Cloning and nucleotide sequence of cDNA for retinochrome, retinal photoisomerase from the squid retina

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The *Rhodopsin-retinochrome* system is essential for the visual photoreception of molluscs. cDNA coding for retinochrome of the squid (*Todarodes pacificus*) was cloned and the nucleotide sequence has been determined. The sequence (2.1 kb) covers the whole coding region of 903 bp. The deduced primary sequence suggests that retinochrome contains seven transmembrane spanning domains. The homology with bovine rhodopsin and the possible retinal binding site are also discussed.

Myeloid body; Photoisomerase; Retinal protein; Retinochrome; Rhodopsin; Visual cell; *Todarodes pacificus*

1. INTRODUCTION

The visual cell of cephalopods [1–3] and gastropods [4,5] contains a pair of photopigments, rhodopsin and retinochrome. Since the molluscan retina has no structure similar to the pigment epithelium found in the vertebrate retina, the visual cells of molluscs provide themselves with biochemical mechanisms necessary for maintaining their visual photoreception. Especially remarkable is the *rhodopsin-retinochrome* system, in which a retinal-binding protein (RALBP) serves as a shuttle during the recycling of retinal [6–9]. In cells, the visual pigment, rhodopsin, is located only in the rhabdomal microvilli of the outer segments, while retinochrome is mainly stored in the myeloid bodies of the inner segments [10–12]. The most marked difference between those photopigments lies in the stereoisomeric form of their chromophores, which is 11-*cis* in rhodopsin but all-*trans* in retinochrome. When irradiated, they are changed into the all-*trans* and the 11-*cis* forms, respectively [2]. Particularly, retinochrome is capable of acting as an effective catalyst in the light to convert various isomers of retinal into the 11-*cis*, the form that is required by opsin to resynthesize rhodopsin [3,13–15]. In order to elucidate mechanisms for the translocation of retinal within a set of the three retinal proteins (rhodopsin, retinochrome and RALBP), first of all, we have been interested in the molecular structure of retinochrome, which severely differs from rhodopsin not only in its stereo-specific photoisomerization of chromophore retinal but also in intracellular location, photolytic behavior and other chemical properties [16–18]. In the present paper, we describe the cDNA cloning for retinochrome of the Japanese common squid, *Todarodes pacificus*. The predicted amino acid sequence indicates the hydrophobic nature of retinochrome molecule. Analysis of the hydropathicity suggests that retinochrome includes seven membrane spanning regions.

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2. MATERIALS AND METHODS

2.1. *Synthesis of primers for polymerase chain reaction (PCR)*

Amino acid sequence of N-terminal region of retinochrome was previously reported with those of its two CNBr peptides [19]. For cloning of retinochrome cDNA, we constructed 23-mer of sense oligonucleotide for the N-terminal region and 18-mer of antisense oligonucleotide for one of the CNBr peptides (Fig. 1a).

2.2. *Preparation of PCR-amplified DNA*

RNA was prepared from the squid (*Todarodes pacificus*) retina using guanidium isothiocyanate method [20]. The RNA (9.3 μg) was suspended in 14 μl of 5 mM Tris-HCl (pH 8.3), incubated at 65°C for 3 min, placed on ice, and used as a template to prepare the first strand DNA. The reaction mixture was composed of the denatured RNA, 72 ng of antisense oligonucleotide (Primer 2), 20 units reverse transcriptase, 50 mM Tris-HCl (pH 8.3), 8 mM $MgCl_2$, 30 mM KCl, 1 mM dithiothreitol and 2 mM dNTP in a total volume of 20 μl and was incubated at 42°C for 1 h. To the obtained first strand, DNA (20 μl), 500 ng of Primer 1, 350 ng of Primer 2, 1.25 unit *Taq* DNA polymerase (Perkin Elmer Cetus), 8 μl of 1.25 mM each dNTP and 5 μl of 10-fold concentrated reaction buffer (500 mM KCl, 100 mM Tris-HCl (pH 8.3), 15 mM $MgCl_2$, 0.1% (w/v) gelatin) were added to make a final volume of 50 μl. After overlaying the sample with mineral oil, 30 cycles of denaturation, annealing and extension were

