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Anti-Bovine Rhodopsin Monoclonal Antibody Recognizing Light-Dependent Structural Change

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ABSTRACT—The antigenic structure of the bovine rhodopsin molecule was investigated by using a bovine rhodopsin-specific monoclonal antibody designated Rh29. Competition assay with sealed intact disks and broken disks indicated that the antibody-binding region was localized in the intradiscal surface. An antigenic peptide obtained by a cyanogene bromide cleavage of rhodopsin was purified and determined as residues 2–39 in the amino acid sequence.

Further analysis suggested that the antigenic determinant included at least residues 21–25. These results were consistent with the structural model for membrane topology of rhodopsin. The antigenicity of the rhodopsin was compared among several states. The antibody bound to both ammonyx LO-solubilized unbleached and bleached rhodopsin. In contrast, upon membrane-embedded rhodopsin, unbleached one was 100-times less antigenic than bleached one. The results suggested that the segment around the determinant of membrane-embedded rhodopsin should undergo a structural change upon absorption of light.

Rh29 detected a band corresponding to bovine, porcine and octopus opsins in immunoblotting. Protein blot of crayfish rhabdome did not show any reactive band. These bands except for crayfish reacted with concanavalin A as well. The N-terminal structure may, therefore, conserved between mammal and erthropoda and diverge between them and cepharopoda.

Key words: rhodopsin, monoclonal antibody, epitope, structural change

INTRODUCTION

Rhodopsin is a photoreceptor protein which initiates the visual transduction in animal eyes. Bovine rhodopsin is composed of 11-cis retinal and apoprotein, opsin, and located in the disk membrane of rod outer segment (ROS).

The primary structures of rhodopsins of vertebrates as well as invertebrates have been determined by the sequence analyses of gene nucleotides (Nathans and Hogness, 1983; Nathans and Hogness, 1984; O'Tousa *et al*.,1985; Takao *et al.,* 1988; Zuker *et al*., 1985.) and protein amino acids (Ovchinnikov *et al*., 1982; Hargrave *et al*., 1983; Pappin and Findlay, 1984), indicating that they are highly homologous. The folding model of rhodopsin moleules in the membrane was estimated (Hargrave *et al*., 1983; Ovchinnikov, 1982; Pappin *et al*., 1984). Recently three dimensional structure of bovine rhodopsin has been analysed by x-ray crystalography and the results suggested that opsin is folded in a membrane with seven helical-spanning segments (Palczewski *et al*. 2000). Experimental information for the rhodopsin structure are also accumulated. N-terminal segment is oriented toward intradiscal space where oligosaccharides attach at Asn2 and Asn15, (Ovchinnikov *et al*., 1982) having affinity for concanavalin A (ConA). C-terminal segment is toward cytoplasmic space where light-dependent phosphorylation occurs (Kuhn and Dreyer, 1972). Exposed segments of the rhodopsin molecule on the membrane are estimated by *in situ* proteolysis (Ovchinnikov, 1982; Hargrave and Fong, 1977; Mullen and Akhtar, 1983).

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In addition to these observations, structural changes of opsin moiety are inferred from the spectroscopic observations (Shichi *et al.*, 1969). However, there are a few evidences for which region of opsin molecule causes conformational changes upon light absorption.

The monoclonal antibodies (mAb) have been applied as a structural probe for rhodopsin (Molday and MacKenzi, 1983) and bacteriorhodopsin (Kimura *et al.*, 1982; Ovchinnikov *et al.*, 1985), proving useful tools for the topological investigations of trans-membrane proteins. On the other hand, mAb has possibility to recognize a specific state of a protein molecule. A selective binding of mAb to photobleached rhodopsin has been found, which is closely related to phosphorylation at C-terminal residues (Molday *et al.*, 1985; Balkema and Draeger, 1985). And the epitope of another mAB, which bound exlusively to the light-activated rhodopsin was mapped to the amino acid sequence 304–311 (Abdulaev and Ridge, 1998).

We previously reported that three clones raised against the purified bovine rhodopsin produced rhodopsin-specific antibodies termed Rh29, Rh112, Rh311 (Tokunaga, *et al.*, 1989). Rh311 had an affinity for ammonyx LO-solubilized rhodopsin not for opsin. Rh112 could bind to both solubilized rhodopsin but not to intact ROS. Rh29 recognized denatured, solubilized or ROS-integrated opsin.

We extended the study of Rh29 and found that the antibody binds to N-terminal segment and that antigenicity of unbleached disks is much lower than that of bleached disks. The antibody recognizes not only mammalian rhodopsin but octopus rhodopsin, indicating that the antigenic structure is conserved in wide animal species.

