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Localization of Binding Sites for Carboxyl Terminal Specific Anti-Rhodopsin Monoclonal Antibodies Using Synthetic Peptides†

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ABSTRACT: The binding sites for four monoclonal antibodies, rho 1D4, rho 3C2, rho 3A6, and rho 1C5, have been localized within the C-terminal region of bovine rhodopsin: Asp$^{18'}$-Glu-Ala$^{16'}$-Ser-Thr-Thr-Val$^{12'}$-Ser-Lys-Thr-Glu$^{8'}$-Thr-Ser-Gln-Val$^{4'}$-Ala-Pro-Ala$^{1'}$. Antibody binding sites were localized by using synthetic C-terminal peptides in conjunction with solid-phase competitive inhibition assays and limited proteolytic digestion of rhodopsin in conjunction with electrophoretic immunoblotting techniques. Binding of the rho 1D4 and rho 3C2 antibodies to immobilized rhodopsin was inhibited with peptides of length 1′–8′ and longer. Antibody rho 1D4 binding was not inhibited by peptides 2′–13′ or 3′–18′, indicating that the C-terminal alanine residue of rhodopsin was required. Similar competitive inhibition studies indicated that the antibody rho 3A6 required peptides of length 1′–12′ and longer whereas rho 1C5 required peptide 1′–18′. Peptide 3′–18′ was as effective as 1′–18′ in inhibiting rho 3A6 binding to rhodopsin, but replacement of glutamic acid in position 8′ with glutamine abolished competition. This substitution had little effect on the binding of antibody rho 1C5. Thus, Glu$^{8'}$ was essential for rho 3A6 binding but not for the binding of the rho 1C5 antibody. Cleavage of the seven amino acid C-terminus from rhodopsin and further cleavage to F_1 (M_r 25 000) and F_2 (M_r 12 000) fragments with *Staphylococcus aureus* V8 protease abolished binding of rho 1D4 antibody to the membrane-bound rhodopsin fragments. Antibodies rho 3A6 and rho 1C5, however, were found to bind both rhodopsin lacking the seven amino acid C-terminus and the corresponding F_2 fragment. These results indicate that a peptide longer than 1′–6′ is required for antibodies rho 1D4 and rho 3C2 binding, 8′–12′ for antibody rho 3A6 binding, and 9′–17′ or 9′–18′ for antibody rho 1C5. Since longer peptides and rhodopsin are more effective competitors, other factors such as conformation also affect binding reactivity. These studies in conjunction with related studies on the binding of these antibodies to membrane-bound and detergent solubilized bleached and unbleached rhodopsin indicate that the carboxyl-terminal 1′–18′ segment of rhodopsin is highly accessible to these immunological probes and relatively insensitive to the state of bleaching and solubilization of rhodopsin. The cross-reactivity of these monoclonal antibodies as well as monoclonal antibodies against other regions of bovine rhodopsin was also studied. Results indicate that the C-terminal segment (as specified by the rho 1D4 and rho 3C2 antibodies), the F_1–F_2 linking region, and the N-terminal region of rhodopsin are highly conserved in pig, dog, cat, rat, rabbit, and frog rhodopsins. Segments farther in from the C-terminus as specified by the rho 3A6 and rho 1C5 antibodies are more variable.

Rhodopsin is the photoreceptor protein of vertebrate rod cells. It consists of a polypeptide chain of M_r 39 000 linked to a molecule of 11-*cis*-retinal [see Hargrave (1982) for a recent review]. The complete covalent sequence of the protein has recently been determined (Ovinchinnikov et al., 1982; Hargrave et al., 1983). Topographic studies carried out in a number of laboratories have shown that the carboxyl-terminal segment of rhodopsin is exposed on the cytoplasmic side of the disk membrane and the amino-terminal segment containing two carbohydrate chains is exposed on the opposite (intradisk) side (Hargrave, 1982). On the basis of numerous labeling and limited proteolysis studies, a model has been constructed in which the polypeptide chain of rhodopsin is visualized as traversing the phospholipid bilayer in seven predominantly hydrophobic, α-helical segments connected by hydrophilic linking regions (Ovinchinnikov et al., 1982; Hargrave et al., 1983).