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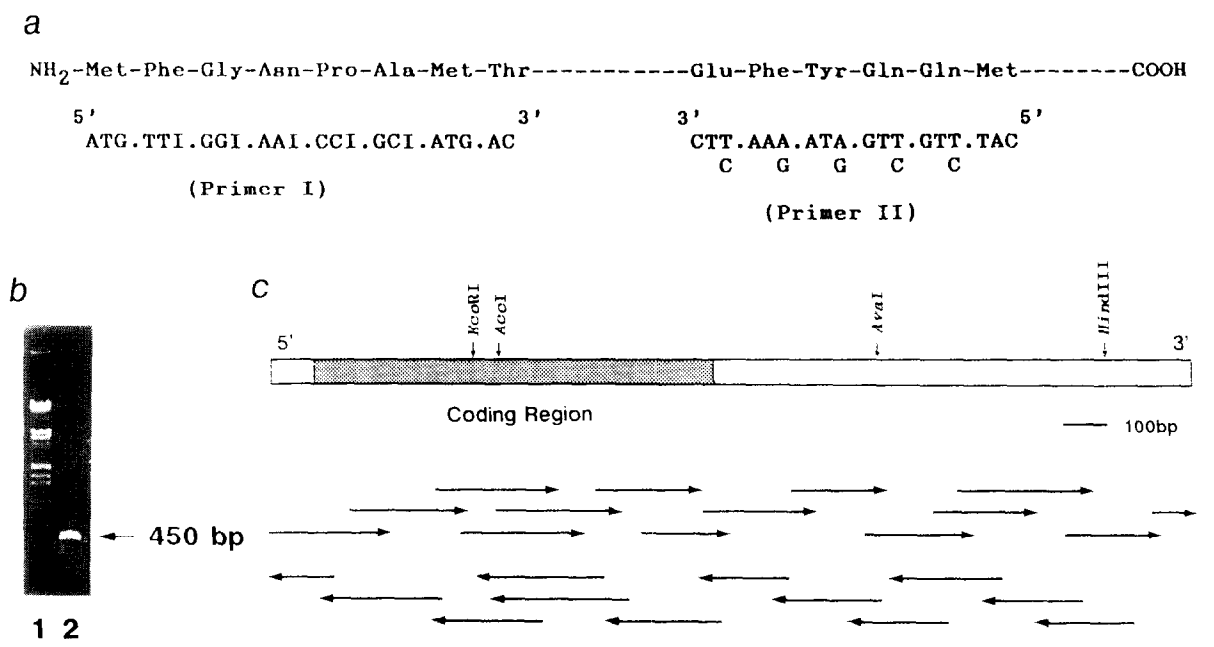

Fig. 1. (a) Synthetic oligonucleotides for amplification of retinochrome cDNA fragment by PCR. Synthetic sense (Primer 1) and antisense (Primer 2) oligonucleotides correspond to amino acid sequences for N-terminus and an internal CNBr peptide of retinochrome, respectively. (b) Amplified cDNA fragment for retinochrome. Lane 1, λ phage DNA digested with *Eco*RI and *Hin*dIII. Lane 2, PCR-extended product resolved by agarose gel electrophoresis and visualized after staining with ethidium bromide. The size of amplified DNA was approximately 450 bp. (c) The sequencing strategy for the squid retinochrome cDNA.

completed. Annealing reaction was performed at 50°C for 2 min, followed by extension at 72°C for 3 min and denaturation at 94°C for 1.5 min.

The PCR-extended product which was precipitated by ethanol and 2 M ammonium acetate twice was labeled by 10 cycles of PCR reaction using the same reaction mixture except for using [α-^{32}P]dATP instead of cold dATP in a total volume of 20 μl, with the result that more than 90% of radioactivity was incorporated into the DNA. The labeled DNA was used as a probe for screening of cDNA library.