MATERIALS AND METHODS

Preparation of photoreceptor membrane

All preparations were made under dim-red light. Bovine ROS was prepared as described by Smith *et al.*, (1975). Disk membrane was isolated by Ficoll floatation methods (Smith *et al.*, 1975). In order to obtain sealed intact disks, 3 ml of disks suspension containing 15 mg of rhodopsin suspended in TBS (50 mM Tris-HCl, pH 7.4, 200 mM NaCl, 2 mM $MgCl_2$) with 1 mM $CaCl_2$ and $MnCl_2$ was mixed with an equal volume of ConA-sepharose (Pharmacia) so that broken and inside-out disks bind to the resin. The mixture was gently agitated overnight at 4°C and centrifuged to separate unbound disks. The supernatant (termed sealed intact disk) was stored at 0°C until use. A portion of sealed intact disks was frozen-thawed for 5 times between liquid nitrogen and room temperatures to obtain unsealed disks (Balkema and Draeger, 1985). Porcine ROS was prepared by the same procedure as bovine. Photoreceptor membranes from octopus and crayfish retina were prepared according to Shichida *et al.* (1978) and Hamacher and Stieve (1984), respectively. Rhodopsin content in each membrane preparation was estimated from a difference absorbance before and after bleaching in the presence of hydroxylamine. Molecular weights and the extinction coefficients of the above animal rhodopsins are assumed to have similar values to bovine (MW=41399, $\varepsilon_{500\ nm}$= 42,000) (Shichi, 1983).

Monoclonal Antibody Binding Assay

Rhodopsin-specific mAb, Rh29, was purified from ascites fluid by protein A column chromatography (Bio-Rad). Enzyme-linked immunosorbent assay (ELISA) was employed for the detection of the antigenic fragment and for the competitive binding study. Bleached ROS was used as an immobilized antigen for the competitive binding assay after frozen-thawing five times to expose the antigenic determinant for Rh29. Microtiter plate wells (Nunc) were coated by means of incubating 50 µl of ROS (3 µg rhodopsin/ml) for 2 hr at 37°C and subsequently 100 µl of blocking solution (TBS containing 3% BSA (bovine serum albmin), 1% normal goat serum) for 30 min at 37°C. A competitor serially diluted with TBS was mixed with Rh29 (final concentration is 65 ng/ml which gave 50–60% of full binding) and preincubated for 1 hr at 37°C. Each mixture of competitor and antibody (50 µl) was placed in wells and incubated for 1 hr at 37°C. After extensive wash with Tw-TBS (TBS containing 0.05% of Tween20) wells were treated with HRPO-conjugated second antibodies (DAKO) for 1 hr at 37°C, rinsed with extensive Tw-TBS and reacted with o-phenylenediamine for 30 min. The reation was stopped by adding 100 µl of 2 N H_2SO_4 and the absorbance at 492 nm was measured.

When disks solubilized with ammonyx-LO or digitonin (2%) were used as competitors, detergent concentration was decreased to 0.05% in a competitor-antibody mixture to avoid the decrease in binding ability of the antibody. When column fractions were tested for their antigenicity, each 20 µl of elution was dried in microtiter plate well (Sumitomo Bakelite) at 50°C and treated with Rh29 (1–2 µg/ml) and second antibodies, as mentioned above.

ConA-Binding Assay

ConA has affinity for mannose or glucose and also for sugar moiety of rhodopsin or HRPO (horse radish peroxidase). Advantage that ConA forms tetramer at neutral pH was taken for the detection of the oligosaccharides of rhodopsin or its glycopeptide. The assay (termed as ConA-binding assay, hereafter) includes essentially the same steps in ELISA described above. Blocking solution used in ConA-binding assay was free from ConA-reactive fractions by passing through ConA-sepharose column. Instead of mAb and second antibodies in ELISA, the wells were treated with ConA and HRPO dissolved in TBS containing 1 mM $CaCl_2$ and $MnCl_2$.

Immunoblotting and ConA-blotting

SDS-PAGE was carried out by the discontinuous slab gels (Laemmli, 1970). Routinely, 2–10 µl of disks or photoreceptor membranes (1 µg rhodopsin/µl) was loaded on 15% gel for proteolysis study or 10% gel for experiments of cross-reaction. Proteins were stained by Coomassie blue (CB), or by amido black after transferred to nitrocellulose filter. Protein blots were also sequentially treated with mAb and second antibodies (immunoblotting) or with ConA and HRPO (ConA-blotting) like mAb- or ConA-binding assay, respectively. Both blots were developed by diaminobenzidine.

Proteolytic Digestion of Intact Disks or Solubilized Disks

Chymotrypsin and TPCK-trypsin were purchased from Sigma. Papain was purchased from Wako. Unbleached intact disks freshly prepared (1 mg rhodopsin/ml) were digested with 5% (w/w) chymotrypsin (37°C, 24 hr) or 5% (w/w) papain (37°C, 6 hr) in a buffer of 50 mM Tris-acetate (pH 8.0), 2 mM $CaCl_2$ or 50 mM sodium phosphate (pH 7.0), 5 mM cysteine, 2 mM EDTA, respectively. Digestion was stopped by washing the disks three times.

To cleave N-terminal segment of opsin, bleached disks were solubilized with 2% (w/v) digitonin (Wako). After detergent concentration was decreased to 0.5%, disks in TBS containing 1 mM $CaCl_2$ were treated with each 1% (w/w) of TPCK-trypsin or chymotrypsin for 6 hr at 37°C. Reaction was stopped by the addition of DFP. The obtained peptide mixture was subjected to the competitive binding assay. Protection and deprotection of arginine residues with 1,2-cyclohexanedione were according to the method of Patthy & Smith (1975). Protected, deprotected opsin and control sample, which

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was treated along the same procedure without the addition of 1,2-cyclohexanedione, were dialyzed against 0.1 M sodium borate (pH 8.0) and digested with TPCK-trypsin (1%, w/w).