The carboxyl-terminal segment of rhodopsin has been shown to be highly accessible on the cytoplasmic surface of the disk membrane. A variety of proteolytic enzymes including thermolysin (Hargrave & Fung, 1977), trypsin (Molday & Molday, 1979), *Staphylococcus aureus* protease (Findlay et al., 1981), and papain (Fung & Hubbell, 1978) initially cleave small peptides from the carboxyl terminus. The membrane-impermeable chemical-labeling agent (nitroazidophenyl)taurine also preferentially labels amino acids in the C-terminal region of rhodopsin (Mas et al., 1980). A rhodopsin kinase has also been shown to phosphorylate serine and threonine residues along the C-terminus when rod outer segments (ROS)[1] are bleached in the presence of ATP (Wilden & Kühn, 1982).

More recently, monoclonal antibodies to bovine rhodopsin have been produced and used as probes to study the organi-

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[1] Abbreviations: AUFS, absorbance units full scale; Boc, *tert*-butoxycarbonyl; BSA, bovine serum albumin; CHAPS, 3-[(3-cholamidopropyl)dimethylammonium]-1-propanesulfonate; DCC, dicyclohexylcarbodiimide; HOBt, 1-hydroxybenzotriazole monohydrate; HPLC, high-performance liquid chromatography; Ig, immunoglobulin; PAM, hydroxymethylphenylacetamidomethyl; RIA, radioimmunoassay; ROS, rod outer segments; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

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zation of rhodopsin in disk membranes (MacKenzie & Molday, 1982; Molday & MacKenzie, 1983). Two such antibodies, designated as rho 1D4 and rho 3D6, have been shown to be directed against the carboxyl-terminal region of rhodopsin on the basis of the differential reactivity of these antibodies to native and trypsin-treated rhodopsin. More precise localization of the binding sites for such antibodies, however, is needed for their application as probes for the structure and function of rhodopsin. In this paper, we describe the synthesis of various peptides from the sequence of the carboxyl terminus of bovine rhodopsin and their application in more precisely localizing the binding sites of monoclonal antibodies against the carboxyl-terminal region of rhodopsin. The cross-reactivities of these and other anti-bovine rhodopsin monoclonal antibodies with rhodopsins from various vertebrate species are also determined.

Experimental Procedures

ROS Membranes. Bovine ROS disk membranes were prepared as previously described (Molday & Molday, 1979) by employing the method of Smith et al. (1975). Frog, pig, dog, cat, rat, rabbit, and catfish ROS were prepared by the method of McDowell & Kühn (1983). Rhodopsin concentration was determined by the light absorption at 500 nm with an extinction coefficient of 40 000 M^{-1} cm^{-1}. Proteolysis of rhodopsin in disk membranes with trypsin or *S. aureus* V8 protease was carried out as previously described (Molday & MacKenzie, 1983).