2.3. *Construction of cDNA library and its screening*

cDNA library was constructed using vector-primer and poly(A)$^+$ RNA [21]. Poly(A)$^+$ RNA was prepared from the squid retina using the guanidium isothiocyanate method, followed by oligo(dT)-cellulose chromatography. To prepare vector-primer, *Pst*I digested vector pTTQ18 (Amersham) was tailed by TTP and terminal transferase and digested by *Xba*I. The dT-tailed vector-primer was annealed with the poly(A)$^+$ RNA and the first strand cDNA was synthesized by reverse transcriptase according to the method described above. The second strand cDNA replacement was performed by RNase H and DNA polymerase I. After the produced DNA was blunted by T_4 DNA polymerase and ligated, the cDNA was transformed into *E. coli*, epiculian cell SCS-1 (Stratagene). Screening of the cDNA library was carried out by colony hybridization [22] using the labeled PCR-extended cDNA fragment as a probe. Approximately 10^5 independent recombinants were screened.

2.4. *DNA sequence analysis*

The deletion mutants of pRet were constructed by exonuclease III treatment of *Bam*HI- and *Sac*I-digested pRet [23]. The sequencing was carried out by dideoxy chain termination methods of plasmid [24], employing [α-^{32}P]dATP and T_7 DNA polymerase (Pharmacia). For the sequencing of sense strand DNA, the synthetic oligonucleotide primer designed on the known nucleotide sequence from −6 to 11 region of pTTQ18 was used. In order to prepare the deletion mutants for the antisense strand DNA, cDNA insert generated by double digestion of pRet with *Bam*HI and *Sph*I was treated with T_4 DNA polymerase to make blunt-end, and then was recombined into *Sma*I digested pBS-KS. This recombinant, pBS-KS-Ret, was treated with exonuclease III after digestion with *Sac*I and *Kpn*I followed by ligation and transformation to obtain the deletion mutants. The universal primer was used for the sequencing of these mutants.

3. RESULTS AND DISCUSSION

After 30 rounds of amplification by using the combination of the two synthetic oligonucleotides, the formation of a PCR-extended product with 450 bp was detected by agarose gel electrophoresis (Fig. 1b). This amplified DNA was then employed as a probe in order to screen the squid retina cDNA library. From approximately 10^5 independent recombinants, 43 positive clones were collected, and finally a clone with the longest insert of cDNA (2.1 kb) was selected and sequenced (Fig. 1c).

The resultant sequence revealed a single open reading frame of 903 bp encoding 301 amino acids (Fig. 2). The deduced protein contained the amino acid sequences of the above-mentioned N-terminal region and CNBr peptides. The latter peptides each occurred just after the methionine residue on the predicted primary sequence, consistent with cleavage manner by CNBr. It was therefore clear that our present clone was nothing but that for retinochrome. We designated it as pRet.

The protein sequence beginning with the first ATG at nucleotides 93–95 was identical to the N-terminal sequence determined for retinochrome. There was no

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                                                        GC CAAACAAATA ACCAGTGATC 22
CATCGCTCAT CACTCCGGGT GTTATCCTCA AGTTTCATAT TTTGTACTTC GGAATATTCG AAAAGCCATC 92