Isolation of mAb-Specific Peptide

Antigenic peptides were separated from protease-digested and CNBr-cleaved rhodopsin according to Brett and Findlay (1983). ROS (20 mg of rhodopsin) was digested with 5% (w/w) thermolysin (Sigma) at 37°C for 24 hr. Washed material was delipidated, carboxymethylated and chromatographed on Sephadex LH-60. F1 (large fragment of thermolysin-cleaved rhodopsin) was dried and then cleaved with CNBr (300 folds excess of methionine) in 70% trifluoroacetic acid for 24 hr at room temperature. Reaction mixture was separated by Sephadex LH-60 and then G-50 column chromatography. Amino acid composition of each peptide was analyzed by Hitachi 835S Amino Acid Analyzer.

RESULTS

We prepared rhodopsin-specific mAb's raised against purified rhodopsin (Tokunaga *et al.*, 1989). One of them termed Rh29 (IgG) was further investigated in this study.

Rh29 detected light-induced structural change of rhodopsin.

The striking difference in the antigenicity for Rh29 was observed between bleached and unbleached disks (Fig. 1). About 100-folds amount of unbleached disks were necessary for the same degree of binding inhibition with bleached disks (A in Fig. 1). Frozen-thawed disks also showed such a large difference in antigenicity (not shown).

Solubilization with strong detergent increased antigenicity of rhodopsin for Rh29.

Under digitonin solubilized conditions bleached rhodopsin was 20-fold more antigenic than unbleached one (B in Fig. 1). Taking it into account that digitonin is relatively mild detergent which could retain some authentic activities of the membrane-associated rhodopsin such as spectroscopic character and ability of regeneration from opsin and retinal (Hubbard, 1952), the results on both intact and digitonin-solubilized disks indicate that the antigenic region should be exposed to surrounding medium during photo-bleaching. In contrast, opsin and rhodopsin solubilized by ammonyx-LO showed the same degree of inhibition for the binding of Rh29 (C in Fig. 1). This would be accounted for that ammonyx-LO effectively removed lipids from membrane-associated rhodopsin and that the antigenic determinant would be naked to be easily bound by Rh29.

Which side of disks does the antibody bind to?

It was tested which side of disks the antibody bound to. Fig. 2 shows the binding ability of Rh29 for bleached disks with or without freeze-thawing. Frozen-thawed disks were found to compete with the immobilized antigen at 33-times lower concentration (at half maximal inhibition) than sealed intact disks. Since freeze-thawing makes disks unsealed (Clark and Molday, 1979), the result suggests that an antigenic determinant of Rh29 is present in the intradiscal space. The carefully prepared sealed intact disks, although, inhibited the binding of Rh29 to immobilized ROS. It was shown in the Fig. 2 that the much higher amount of opsin as compared to the antibody (65 ng/ml) was required for the inhibition. Thus, the small inhibition of binding with our "sealed intact disks" was probably due to a contaminant of broken disks in the material. Assuming that a freeze-thawing makes completely disrupted disks which can be bound by Rh29, our "sealed intact disks" would contain broken ones up to 3%. Instead of mAb, the competitive ConA-binding

