome on HPLC. Separation of CNBr-cleaved peptides was monitored by absorbances at 215 and 330 nm. The 215-nm record was started slightly later to avoid overlapping.

Squid (*Todarodes*)	retinochrome	PPIMSKTGCALFFLLI	2)
Squid (*Todarodes*)	rhodopsin	PVMFAKASAIHNPMIY	3)
Squid (*Loligo*)	rhodopsin	PVMFAKASAIHNPMIY	7)
Octopus	rhodopsin	PVLFAKASAIHNPIVY	8)
Fly (*Drosophila*)	rhodopsin	GACFAKSAACYNPIVY	9)
Fly (*Calliphora*)	rhodopsin	GACFAKSAACYNPIVY	10)
Crayfish (*Procambarus*)	rhodopsin	GYVFAKANAVYNPIVY	11)
Lamprey (*Lampetra*)	rhodopsin	PAFFAKSSALYNPVIY	12)
Goldfish (*Carassius*)	rhodopsin	PAFFAKTAAVYNPCIY	13)
Frog (*Rana*)	rhodopsin	PAFFAKSSAIYNPVIY	14)
Gecko (*Gekko*)	visual pigment-467	PAFFSKSSSIYNPIIY	15)
	visual pigment-521	PAYFAKSATIYNPVIY	15)
Chicken	rhodopsin	PAFFAKSSAIYNPVIY	16)
	iodopsin	PAYFAKSATIYNPIIY	17)
Bovine	rhodopsin	PAFFAKTSAVYNPVIY	18)
Human	rhodopsin	PAFFAKSAAIYNPVIY	19)
	cone pigment (blue)	PSFFSKSACIYNPIIY	20)
	cone pigment (green)	PAFFAKSATIYNPVIY	20)
	cone pigment (red)	PAYFAKSATIYNPVIY	20)

Fig. 2. Sequence homology in retinal-binding regions of various eye photopigments.

tween retinochrome and visual pigments are probably related to their respective photoisomerization of chromophore and to the characteristic photoisomerase activity of retinochrome as a catalyst for converting all-*trans* retinal to 11-*cis*.

In Fig. 3, a structural model of *Todarodes* retinochrome is proposed, based on the primary protein structure deduced from the cDNA sequence [2] and the retinal-binding site identified by the present study. Just like many visual pigments, squid retinochrome possesses seven transmembrane helices which abounds in aromatic amino acids. The two hydrophilic loops connecting helices IV and V and helices V and VI are larger than the others. Compared with *Todarodes* rhodopsin (448 amino acids and 49,800 Da [3]), retinochrome is fairly small in molecular size (301 amino acids and 33,500 Da [2]), accompanied with very shorter N- and C-terminal domains. Particularly, in the C-terminal cytoplasmic domain, retinochrome, unlike rhodopsin, lacks a long tail of repetitive sequences of Pro-Pro-Gln-Gly-Tyr (PPQGY) and an additional loop created by the presence of double cysteines for palmitoylation near helix VII.

Fig. 4 is presented to facilitate further structural comparison between rhodopsin and retinochrome in *Todarodes pacificus*. In the region from helices I to VII (between residues 35 and 318 in rhodopsin), the two photopigments contained only 23.9% identical amino acids. Higher homology was observed in helices III (42% identity) and VI (42%), while lower homology in helices V (13%) and VII (13%). In both photopigments, most of the intramembraneous prolines are arranged at a homologous position in each of the helices II, IV, V and VII to keep the polyene chain of retinal stable in the membrane. However, retinochrome lacks proline in helix VI, but with one more proline in helix VII (cf. Fig. 2). All the hydrophilic loops show low amino acid identity, with no more than 26% identity in the lumenal loop between helices IV and V.