ATG.TTC.GGA.AAT.CCA.GCA.ATG.ACT.GGT.TTA.CAC.CAG.TTT.ACC.ATG.TGG.GAA.CAT.TAT.TTT 152
Met-Phe-Gly-Asn-Pro-Ala-Met-Thr-Gly-Leu-His-Gln-Phe-Thr-Met-Trp-Glu-His-Tyr-Phe
1
ACC.GGT.AGC.ATT.TAC.TTG.GTG.CTT.GGT.TGC.GTT.GTA.TTT.TCA.CTA.TGT.GGA.ATG.TGT.ATT 212
Thr-Gly-Ser-Ile-Tyr-Leu-Val-Leu-Gly-Cys-Val-Val-Phe-Ser-Leu-Cys-Gly-Met-Cys-Ile
21
ATC.TTC.CTC.GCA.AGA.CAG.AGT.CCC.AAG.CCC.AGG.AGA.AAA.TAT.GCA.ATT.CTT.ATT.CAT.GTT 272
Ile-Phe-Leu-Ala-Arg-Gln-Ser-Pro-Lys-Pro-Arg-Arg-Lys-Tyr-Ala-Ile-Leu-Ile-His-Val
41
CTC.ATC.ACC.GCT.ATG.GCT.GTC.AAT.GGA.GGA.GAT.CCC.GCT.CAC.GCA.TCA.TCT.AGT.ATT.GTT 332
Leu-Ile-Thr-Ala-Met-Ala-Val-Asn-Gly-Gly-Asp-Pro-Ala-His-Ala-Ser-Ser-Ser-Ile-Val
61
GGA.AGA.TGG.CTT.TAC.GGT.AGC.GTT.GGT.TGT.CAA.TTG.ATG.GGT.TTC.TGG.GGA.TTC.TTT.GGT 392
Gly-Arg-Trp-Leu-Tyr-Gly-Ser-Val-Gly-Cys-Gln-Leu-Met-Gly-Phe-Trp-Gly-Phe-Phe-Gly
81
GGA.ATG.TCT.CAT.ATT.TGG.ATG.CTT.TTC.GCA.TTC.GCA.ATG.GAA.CGT.TAT.ATG.GCT.GTA.TGT 452
Gly-Met-Ser-His-Ile-Trp-Met-Leu-Phe-Ala-Phe-Ala-Met-Glu-Arg-Tyr-Met-Ala-Val-Cys
101
CAC.CGA.GAA.TTC.TAT.CAA.CAG.ATG.CCT.TCA.GTT.TAC.TAT.TCA.ATT.ATC.GTT.GGA.TTG.ATG 512
His-Arg-Glu-Phe-Tyr-Gln-Gln-Met-Pro-Ser-Val-Tyr-Tyr-Ser-Ile-Ile-Val-Gly-Leu-Met
121
TAT.ACT.TTT.GGA.ACC.TTT.TGG.GCT.ACT.ATG.CCT.CTA.CTT.GGA.TGG.GCA.TCA.TAT.GGT.TTG 572
Tyr-Thr-Phe-Gly-Thr-Phe-Trp-Ala-Thr-Met-Pro-Leu-Leu-Gly-Trp-Ala-Ser-Tyr-Gly-Leu
141
GAA.GTA.CAT.GGA.ACT.TCT.TGC.ACC.ATC.AAC.TAC.AGT.GTG.TCT.GAC.GAG.AGC.TAC.CAA.TCC 632
Glu-Val-His-Gly-Thr-Ser-Cys-Thr-Ile-Asn-Tyr-Ser-Val-Ser-Asp-Glu-Ser-Tyr-Gln-Ser
161
TAT.GTC.TTT.TTC.TTG.GCT.ATA.TTT.TCA.TTC.ATT.TTC.CCA.ATG.GTC.TCC.GGC.TGG.TAT.GCC 692
Tyr-Val-Phe-Phe-Leu-Ala-Ile-Phe-Ser-Phe-Ile-Phe-Pro-Met-Val-Ser-Gly-Trp-Tyr-Ala
181
ATC.AGC.AAG.GCT.TGG.TCT.GGA.CTT.AGC.GCC.ATC.CCC.GAC.GCT.GAA.AAG.GAA.AAA.GAC.AAG 752
Ile-Ser-Lys-Ala-Trp-Ser-Gly-Leu-Ser-Ala-Ile-Pro-Asp-Ala-Glu-Lys-Glu-Lys-Asp-Lys
201
GAC.ATC.CTG.TCA.GAA.GAA.CAA.TTA.ACT.GCT.CTT.GCT.GGT.GCC.TTC.ATC.CTC.ATC.TCT.CTG 812
Asp-Ile-Leu-Ser-Glu-Glu-Gln-Leu-Thr-Ala-Leu-Ala-Gly-Ala-Phe-Ile-Leu-Ile-Ser-Leu
221
ATC.TCT.TGG.TCA.GGT.TTC.GGC.TAC.GTC.GCA.ATT.TAC.AGC.GCC.CTC.ACC.CAC.GGT.GGT.GCT 872
Ile-Ser-Trp-Ser-Gly-Phe-Gly-Tyr-Val-Ala-Ile-Tyr-Ser-Ala-Leu-Thr-His-Gly-Gly-Ala
241
CAA.CTC.AGC.CAT.CTG.CGT.GGT.CAT.GTG.CCC.CCA.ATC.ATG.AGC.AAA.ACT.GGT.TGT.GCT.CTC 932
Gln-Leu-Ser-His-Leu-Arg-Gly-His-Val-Pro-Pro-Ile-Met-Ser-Lys-Thr-Gly-Cys-Ala-Leu
261
TTC.CCA.CTT.CTC.ATC.TTT.CTC.CTT.ACT.GCC.CGT.AGC.CTT.CCC.AAA.AGT.GAC.ACC.AAG.AAG 992