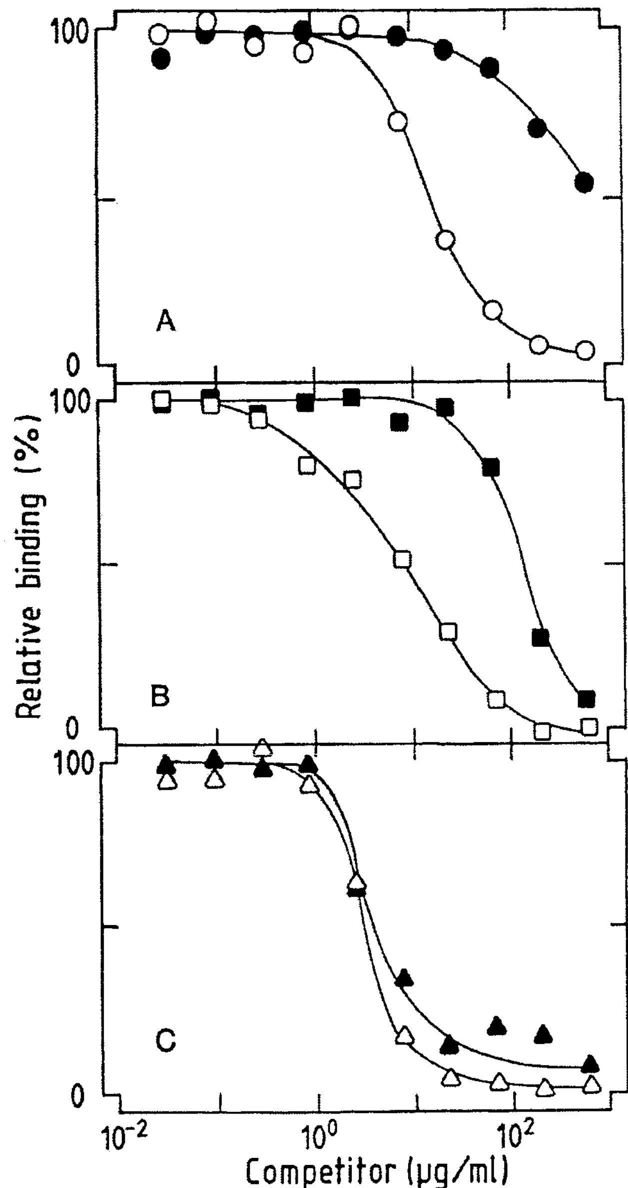

Fig. 1. Effect of photo-bleaching on the binding of Rh29 to membrane-bound rhodopsin and solubilized rhodopsin. Competitive binding assay was made to examine the binding character of intact disks (A), disks solubilized by digitonin (B) and by ammonyx-LO (C). Open and closed symbols represent bleached and unbleached state of rhodopsin, respectively. Each point is a mean value of 3–4 wells.

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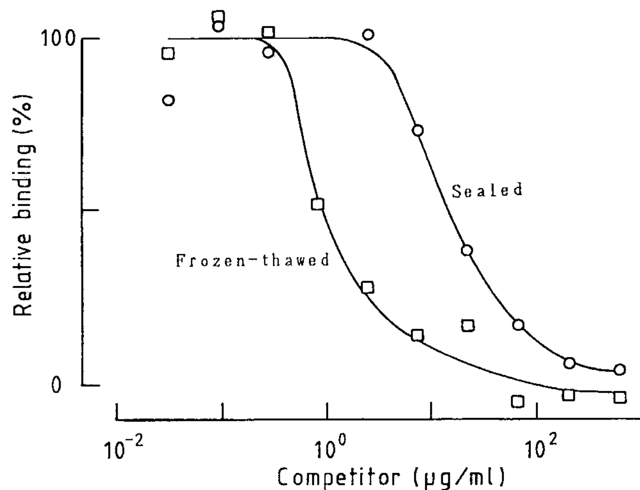

Fig. 2. Competitive inhibition by sealed intact disks (circles) and frozen-thawed disks (squares) in the binding of Rh29 to immobilized antigen. The microtiter plate wells were coated with bleached ROS. Rh29 was preincubated with competitors; bleached sealed intact disks or bleached frozen thawed disks. The wells were sequentially treated with the preincubated mixture and second antibodies. The percentage of 0 or 100 was defined as values obtained along the same treatment without Rh29 or competitor, respectively. Each point represents a mean value of 4 wells.

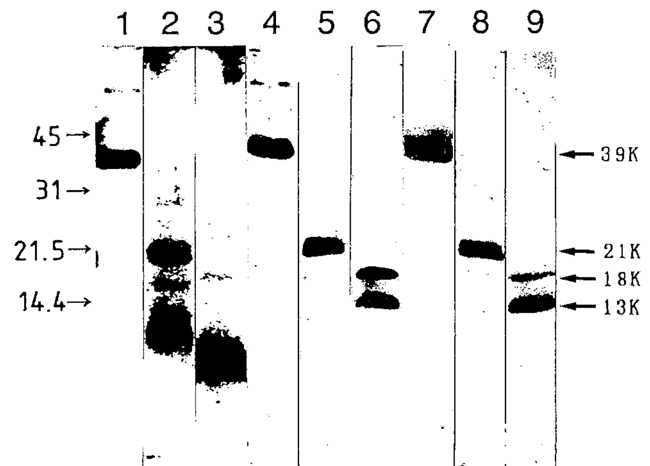

Fig. 3. Immunoblotting and ConA-blotting of proteolytic intact disks. Approximately 10 μg of rhodopsin undigested (lane 1, 4 and 7), digested with chymotrypsin (lane 2, 5 and 8) and those with papain (lane 3, 6 and 9) were run in a 15% polyacrylamide gel and were stained with Coomassie blue (CB), with Rh29 (1.3 μg/ml) and the second antibodies (2 μg/ml) (Rh29) or with ConA (10 μg/ml) and HRPO (20 μg/ml)(ConA).

assay also resulted in the increase in accessibility of ConA to frozen-thawed disks (data not shown) in agreement with the previous report (Molday and MacKenzie, 1983). On the basis of the rhodopsin structural model (Hargrave *et al.*, 1983; Ovchinnikov, 1982), three loops (connecting segments) and N-terminal segment are exposed toward intradiscal space.

The epitope of Rh29 is N-terminal region of rhodopsin molecule.

To survey the antigenic determinants, intact disks treated with chymotrypsin or papain were analyzed by immunoblotting (Fig. 3). A pattern of intact disks stained with CB indicated opsin being major protein which banded at 39kDa. This band was recognized by Rh29 (lane 4 in Fig. 3).

When intact disks were digested with chymotrypsin, the resultant fragments migrated in the region of 12–30 kDa. In these fragments, a band of 21 kDa reacted with Rh29. Papain cleaved rhodopsin in intact disks into 9.5 k, 11 k, 13 k and 18 kDa fragments. The band of 13 kDa was faint in the CB stain. However, this and a band at 18 kDa were recognized by Rh29 (lane 6 in Fig. 3). Upon the ConA-blotting, the 21 kDa of chymotryptic and the 13 k and 18 kDa of papainic fragments were detected (lanes 7, 8 and 9 in Fig. 3). Thus, these antigenic peptides should contain rhodopsin N-terminal segment attached by oligosaccharides.