Although retinochrome, unlike rhodopsin, does not

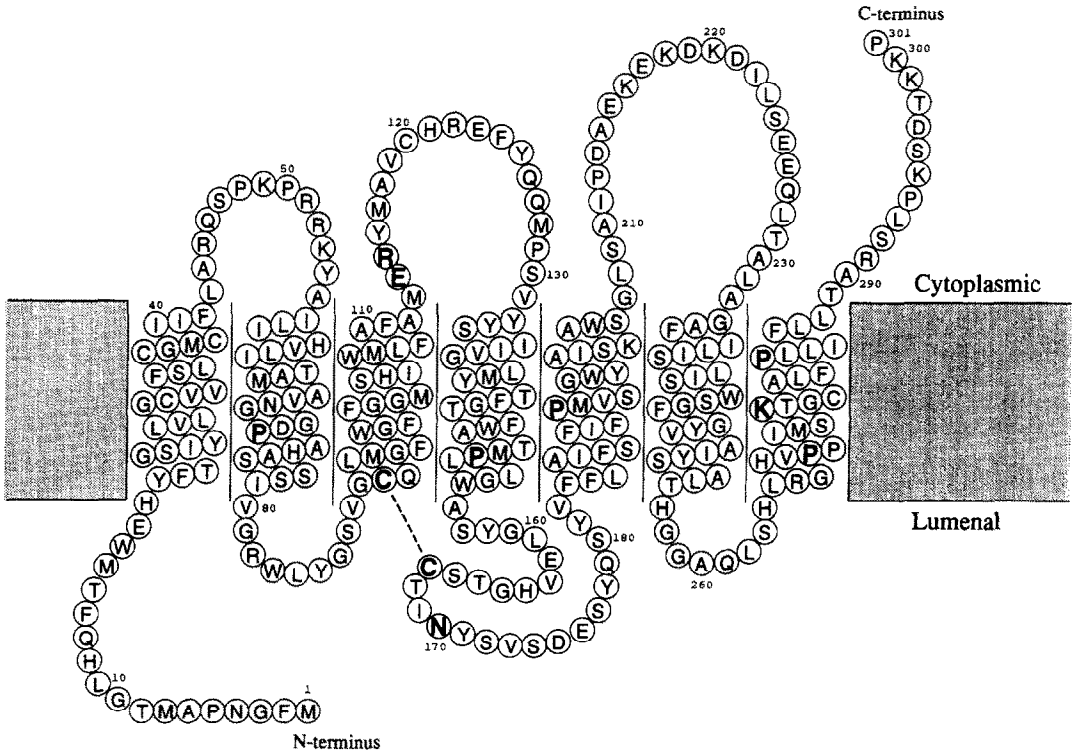

Fig. 3. Structural model of *Todarodes* retinochrome, constructed on the basis of the proposal for bovine rhodopsin [18,21].

Fig. 4. Comparison of amino acid sequences between *Todarodes* retinochrome and rhodopsin. The homology was analyzed by DNASIS (Hitachi). Dotted areas indicate transmembrane domains. ':' represents identical amino acid.

bind to concanavalin A, one possible *N*-linked glycosylation site is observed at residue Asn-170 in the lumenal loop IV–V [21]. The two cysteines, Cys-90 and Cys-167, may form a disulfide bond required for proper protein structure of retinochrome [22]. In cephalopod rhodopsins, the tyrosine in helix III seems to serve as the retinal Schiff's base counterion [3,7], but glutamic acid, aspartic acid or tyrosine as a candidate for counterion was not found within helix III in retinochrome. At the cytoplasmic end of helix III, retinochrome contains the charged pair of Glu-Arg which has been known to be important for G-protein binding in bovine rhodopsin [23] (Asp-Arg in squid rhodopsin). In the C-terminal domain, serine and threonine are scattered for putative phosphorylation sites [24], 8 residues in rhodopsin but only 4 in retinochrome.

As is well known, the retinal–protein interaction in retinochrome is far weaker than in rhodopsin. For instance, rhodopsin is stable when treated with sodium borohydride or hydroxylamine, whereas retinochrome readily react with these reagents to form reduced pigment or retinaloxime, respectively [4]. Furthermore, retinochrome is regenerated far more quickly than rhodopsin on addition of retinal to meta- or apo-protein. These particular properties of retinochrome seem to be derived from the shorter C-terminal tail and the absence of palmitoylated double cysteines in this region. Such an idea would also be applicable to understanding similar differences between rod and cone pigments in vertebrates [17].

In the rhodopsin-retinochrome system, the 11-*cis*-retinal of metaretinochrome is sent to metarhodopsin by RALBP to form rhodopsin in the rhabdomal microvilli, and the all-*trans*-retinal of metarhodopsin is carried to metaretinochrome to regenerate retinochrome in the myeloid bodies. RALBP is capable not only of acting as an intracellular shuttle of retinal but also of reacting with membrane-bound meta-pigment to exchange retinal [1]. In the loop between helices V and VI of retinochrome, the region from Asp-213 to Glu-225 appears to form a possible domain for calcium binding. This suggests the possibility that calcium ion may contribute to the retinal exchange between RALBP and metaretinochrome to regulate retinochrome regeneration. Since we previously reported another similar domain in the C-terminus of squid rhodopsin [3], experiments on the role of calcium in the rhodopsin-retinochrome system are now in progress.

