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Insight into Rhodopsin Diversity from Viewpoint of Counterion

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In vertebrate rhodopsins the glutamic acid at position 113 serves as a counterion to stabilize the protonated retinylidene Schiff base linkage and to shift the spectrum to the visible region. Invertebrate rhodopsins and retinochrome have the amino acid residue different from glutamic acid or asparatic acid at this position and therefore, these pigments may have a counterion at different position. We first investigated the counterion in retinochrome by site specific mutagenesis. The results showed that the counterion is the glutamic acid at position 181, where almost of all the pigments including vertebrate and invertebrate rhodopsins in the rhodopsin family have a glutamic acid or an aspartic acid. In vertebrate rhodopsins, however, Glu 181 does not act as a counterion, and the red-sensitive cone pigments have a histidine at this position, which serves as a chloride-binding site for red-shift of the absorption spectrum. These findings suggested that the role of Glu181 as a counterion may be weakened by the newly acquired counterion at position 113. Taken together with our recent studies on an invertebrate-type rhodopsin, the rhodopsin diversity was discussed from viewpoint of counterion.

Key words: vision, rhodopsin family, retinal chromophore, retinylidene Schiff base, counterion

INTRODUCTION

Most of animals capture visible light by rhodopsin or its related molecules. They are seven-transmembrane alpha-helical proteins, each of which contains a retinal as a chromophore. More than 200 kinds of rhodopsin-related proteins have been identified thus far and defined as rhodopsin family. The members of rhodopsin family divided into at least five subgroups based on amino acid sequence similarity; the vertebrate transducin-coupled, the invertebrate Gq-coupled and the scallop Go-coupled rhodopsins as well as cephalopod retinochrome/ mammalian RGR (photo-isomerase) and mammalian peropsin [1, 2].

It has been well known that the retinal chromophore free in solution exhibits absorption maximum in ultra-violet region and protonation of retinylidene Schiff base is a major mechanism to shift its maximum to the visible region (Fig. 1). Like the vertebrate rhodopsins, the retinal chromophore in other members for visible light absorption also binds to the lysine residue at position 296[1] in the helix VII (Fig. 2) possibly through a protonated Schiff base linkage. The positive charge on the protonated Schiff base is energetically unstable in the protein interior and therefore, a negatively charged amino acid residue called *counterion* should be present in the protein. In vertebrate visual pigments such as bovine rhodopsin, the glutamic acid at position 113 in the helix III serves as a counterion to stabilize the protonation of the retinylidene Schiff base [3-5].

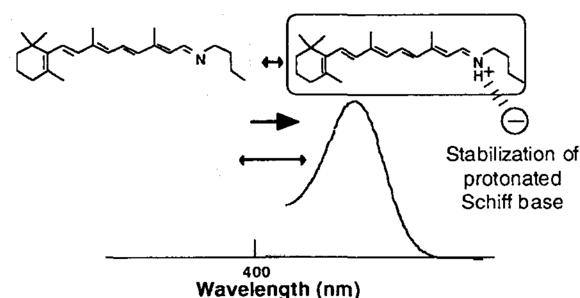

Fig.1: Schematic representation showing importance of counterion for the stabilization of a protonated Schiff base linkage and visible light absorption.

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[1] Bovine rhodopsin numbering system.

However, the other pigments including invertebrate rhodopsins and retinochrome have the amino acid residue different from glutamic acid or asparatic acid at this position [6,7] and therefore, these pigments should have a counterion at different position. However there have not been any lines of the direct evidence to elucidate the counterion in the other pigments because expression of mutant proteins other than vertebrate rhodopsins has not been successful.

We have recently established the expression system of retinochrome, a member of rhodopsin family [2]. Retinochrome has an amino acid sequence ~20 % identical to those of vertebrate and invertebrate rhodopsins [8] and exhibits an absorption maximum (495 nm) similar to those of rhodopsins. Retinochrome contains an all-*trans*-retinal as a chromophore and produces an 11-*cis*-retinal upon absorption of a visible light [9] and serves as a retinal-isomerase. The physiological roles of retinochrome and rhodopsin are different but they function with absorption of a visible light that isomerizes the retinal chromophore, the common mechanism in rhodopsin family. Thus we first tried to identify the residue that acts as a counterion in retinochrome by site-directed mutagenesis [2]. Moreover we also analyze one of the amphioxus rhodopsins (Koyanagi et al., manuscript in submitted) as a model for invertebrate-type rhodopsins.