In the next stage, we obtained a Rh29-reactive CNBr-peptide of rhodopsin. Unbleached ROS was digested with thermolysin which produced major two peptide fragments, F1 (1–240) and F2 (241–327) (Pober and Stryer, 1975).

After delipidation and carboxymethylation, F1 and F2 were separated on Sephadex LH-60 column. Rh29 and ConA reacted with a fraction of F1 fragment. The pool containing F1 fragment was cleaved by CNBr and chromatographed on Sephadex LH-60 column.

After the chromatography, Rh29 and ConA also reacted with the same fractions. These fractions were finally rechromatographed on Sephadex G-50 (Fig. 4). Rh29-binding assay showed that the first peak appeared in the void volume and the second peak included the antigenic peptide (the middle trace in Fig. 4). ConA also reacted with the same fractions (the bottom trace in Fig. 4). The second peak was revealed to contain a single peptide corresponding to residues 2-39 by amino acid analysis (Table 1).

The first peak did not contain any peptide expected from the digestion of rhodopsin with CNBr and seemed to be an aggregate of partially cleaved materials.

Based on the structural model, N-terminal residues up to 36 are thought to be localized in the intradiscal space (Hargrave *et al.*, 1983). This agreed with our results that Rh29 recognized amino acids residues 2–39 and that unsealed disks became more antigenic than sealed intact disks.

In order to elicit the essential amino acid residues for the binding of Rh29, we examined the effect of proteolytic digestion of the solubilized disks on the binding to the antibody. Digitonin-solubilized disks were treated with trypsin or chymotrypsin which cleaves N-terminal region of opsin at several sites (Ovchinnikov, 1982; Pober and Stryer, 1975). The resultant fragment mixture was subjected to competitive binding assay. Each tryptic or chymotryptic fragment was found not to be an effective competitors for binding to Rh29 (Fig. 5A). In both cases digested disks required for 50% inhi-

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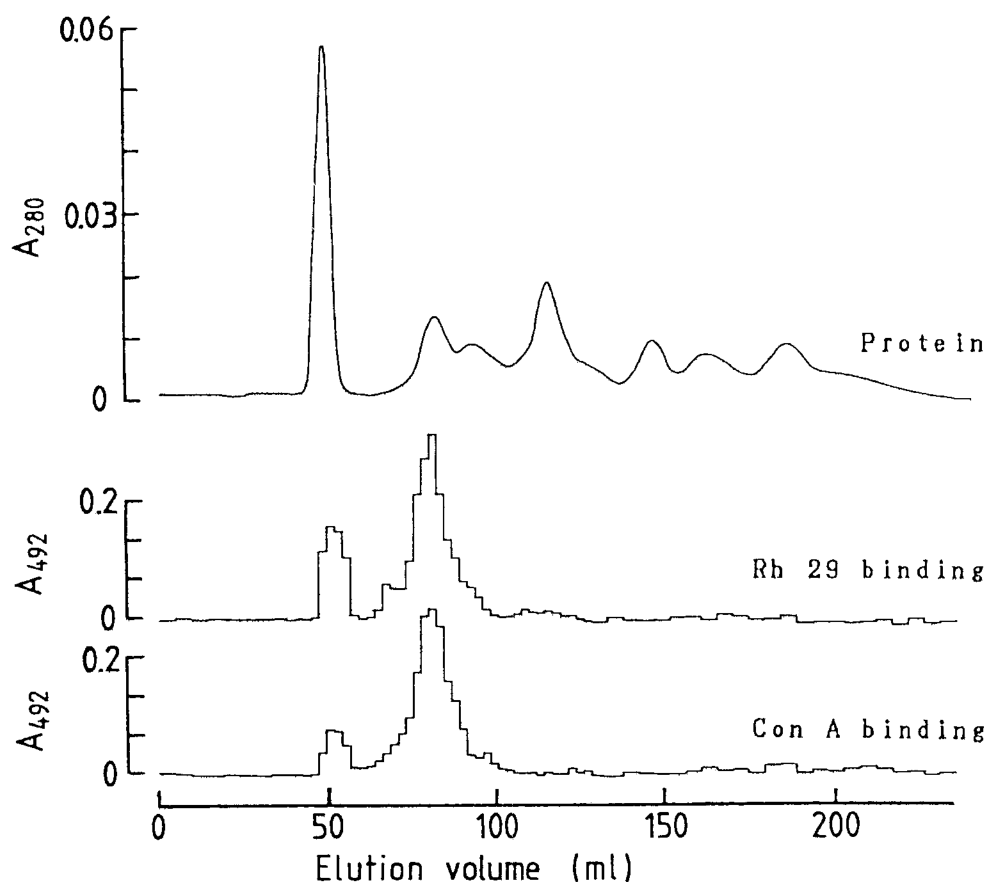

Fig. 4. Isolation of Rh29-reactive CNBr-fragment by Sephadex G-50 column chromatography. A large thermolitic fragment of opsin (F1; 1–249) was cleaved with CNBr and developed on Sephadex LH-60 column. Rh29-reacted fractions were collected and then rechromatographed on Sephadex G-50 column (upper trace). Portions of fractions were dried in the microtiter plate wells and reacted with Rh29 (middle trace) or with ConA (lower trace).