Synthesis of Carboxyl-Terminal Rhodopsin Peptides. Synthesis of carboxyl-terminal peptides of rhodopsin was carried out manually by the modified solid-phase procedure of Merrifield (1963). The side-chain functional groups of Ser, Thr, Glu, and Asp were protected by benzyl groups, and the ϵ-amino group of lysine was protected by the 2-chlorobenzyloxycarbonyl group. Synthesis of 1′–4′ to 1′–18′ peptides was initiated with 1 mmol of Boc-Ala-PAM resin (0.59 milliequiv of Ala/g of resin) prepared (Mitchell et al., 1978) from poly(styrene-*co*-1% divinylbenzene) (Bio-Rad). All couplings were performed by the DCC/HOBt method, and double coupling was employed at every cycle. One cycle of the synthesis consisted of the following operations: (1) CH_2Cl_2 wash (3 × 1.5 min); (2) deprotect with 50% CF_3COOH containing 0.1% indole (1 × 1.5 min, 1 × 30 min); (3) CH_2Cl_2 wash (6 × 1.5 min); (4) neutralize with 10% diisopropylethylamine in CH_2Cl_2 (2 × 1.5 min); (5) CH_2Cl_2 wash (6 × 1.5 min); (6) equilibrate with Boc-protected amino acid and HOBt (2.5 equiv each in CH_2Cl_2–DMF, 1:1, 5 min); (7) without filtration add DCC (2.5 equiv); (8) couple, 2 h; (9) repeat steps 3–8. Unreacted chains were terminated (1 mL of acetic anhydride–pyridine mixture, 1:1 v/v, 30 min) when necessary as judged by the ninhydrin test (Kaiser et al., 1970). For coupling of radioactive alanine at position 3′, only 1 equiv of Boc-[3-^{3}H]Ala (1 mCi) was used in the first coupling (incorporation 73%), and the same mixture was used in the second coupling with neutralized and washed resin (incorporation 12.5%). Unlabeled Boc-Ala (2.5 equiv) was used in the third coupling to help ensure complete reaction. About 0.1 mmol of each protected peptide–resin was treated with HF (9 mL) in the presence of anisole (1 mL) at 0 °C for 45 min as previously described (Sakakibara et al., 1967). Peptides 3′–18′ and 3′–18′ ($Glu^{8'} \rightarrow Gln^{8'}$) were initiated with 0.6 mmol of the same Boc-Ala-PAM resin, and the 2′–13′ peptide was initiated with 0.3 mmol of Boc-Pro-PAM resin (0.65 milliequiv of Pro/g of resin).

The crude peptides were desalted on a Bio-Gel P-2 column (2.5 × 100 cm) in 50% acetic acid. The peptides were purified by preparative HPLC on a Whatman ODS-3 (Magnum 20) column with either a linear gradient or an isocratic system (0.1% TFA in water vs. methanol at 10 mL/min with the eluant monitored at 220 nm with 2.0 AUFS). The purity of the peptides was determined by reverse-phase HPLC either on a Varian MCH5-N-CAP (4.5 × 150 mm) column developed with a linear gradient of 0–30% acetonitrile in 0.05 M KH_2PO_4, pH 3.0, or on a Whatman ODS-3 (5 × 250 mm) column equilibrated with 0.1% H_3PO_4 in acetonitrile. The composition and concentration of the purified peptides were determined by amino acid analysis.

Production of Monoclonal Antibodies. Hybridoma cell lines were obtained by the fusion of NS-1 mouse myeloma cells with spleen cells from mice immunized with bovine ROS disks as previously described (MacKenzie & Molday, 1982). Culture supernatants from cloned hybridoma cells were used in most experiments.

Solid-Phase Radioimmunoassay. The binding of monoclonal antibodies to ROS membranes was measured by using indirect solid-phase RIA as previously described (MacKenzie & Molday, 1982). Briefly, bleached ROS membranes were solubilized with Triton X-100, and 25 μL of a 0.25 mg/mL solution was dried onto flex vinyl microtiter wells at 60 °C. The wells were rinsed in RIA buffer (phosphate-buffered saline containing 1% BSA, 1% fetal calf serum, and 0.1% NaN_3) and incubated with 25 μL of hybridoma culture fluid for 60 min at 23 °C. The wells were then rinsed in phosphate-buffered saline and incubated with 25 μL of ^{125}I-labeled goat anti-mouse Ig [10–30 μg/mL; (1–2) × 10^6 dpm/μg] in RIA buffer for 30–60 min at 23 °C. The plates were then rinsed in phosphate-buffered saline, and individual wells were counted in a Beckman 8000 gamma counter.