Phe-Pro-Leu-Leu-Ile-Phe-Leu-Leu-Thr-Ala-Arg-Ser-Leu-Pro-Lys-Ser-Asp-Thr-Lys-Lys
281
CCC.TAAACAGACA ACGGCCTCTT AGTTTTACTA AAAAAGAAGG TGCTGGCTCT GGGAGAAACT GATTTCTCAA 1065
Pro ***
301
GTTCAGAATA AAACGTCCAT TATTTTATTT TCTGGCCAGT AAAAAAAATA TCTGTTACAA CTAAAACAAA 1135
GACTGAAAAA GTGAAAACCA CATTCCATGA ATATTTGATT CTAAATTGCT TTTTGATAGT TTTAGTTGCA 1205
TCCAAAGATT TTCTGGTAAA ACCGAACTTG TTGTCAAAGT TATTTTTGTT AGCTCGTGCG AATTTTCGGC 1275
CGAATCGTTC AGCGGTCTCT TTCGGATGTA CCATGCGTAT GCAGATGGGT GTACTTTTTT CAAGTATGAA 1345
AAGTTTTTCA TCTTTGCGTA CGGATATTAC ACCCGAGGGG GGCCATTGAA TGTATAGGCT GAAAAATCGC 1415
ATGAAAAAAA AAAAAAAATA ACAAGGGAGA CGAGTTCAAT TTTCCGGAAA ACTAATAGAA TGTTAATGAC 1485
CCTTCTCGAA ATTATATATA ATTTCTTATA GTTCAATCGT CATGTAGAAT TGTCATGTTT AATTTTTTTT 1555
TGCTCGAAGT GAGCCTGTTT TATTTTCAAT GCATTCGAAT GTGTGGCAAG CTCAACCGAA ATAAAGCTCA 1625
TCTTAATGTT AATGTTCAGT CCTCAGTGGG TGTTCGCATC TCTCTGCGAT GAACCATATT CTTGCCACTG 1695
TAAAACACCT TATTTTTGCA CAAACCACTT TCTAATGCCT TTCGCACTGA GAAGATGTCA TTTTGAATCA 1765
TCCAAAGATC GAATGAGGGC AGAGAATAAA TTGAAATTCC AACTGCCAGG ACACTCATTC CCCTAGCGAG 1835
TGTCCATGTA ACAACAGGGG ATCTTGCACG AATATGCAAA AGCTTTTAAG GCAACCTTTT GCGTATTTGA 1905
AAATATTATT TTTCGTAAAT GCTTTTAAAT GCAGTATATG AGCTTTGGAG AATAGAATTT ATCTATTAAC 1975
ATTGTAACTT CTCAGATTTT TACTGACATG ACAAGGAGAT TTTGATTAGT AAAATATATT TTGATTCTAA 2045
AAC poly(A)
```

Fig. 2. cDNA nucleotide sequence and deduced amino acid sequence of the squid retinochrome. The nucleotides are numbered on the right of each line, while the predicted amino acid sequence is numbered to the left of each line. Underlined amino acid sequences agree with those of N-terminal region and the CNBr peptides of retinochrome reported previously [19]. Underlined nucleotides at 3′-noncoding region represent the possible signals for polyadenylation.

more ATG upstream from nucleotide 93, but a stop codon TGA at nucleotides 18–20 in frame. This indicated that the ATG for the N-terminus was the initiation codon itself, and any processing of leader sequence did not occur. It was noted that the initiator methionine residue was not cleaved from the protein. Since the

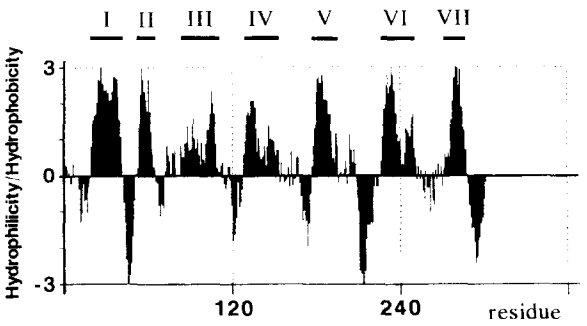

Fig. 3. Hydropathicity profile of the deduced amino acid sequence of squid retinochrome. Hydrophobicity values were calculated according to Kyte and Doolittle [28] for a window size of 8 amino acid residues. Numbers on the abscissa denote those assigned for the deduced amino acid sequence in Fig. 2.