Table 1. Amino acid composition of Rh29- and ConA-bound peptide

Amino acid	PeakII* expected (mol/mol fragment)	found
Asp	3	3.3
Thr	2	1.7
Ser	3	3.0
Glu	5	4.7
Gly	3	4.1
Ala	2	2.1
Val	3	2.7
Ile	0	0.4
Leu	1	1.4
Tyr	3	2.3
Phe	4	3.2
Lys	1	1.1
His	0	0.3
Arg	1	0.9
Pro	5	4.4

*Second peak in Fig. 4.

bition were over thousand times higher than undigested one.

The carboxylic side of Lys16 or Arg21 was reported to be attacked by trypsin (Ovchinnikov, 1982). The chemical modification of arginine with 1,2-cyclohexanedione prevent tryptic cleavage and the cleavage sites can be restricted within lysine (Patthy and Smith, 1975). We, thus, modified the arginine residues of solubilized disks and digested with trypsin. The arginine-protected opsin could bound Rh29 regardless of trypsin treatment, while unprotected and deprotected opsin apparently lost their anitigenicity with trypsin digestion (Fig. 5B). Therefore, the cleavage between Arg21 and Ser22 should be responsible for the loss of antigenicity.

The epitope for Rh29 was conserved among wide species.

Finally, we tested the cross-reactivities of Rh29 to rhodopsins of several animals (Fig. 6). Rh29 recognized opsins (molecular weight 39–41 kDa) and its oligomer of bovine and porcine ROS, and octopus microvilli. Analysis of ConA-blots also elicited that these opsins had ConA-specific oligosaccharides. Residual ConA-specific proteins were

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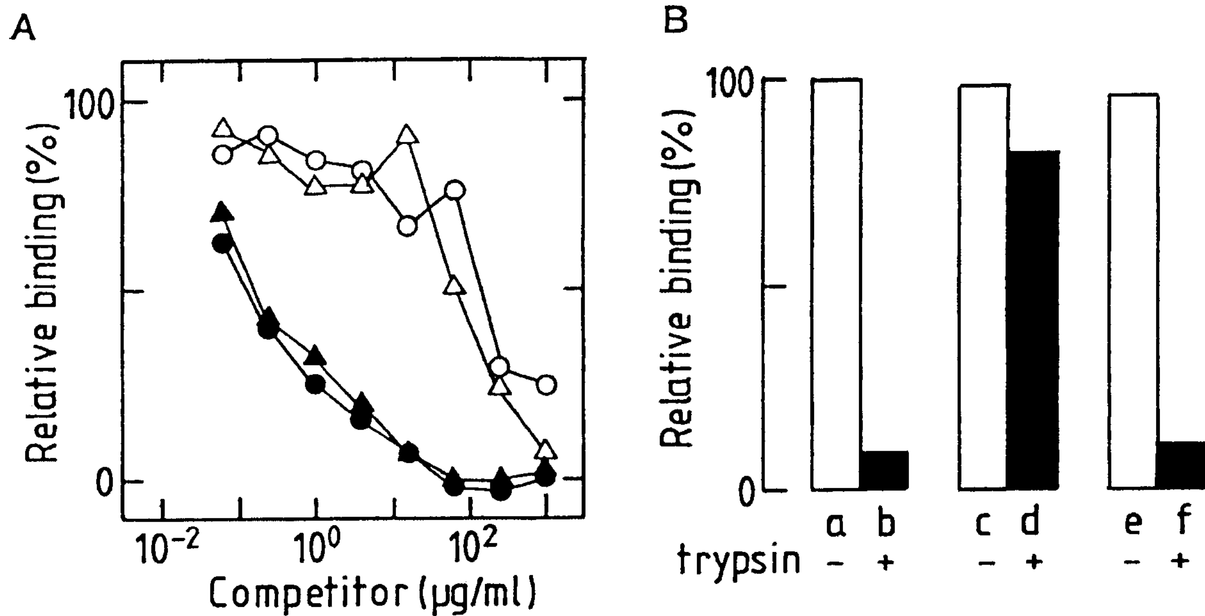

Fig. 5. (A): Effect of proteolytic digestion of solubilized opsin on the binding of Rh29. Bleached disks were solubilized by digitonin and incubated with TPCK-trypsin (triangles) or chymotrypsin (circles). The reaction was stopped by DFP and then the antigenicity of the peptide mixture was examined by competitive binding assay. Closed symbols represent controls incubated with the each enzyme and DFP. DFP-treated trypsin and chymotrypsin did not affect the binding of Rh29 to antigen. (B): Effect of modification of arginine residue. Digitonin-solubilized bleached disks (1 mg opsin; a and b) were treated with 1,2-cyclohexanedione (0.15 M; c and d) and then with hydroxylamine for deprotection of modified arginine (e and f). Each samples were then incubated with 1% TPCK-trypsin (b, d and f) or with TPCK-trypsin and DFP (a, c and e).