Antigen Competitive Inhibition Assays. The following competitors were tested for their effectiveness in inhibiting the binding of antibodies to immobilized rhodopsin in competition assays (Molday & MacKenzie, 1983): synthetic peptide analogues of rhodbopsin's C-terminus, rhodopsin in disk membranes, or rhodopsin solubilized in 1% Triton X-100 or CHAPS. A fixed concentration of monoclonal antibody was treated with serial dilutions of free antigens (peptide or disk membranes) in microtiter wells containing immobilized rhodopsin as described above. Incubation was carried out for 1 h at 23 °C after which the wells were rinsed with phosphate-buffered saline to remove soluble antibody–antigen complexes. The wells were then treated with ^{125}I-labeled goat anti-mouse Ig, washed, and counted as described above.

$NaDodSO_4$–Polyacrylamide Gel Electrophoresis. Bovine ROS disk membranes (4 mg/mL) were solubilized with an equal volume of denaturing solution containing 5% $NaDodSO_4$, 0.01 M Tris, pH 6.8, 10% β-mercaptoethanol, and 4% bromophenol blue. A total of 10 μL was applied to each well of a mini slab gel (0.75-mm thickness × 3-cm length) in the buffer system of Lammeli (1970). Gels were either stained with Coomasie blue or transferred to CNBr-activated paper as previously described (Molday & MacKenzie, 1983).

Transfer papers were rinsed in RIA buffer, incubated with 5 mL of hybridoma culture fluid for 1 h at 23 °C, and washed in phosphate-buffered saline containing 0.4% *N*-lauroylsarcosine. Finally, the papers were incubated with 5 mL of ^{125}I-labeled goat anti-mouse Ig (1 μg/mL) for 1 h at 23 °C, washed in phosphate-buffered saline, and subjected to autoradiography (Molday & MacKenzie, 1983).

Results

Monoclonal Antibodies to Bovine Rhodopsin. The fusion of mouse myeloma cells with spleen cells from mice immunized

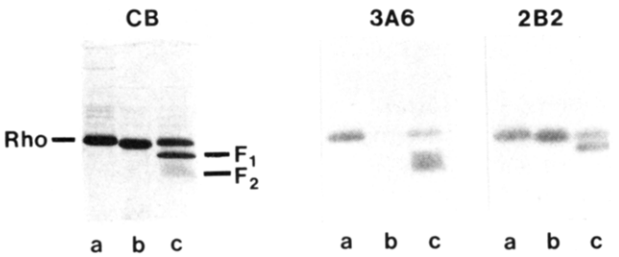

FIGURE 1: $NaDodSO_4$–polyacrylamide gel and immunoblots of rhodopsin and its proteolytic fragments. Undigested bovine disk membranes (gels a) and disk membranes digested with trypsin (gels b) or *S. aureus* V8 protease (gels c) were subjected to $NaDodSO_4$ gel electrophoresis on slab gels and either stained with Coomasie blue (CB) or electrophoretically transferred to CNBr-activated paper. The transfer papers were labeled with rho 3A6 or rho 2B2 hybridoma culture supernatant followed by ^{125}I-labeled goat anti-mouse Ig and subjected to autoradiography.

with bovine ROS disk membranes has generated a series of monoclonal antibodies against different segments of the rhodopsin polypeptide chain. Three antibodies (rho 3A6, rho 1C5, and rho 3C2) were similar to the rho 1D4 antibody (Molday & MacKenzie, 1983) in that they bound to undigested rhodopsin but not trypsin-digested rhodopsin lacking the nine amino acid C-terminal peptide. This is illustrated in Figure 1 for the rho 3A6 antibody by the immunoblotting technique. On this basis, these antibodies appear to bind to antigenic sites located along the C-terminal region of rhodopsin.