MATERIALS AND METHODS

Preparation of Retinochrome and Rhodopsin Mutants. Retinochrome cDNA [8] was generous gift from Profs. Ikuko Hara-Nishimura (Kyoto University) and Mikio Nishimura (National Institute for Basic biology). Lancelet rhodopsin cDNA (LOP1A) was cloned by Koyanagi et al (Kyoto University, manuscript in submitted). The coding region of cDNAs was isolated by PCR with being tagged by the monoclonal antibody rho 1D4 epitope-sequence (ETSQVAPA) [10]. The tagged cDNA was inserted into a plasmid vector SRα [11]. The method for the construction of the squid retinochrome was described earlier [2]

Expression and Purification of Retinochrome and Rhodopsin. cDNA was transfected into HEK 293S cells by calcium-phosphate methods according to the previous report [2, 12]. Absorption spectra were recorded at 0°C with a Shimadzu Model MPS-2000 spectrophotometer interfaced with an NEC PC-9801 computer [13].

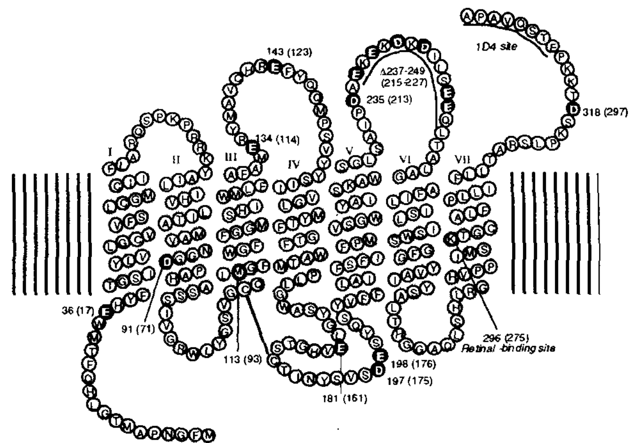

Fig. 2: Secondary structural model of retinochrome showing the location of Glu and Asp residues (white letter). For affinity purification using an anti-rhodopsin antibody, the amino acid sequence of monoclonal antibody Rho1D4 epitope (ETSQVAPA) is introduced to the C-terminus of retinochrome. On the basis of sequence alignment, the bovine rhodopsin amino acid residue numbering system is used. The retinochrome numbering system is shown in parentheses

RESULTS AND DISCUSION

Because retinochrome contains 9 Glu and 6 Asp in its sequence (Figure 2), we speculated that one of these residues would act as a counterion in retinochrome. Thus we have expressed single amino acid mutants of retinochrome where each Glu or Asp in the molecule except in the V-VI cytoplasmic loop region was replaced with Gln or Asn. To examine the possible contribution of the residues in the V-VI loop as a candidate of counterion, we prepared a deletion mutant (Δ 237-249) that lacks the region.

Figure 3 shows absorption spectra of the expressed wild-type and mutants of retinochrome in the absence of chloride because chloride can serve as a surrogate counterion in counterion mutants of bovine rhodopsin [3-5]. The deletion mutant did not form an active protein and therefore the spectra of this mutant are not shown in the figure. All the active mutants except D91N and E181Q exhibited almost the same absorption maxima (493 nm) as wild-type. D91N mutant showed

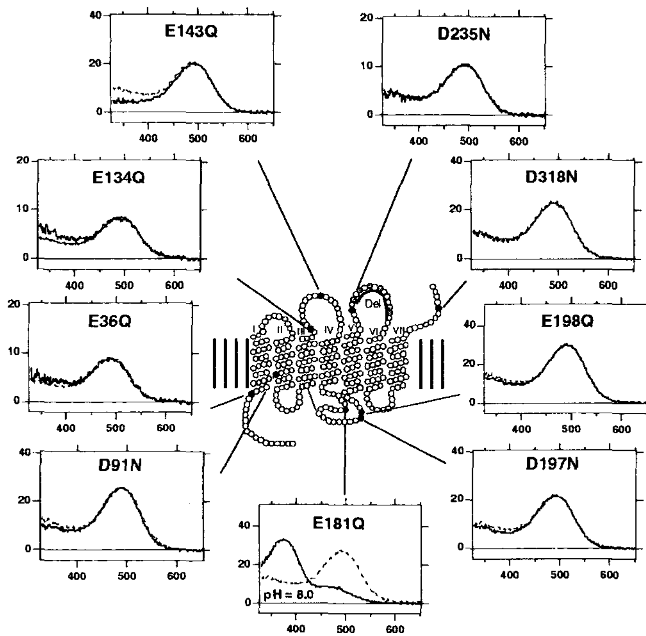

Fig. 3: Absorption spectra of mutant retinochromes. Solid and dotted lines show the spectra of mutant and wild-type rhodopsins, respectively. The numbering is shown as bovine rhodopsin system.