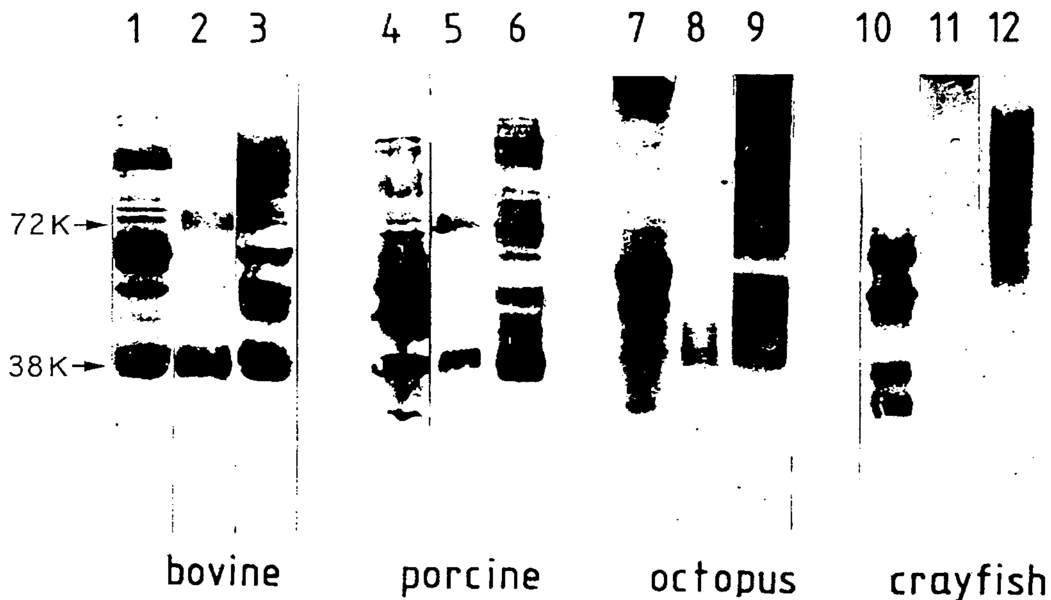

Fig. 6. Immunoblotting and ConA-blotting of photoreceptor membranes of several animals. SDS-PAGE using 10% polyacrylamide gel was made for bovine, porcine ROS, octopus microvilli and crayfish rhabdome. Proteins were transferred on nitrocellulose paper and stained with amido black (1, 4, 7 and 10), or treated with Rh29 (2, 5, 8 and 11) or ConA (3, 6, 9 and 12). The conditions for the reaction were same as in Fig. 3.

banded in higher molecular weight region. In crayfish rabdome, no bands were detected with Rh29, although applied membrane samples contained as much opsin as the other samples (>3 μg determined by visible absorbance measurements). In addition, ConA did not bind to the protein smaller than 50 kDa in crayfish, suggesting that the primary structure around N-terminal segment and the sugar structure might be altered in arthropoda.

DISCUSSION

Monoclonal antibody is useful tool for structural studies

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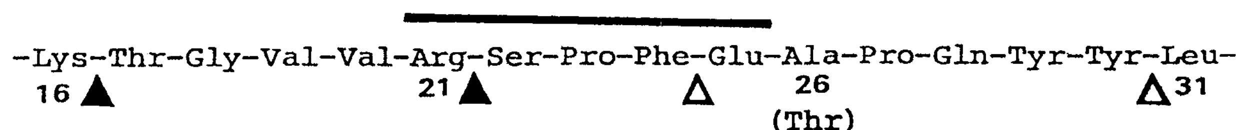

Fig. 7. Antigenic structure of Rh29 monoclonal antibody. Approximate location of Rh29 binding region (bar) was estimated by proteolytic study. Triangles represent the cleavage sites by TPCK-trypsin (losed triangles) and chymotrypsin (open triangles) according to Ovchinnikov.9 Ala26 is substituted by Thr in porcine (Pappin and Findley, 1984).

of membrane proteins (Ovchinnikov *et al.*, 1985; Yelton and Scharff, 1981; Eisenbrath, 1981). We used mAb in order to examine the membrane topology of rhodopsin, to attempt to detect the structural change during photo-bleaching and to survey the cross-reactivity in several animal photorceptor membranes.

The antigenic peptide of Rh29 generated by CNBr-cleavage was determined as Met2-Ser39 in bovine rhodopsin. We demonstrated that the peptide bond between Arg21 and Ser22 is essential for binding to Rh29. Moreover, it was found that the binding region of Rh29 was cleaved by chymotrypsin (Fig. 4). Cleavage sites of chymotrypsin near N-terminal segment of bovine rhodopsin have been determined as Thr10, Phe13, Phe24 and Lys30 (Ovchinnikov, 1982). Combined with this and the necessity of the peptide bond of Arg21-Ser22 not of Lys16-Thr17 for the binding to Rh29, the peptide bond of Phe24-Glu25 and/or Lys30-Leu31 should be also required for the binding to Rh29 as well as Arg21-Ser22. Thus, we concluded that the determinant for Rh29 encompassed at least Arg21-Glu25 (Fig. 7).