The binding of these antibodies to rhodopsin digested with *S. aureus* V8 protease was also studied. This enzyme has been shown to release a seven amino acid peptide from the C-terminus of rhodopsin in disk membranes and further cleave rhodopsin into membrane-bound fragments F_1 and F_2 having approximate M_r values of 25 000 and 12 000, respectively (Findlay et al., 1981). As shown in Figure 1, antibody rho 3A6 binds both to *S. aureus* V8 digested rhodopsin (lacking the seven amino acid terminal peptide) and to the corresponding F_2 fragment but not to the F_1 fragment. The immunoblot of the rho 1C5 antibody was identical with that for the rho 3A6 shown in Figure 1 (data not shown). In contrast, the rho 1D4 antibody does not bind to any of the fragments of rhodopsin generated by digestion with *S. aureus* V8 protease (Molday & MacKenzie, 1983). Binding of the rho 3C2 antibody to *S. aureus* V8 digested rhodopsin was not determined.

Another monoclonal antibody, rho 2B2, shows different binding properties. This antibody was observed to bind to rhodopsin, to trypsin-digested rhodopsin, and to the F_1 fragment of *S. aureus* V8 protease digested rhodopsin (Figure 1). On the basis of these results, the binding site for this antibody appears to be localized along the amino-terminal two-thirds of the rhodopsin chain.

C-Terminal Peptides. In order to more precisely localize the peptide binding determinants of the C terminal specific anti-rhodopsin antibodies, a series of eight peptides from 1′–4′ to 1′–18′, each differing by two amino acids in length, was synthesized on the basis of the known amino acid sequence of the carboxyl-terminal region of bovine rhodopsin (Hargrave, 1977):

-Asp-Glu-Ala$^{16'}$-Ser-Thr-Thr-Val$^{12'}$-Ser-Lys-Thr-Glu$^{8'}$-
Thr-Ser-Gln-Val$^{4'}$-Ala-Pro-Ala$^{1'}$-COOH

Peptides isolated by preparative HPLC were over 90% pure as determined by analytical HPLC (Figure 2) and gave the expected molar ratios of amino acids by amino acid analysis. Peptides lacking the C-terminal alanine (peptide 2′–13′) and the C-terminal Pro-Ala group (peptide 3′–18′) were also

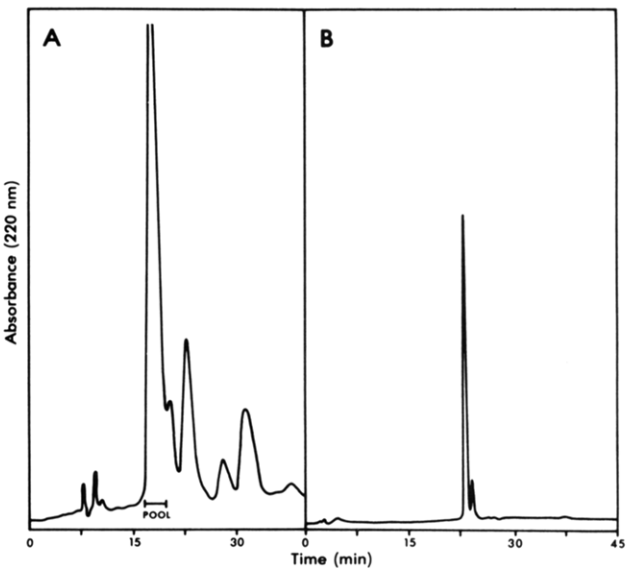

FIGURE 2: HPLC of synthetic rhodopsin peptide 1′–12′. (A) Preparative HPLC of peptide (14.7 mg) on a Whatman ODS-3 (Magnum 20) column in 75% buffer A (0.1% TFA in water) and 25% buffer B (methanol) at 10 mL/min with 2.0 AUFS. Fractions were pooled as indicated. (B) Analytical HPLC of purified peptide on a Whatman ODS-3 column (0.5 × 25 cm) in 0–30% buffer B over 40 min where buffer A was 0.1% H_3PO_4 in H_2O and buffer B was 0.05% H_3PO_4 in CH_3CN.