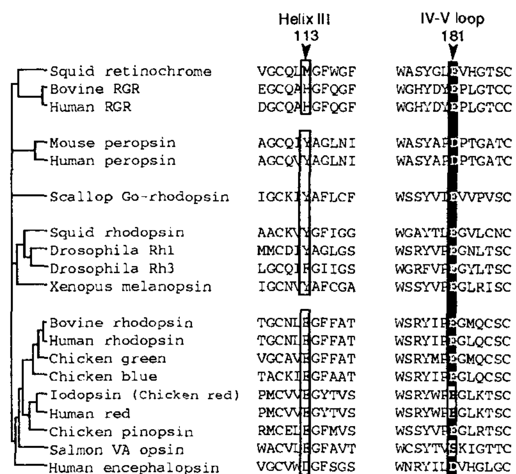

Fig. 4: Comparison of amino acids at position 113 and 181. Phylogenetic relationship among the members of rhodopsin family is schematically represented based on the previous reports [2]. All of the members of vertebrate rhodopsin group possess glutamic or aspartic acid at position 113 in helix III, while retinochrome or invertebrate rhodopsin groups do not. On the other hand, all of the members have glutamic or aspartic acid at position 181 in the extracellular IV-V loop except for red-sensitive cone visual pigments and VA-opsins. Note that Tyr 113 is neutral in the case of invertebrate octopus rhodopsin [6].

absorption maximum (490 nm) slightly different from that of the wild-type. Our results clearly showed that the replacement of this residue did not match the criteria of counterion, although this residue may be located near the chromophore. The criteria of counterion are clearly applicable to the E181Q mutant; that is, the mutant showed absorption maximum at about 380 nm in the absence of chloride and showed it in visible region (480 nm) in the presence of chloride (Figure 3). These results strongly suggested that the glutamic acid at position 181 acts as a counterion in retinochrome.

To eliminate the possibility that E181Q mutant is denatured in the absence of chloride, we added chloride in the chloride-free sample and observed an increase in absorbance at visible region (data not shown). Furthermore, the pKa of the Schiff base was estimated to be 7.0 by changing the pH of the sample in the absence of chloride (data not shown). The value is very similar to that observed in the counterion-depleted mutant of bovine rhodopsin (pKa = ~6.0) [3].

The above results indicated that the glutamic acid at position 181 in the extracellular loop connecting the transmembrane helices IV and V acts as the counterion in retinochrome, whereas in vertebrate rhodopsins, the counterion is the glutamic acid at position 113 in the transmembrane helix III. Interestingly, vertebrate rhodopsins also have the glutamic acid at position 181 (Figure 4). Furthermore, almost all the pigments in the rhodopsin family whose amino acid sequences have been reported so far have the glutamic acid or the aspartic acid at this position. In bovine rhodopsin crystal structure [14, 15], one of the oxygen atoms of the glutamic acid at position 181 is situated close (about 4.5 Å) to the C12 of the retinal chromophore. E181Q mutation caused about 10 nm red-shift of absorption maximum but addition of chloride recovered the maximum to that of the wild-type (data not shown). These results suggested that Glu181 in bovine rhodopsin is not the counterion but is located near the retinal chromophore.

From the result of retinochrome we can not conclude whether or not the glutamic or the aspartic acid at position 181 acts as the counterion in invertebrate rhodopsins because retinochrome is not

the invertebrate "rhodopsin".

Recently we have been successful to express one of the amphioxus opsins which is close to scallop Go-coupled rhodopsin in the molecular phylogenetic tree (Koyanagi et al., manuscript in submitted). Its amino acid residues at position 113 and 181 are Tyr and Glu, respectively. This combination is identical to not only the scallop Go-coupled rhodopsin but also the invertebrate Gq-coupled rhodopsins. The expressed amphioxus "rhodopsin" binds 11-cis-retinal and exhibits its absorption maximum at about 480 nm. The mutation of Tyr 113 to Phe in this rhodopsin scarcely affected its absorption characteristic. The mutation Glu181 to Gln, however, increases of the 380 nm-peak and decrease of the visible peak. This finding strongly suggested that the counterion of the amphioxus rhodopsin is glutamic acid at position 181.

Almost all the pigments in the rhodopsin family whose amino acid sequences have been reported so far have the glutamic acid or the aspartic acid at position 181 (Figure 4). It is of interest to note that some change in amino acid residue is seen at position 181 in only the vertebrate visual pigments. Moreover Glu 181 is not a counterion in vertebrate rhodopsin group. It should be noted that the histidine at position 181 of red-sensitive cone visual pigments is responsible for the chloride-binding site [16] and binding of chloride causes about 40 nm-red shift of the pigments [17]. These facts suggest that the role of Glu 181 as a counterion may be weakened by the newly acquired counterion Glu 113. In other words, acquirement of Glu 113 may be one of the key mutations for emergence of a red-sensitive cone visual pigment.

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