It must be noted that the attached oligosaccharids are not antigenic for this antibody. Like Rh29, numerous antibodies specific for N-terminal segment prepared in several laboratories are also directed against peptide residues not against oligosaccharide chains (Hargrave *et al.*, 1986).

Our present data that Rh29 binding region including Arg21- Glu25 becomes more accessible to the antibody by freeze-thawing agreed with the fact that N-terminal segment of rhodopsin locates in intradiscal space and with the structural model of the seven transmembrane helices based on the hydrophobicity along the amino acid sequence (Hargrave *et al.*, 1983: Ovchinnikov, 1982). The connecting loops on cytoplasmic surface are studied by in situ proteolysis. Using intact disks or ROS, Ser240 in the loop between transmembrane helices V and VI (loop V/VI) is nicked by thermolysin (Ovchinnikov, 1982). Chymotrypsin cleaves at Phe146 in another loop III/IV (Ovchinnikov, 1982). There is, however, no evidence for that a loop I/II is exposed on cytoplasmic surface. We showed that the 21 kDa chymotryptic-fragment and 13 kDa papainic fragment contained both Rh29-binding and sugar-attachment region. The 21 kDa band obtained by chymotryptic digestion is larger in apparent molecular weight than that expected from amino acid sequences 1–146 (16.4 kDa). It may be resulted from two oligosaccaride chains in N-terminal segment and high hydrophobicity of the segments affecting on the migration in SDS-polyacrylamide gel. The real size of papainic fragment, thus, may be smaller than that observed on SDS-polyacrylamide gel; 13 kDa. If the molecular weight of papainic fragment was overestimated for the same degree as chymotryptic fragment, the cleaved site with papain is located at about 72'nd residue. This 72'nd residue is localized just in loop I/II of the model. Therefore, our data suggest that loop I/II may protrude toward cytoplasm and be cleaved by papain.

Some antibodies recognize the surface structure formed by protein higher order structure. The binding of such antibodies to antigenic protein is easily lost when the antigen is exposed to denaturing conditions. Rh311 and Rh112 are antibodies of this type, which did not bind opsin band in SDS-polyacrylamide gel (Tokunaga *et al.*, 1989).

In contrast to this, Rh29 recognized SDS-treated opsin, heat-denatured opsin (not shown) and CNBr-cleaved opsin fragment. This indicates that Rh29 recognizes a specific peptide sequence including residues 21–25 rather than a surface structure of intact conformation. On the other hand, we showed that opsin was 100-times more antigenic for Rh29 than rhodopsin in the disk membrane. This means that Rh29 probes a light-dependent structural change specifically around the antigenic determinant of rhodopsin. A clue to know what kind of change takes place comes from the result that solubilization of disks by ammonyx-LO makes unbleached rhodopsin to be more antigenic. It suggests that N-terminal segment of unbleached rhodopsin in ammonyx-LO solution has a conformation similar to that of bleached rhodopsin. The effect of ammonyx-LO might be a partial delipidation of disk membranes. That is, the antigenic segment of membrane-bound rhodopsin is probably embedded in or tightly bound to lipid bilayer. As a result of partial delipidation due to ammonyx-LO, Rh29 could bind to the segment. The conformational change upon light absorption in N-terminal region of rhodopsin in disk membeane should be, therefore, like a protruding of antigenic segment from the lipid bilayer to water phase.

Light-dependent structural changes have been observed in and near cytoplasmic loops. Residues in loop III/IV and V/VI become more accessible to several protease in bleached disks than in unbleached disks (Pellicone *et al.*, 1985a).

Methionine residues at the position 155 and 253 are attacked by CNBr on illuminating disks (Pellicone *et al.*, 1985b). These increases in accessiblity suggest that some helical segments may shift toward cytoplasmic space. Our data lead to a conclusion that structural changes initiated by light-absorption at chromophore propagate toward intradis-

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cal N-terminal residues. Therefore, both cytoplasmic and intradiscal segments may be involved in light-induced conformational changes of rhodopsin.

Porcine rhodopsin showed higher antigenicity to Rh29 in spite of Ala26 being replaced by Thr (Pappin *et al*., 1984), suggesting that the determinant should not exceed the position 26 (Fig. 7). Rh29 binds the rhodopsins of other vertebrates such as bullfrog, newt, and lamprey. On the other hand, no antigenicity was observed in crayfish (Hariyama *et al*., 1993; Crandal and Cronin, 1997). In concern with this, Drosophila opsin does not show an identical sequence to bovine with more than three continuous residues (O'Tousa *et al*., 1985; Zuker *et al*., 1985). Thus, the sequence at N-terminal segment may diverge from cepharopoda. Unlike crayfish, octopus rhodopsin is recognized by Rh29 as much as bovine. The cross-reaction to this invertebrate rhodopsin suggests that the sequence at N-terminal segment might be highly conserved between mammals and erthropods. The amino acid sequence is not the same at corresponding region in Octopus (Ovchinnikov *et al.*, 1988). Although the antigenic segment in Octopus rhodopsin is not determined at present, the fact that the antigenic segment is conserved in wide varaiety of animals suggests that the segment might have unknown physiological meaning(s) for rhodopsin.

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