Table I: Concentrations (μM) of C-Terminal Peptides or Rhodopsin That Inhibit by 50% (I_{50}) the Binding of Monoclonal Antibodies to Immobilized Rhodopsin

competing antigen	rho 1D4	rho 3C2	rho 3A6	rho 1C5	rho 2B2[a]
1′–4′					
1′–6′					
1′–8′	52.5	525			
1′–10′	2.51	52.4			
1′–12′	0.58	15.8	63.1		
1′–14′	0.46	ND	11.0		
1′–18′	0.17	3.31	3.0	3.3	
2′–13′		ND	13.2	ND	ND
3′–18′			7.6	3.3	
3′–18′ (Glu$^{8'}$ → Gln$^{8'}$)			>251.0	7.6	
disk membranes					
bleached	0.05	0.002	0.06	0.25	
unbleached	0.06	ND	0.05	0.20	
solubilized disks					
bleached (Triton X-100)	0.06	0.003	0.04	1.0	0.03
unbleached (Triton X-100)	0.04	ND	ND	ND	1.58
bleached (CHAPS)	ND[b]	ND	ND	0.76	2.30
unbleached (CHAPS)	0.04	ND	ND	ND	145

[a] Monoclonal antibody against the N-terminal region. [b] ND, not determined.

prepared. In addition, a peptide in which the glutamic acid residue in position 8′ was replaced with a glutamine residue [peptide 3′–18′ (Glu$^{8'}$ → Gln$^{8'}$)] was also synthesized for use in determining the specificity of the C-terminal anti-rhodopsin antibodies.

C-Terminal Peptides as Competitive Inhibitors. Inhibition of the binding of monoclonal antibodies to immobilized bovine rhodopsin by synthetic peptides was determined by solid-phase competition assays. Competitive inhibition curves of carboxyl-terminal peptide analogues for three monoclonal antibodies (rho 1D4, rho 3A6, rho 1C5) are illustrated in Figures 3 and 4. The concentrations of the peptides and rhodopsin that produce half-maximum inhibition (I_{50}) for four C-terminal

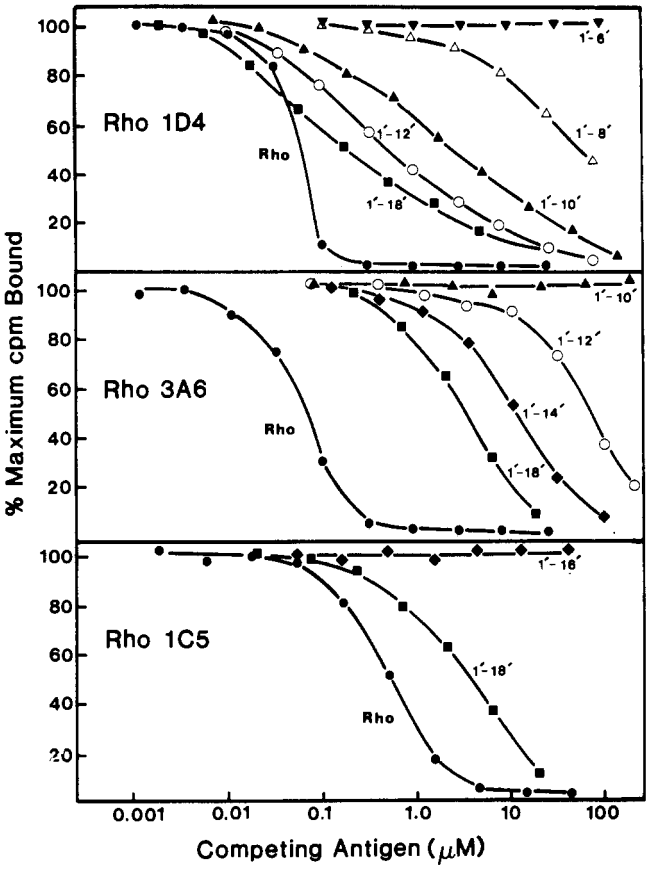

FIGURE 3: Competitive inhibition of rho 1D4, rho 3A6, and rho 1C5 antibody binding to bovine rhodopsin by synthetic C-terminal peptides of various lengths. Culture supernatants containing the monoclonal antibodies were incubated with serial dilutions of the peptides or disk membranes containing bleached bovine rhodopsin (rho) in microtiter wells coated with rhodopsin. After 60 min, the wells were washed in RIA buffer, treated with ^{125}I-labeled goat anti-mouse Ig, and counted.