translation product showed no cleavable signal sequence, internal signals would be involved in insertions of the protein into the myeloid membrane, just like start and stop transfer signals that often appear in the other integral membrane proteins such as bacteriorhodopsin [25]. Based on the predicted sequence, the molecular weight of retinochrome was calculated to be 33490, fairly larger than that (24000) estimated by SDS-PAGE [26]. Such a discrepancy has also been reported for other membrane proteins [27].

While three hexanucleotides AATAAA occur at nucleotides 1072–1077, 1615–1620 and 1790–1795 in the 3′-noncoding region, a distance even from the third AATAAA to a poly(A) stretch is as far as 253 nucleotides. The possibility that the analogous sequence AAAATA preceding poly(A) tail before 17 nucleotides is implicated as a signal for polyadenylation cannot be ruled out.

The amino acid composition of the predicted protein coincides with that obtained from purified retinochrome containing a large number of hydrophobic amino acids [17]. The analysis of hydropathicity for the amino acid sequence with Kyte and Doolittle [28] clearly shows that seven repeating clusters of hydrophobic sequences, 20–30 residues long, with a high proportion of aromatic amino acids occurred in the retinochrome (Fig. 3) as reported on rhodopsins derived from various species and several kinds of receptor proteins [29]. The hydropathicity profile and homology with bovine rhodopsin (Fig. 4) strongly suggests that squid retinochrome possesses seven membrane spanning helices as has been ascertained for bacteriorhodopsin [30] and rhodopsin [31].

The amino acid sequence homology between squid retinochrome and bovine rhodopsin [31] is shown in Fig. 4. Retinochrome contains 22.8% identical amino acids in the region of 34 to 301 amino acid residues of bovine opsin. The seven domains for postulated transmembrane helices of bovine opsin are also indicated in Fig. 4. High homologies of amino acid sequences were observed in one transmembrane domain, helix III, and in three hydrophilic regions between the helices II–III, IV–V and VI–VII of bovine opsin. These three homologous regions of bovine opsin are known to be located in the lumenal side of disk membrane in rod outer segment. One possible N-linked glycosylation site is observed at residue Asn 170 in