REFERENCES

[1] Hara, T. and Hara, R. (1991) in: Progress in Retinal Research (N.N. Osborne and G.J. Chader eds.) vol. 10, Pergamon, Oxford, pp. 179–206.
[2] Hara-Nishimura, I., Matsumoto, T., Mori, H., Nishimura, M., Hara, R. and Hara, T. (1990) FEBS Lett. 271, 106–110.
[3] Hara-Nishimura, I., Kondo, M., Nishimura, M., Hara, R. and Hara, T. (1993) FEBS Lett. 317, 5–11.
[4] Hara, T. and Hara, R. (1982) in: Methods in Enzymology, Biomembranes, Part H (L. Packer ed.) Visual Pigments and Purple Membranes, vol. 81, Academic Press, New York, 1982, pp. 827–833.
[5] Hall, M.O. and Bok, D. (1982) in: Methods in Enzymology, Biomembranes, Part H (L. Packer ed.) Visual Pigments and Purple Membranes, vol. 81, Academic Press, New York, 1982, pp. 290–293.
[6] Seidou, M., Kubota, I., Hiraki, K. and Kito, Y. (1988) Biochim. Biophys. Acta 957, 318–321.
[7] Hall, M.D., Hoon, M.A., Ryba, N.J.P., Pottinger J.D.D., Keen, J.N., Saibil, H.R. and Findlay, B.C. (1991) Biochem. J. 274, 35–40.
[8] Ovchinnikov, Yu.A., Abdulaev, N.G., Zolotarev, A.S., Artamonov, I.D., Bespalov, I.A., Dergachev, A.E. and Tsuda, M. (1988) FEBS Lett. 232, 69–72.
[9] Zuker, C.S., Cowman, A.F. and Rubin, G.M. (1985) Cell 40, 851–858.
[10] Huber, A., Smith, D.P., Zuker, C.S. and Paulsen, R. (1990) J. Biol. Chem. 265, 17906–17910.
[11] Hariyama, T., Ozaki, K., Tokunaga, F. and Tsukahara, Y. (1993) FEBS Lett. 315, 287–292.
[12] Hisatomi, O., Iwasa, T., Tokunaga, F. and Yasui, A. (1991) Biochem. Biophys. Res. Commun. 174, 1125–1132.
[13] Johnson, R.L., Grant, K.B., Zankel, T.C., Boehm, M.F., Merbs, S.L., Nathans, J. and Nakanishi, K. (1993) Biochemistry 32, 208–214.
[14] Pittler, S.J., Fliesler, S.J. and Baehr, W. (1992) FEBS Lett. 313, 103–108.
[15] Kojima, D., Okano, T., Fukuda, Y., Shichida, Y., Yoshizawa, T. and Ebrey, T.G. (1992) Proc. Natl. Acad. Sci. USA 89, 6841–6845.

[16] Takao, M., Yasui, A. and Tokunaga, F. (1988) Vision Res. 28, 471–480.
[17] Kuwata, O., Imamoto, Y., Okano, T., Kokame, K., Kojima, D., Matsumoto, H., Morodome, A., Fukuda, Y., Shichida, Y., Yasuda, K., Shimura, Y. and Yoshizawa, T. (1990) FEBS Lett. 272, 128–132.
[18] Hargrave, P.A., McDowell, J.H., Curtis, D.R., Wang, J.K., Juszczak, E., Fong, S.-L., Rao, J.K.M. and Argos, P. (1983) Biophys. Struct. Mech. 9, 235–244.
[19] Nathans, J. and Hogness, D.S. (1984) Proc. Natl. Acad. Sci. USA 81, 4851–4855.
[20] Nathans, J., Thomas, D. and Hogness, D.S. (1986) Science 232, 193–202.
[21] Hargrave, P.A. (1982) in: Progress in Retinal Research (Osborne, N.N. and Chader, G.J. ed.) vol. 1, pp. 1–51, Pergamon Press, Oxford.
[22] Karnik, S.S., Sakmar, T.P., Chen, H.-B. and Khorana, H.G. (1988) Proc. Natl. Acad. Sci. USA 85, 8459–8463.
[23] Franke, R.R., Konig, B., Sakmar, T.P., Khorana, H.G. and Hofmann, K.P. (1990) Science 250, 123–125.
[24] Findlay, J.B.C. and Pappin, D.J.C. (1986) Biochem. J. 238, 625–642.