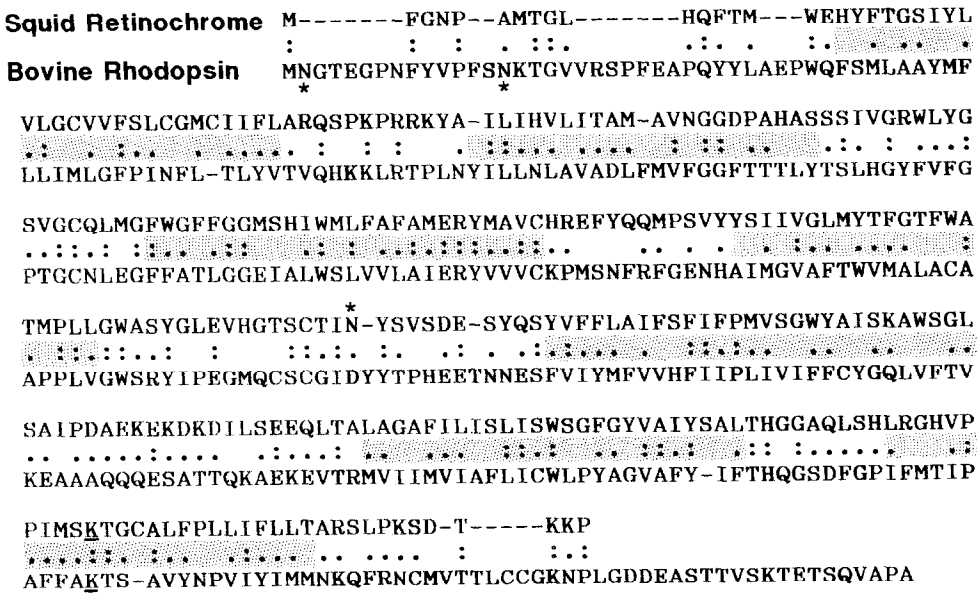

Fig. 4. Comparison of amino acid sequences between squid retinochrome and bovine rhodopsin. The homology was analyzed by DNASIS (Hitachi). (:) represent identical amino acid and (·) homologous amino acid. Putative N-glycosylation site is indicated by asterisk. The underlined Lys-296 of bovine rhodopsin is involved in the formation of Schiff's base with retinal. Lys-275 of retinochrome corresponds to Lys-296 of the opsin. Dotted areas indicate transmembrane domains in bovine rhodopsin [31].

retinochrome, while bovine rhodopsin has two glycosylation sites in its N-terminal region [32]. It should be noted that glycosylation sites of both proteins are located in the lumenal sides of each membrane.

On the contrary, the cytosolic regions of bovine rhodopsin have low homologies with those of retinochrome, although bovine rhodopsin is reported to be interactive with transducin on its cytosolic domains [33]. Phosphorylation has an important role in the regulation of rhodopsin which is to multiply phosphorylated serine and threonine residues in its C-terminal region localized in a cytosolic space [34]. However, retinochrome lacks the sequences corresponding to a C-terminal region of bovine opsin (Fig. 4) and the consensus sequence for protein kinase A and C sites.

Chromophore retinal is known to be bound to Lys 296 in the seventh transmembrane domain in bovine rhodopsin [35]. When the sequence of this opsin domain was aligned with that of the analogous region of retinochrome (Fig. 4), Lys 275 of retinochrome corresponded to Lys 296 of the opsin, suggesting that retinal could be made Schiff's base with Lys 275 in the seventh transmembrane domain of retinochrome. Sequence homology of retinal binding regions between retinochrome and rhodopsin is lower than those among various rhodopsins. The low homology between the two photopigments could explain the different stereoisomeric forms of their chromophores, the different photoisomerization mechanism and the characteristic photoisomerase activity of retinochrome. Further characterization of protein-engineered retinochrome, rhodopsin and their hybrid protein will reveal the molecular mechanism of isomerization and translocation of retinal in the *rhodopsin-retinochrome* system.

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