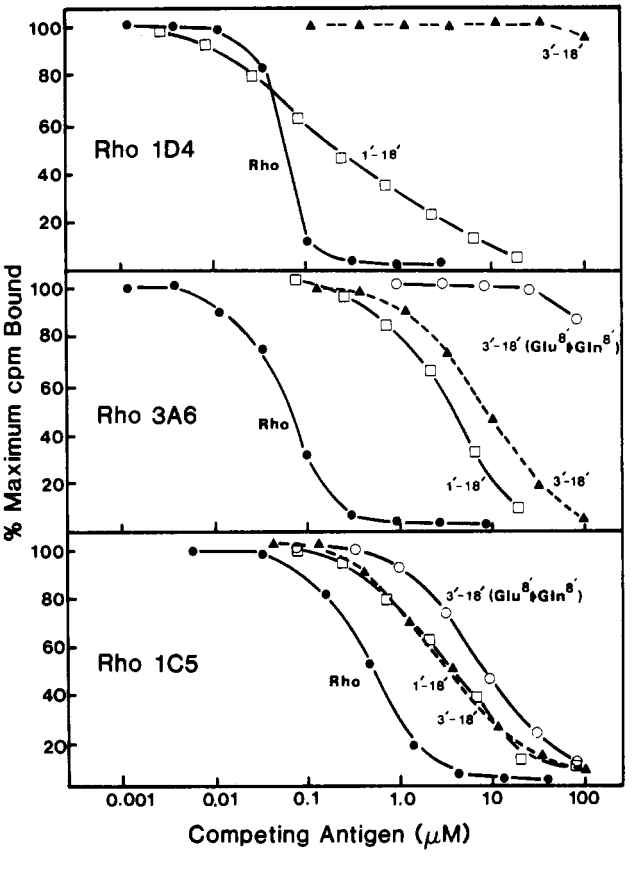

FIGURE 4: Competitive inhibition of rho 1D4, rho 3A6, and rho 1C5 antibody binding to rhodopsin by synthetic C-terminal peptide analogues. Culture supernatants containing the monoclonal antibodies were incubated with serial dilutions of the indicated peptides or disk membranes containing bleached bovine rhodopsin in microtiter wells coated with rhodopsin. After 60 min, the wells were washed in RIA buffer, treated with ^{125}I-labeled goat anti-mouse Ig, and counted.

antibodies (rho 1D4, rho 3C2, rho 3A6, and rho 1C5) and one N-terminal antibody (rho 2B2) serving as a control are summarized in Table I.

Binding of antibody rho 1D4 to rhodopsin was inhibited by peptides of length 1′–8′ and longer but not by 1′–4′ or 1′–6′ peptides even at concentrations of 100 μM. An increase in inhibition was observed as the length of the peptide was extended from 1′–8′ to 1′–18′. A similar pattern of binding inhibition by peptides of various lengths was observed for the rho 3C2 antibody. Rhodopsin was found to be a more effective inhibitor than the 1′–18′ peptide by over 3 orders of magnitude for this antibody (Table I).

Competitive inhibition of rho 3A6 was only observed for peptides of length 1′–12′ and longer over the concentration range employed. Rhodopsin, however, was almost 50 times more effective as an inhibitor than peptide 1′–18′ (Table I). Antibody rho 1C5 was only inhibited by the longest peptide (1′–18′). Binding of antibody rho 2B2 to immobilized rhodopsin was not inhibited by any of the C-terminal peptides.

The effect of removing the carboxyl-terminal Ala and Pro-Ala on antibody binding was also studied (Figure 4 and Table I). Binding of antibodies rho 1D4 and rho 3C2 to rhodopsin was not inhibited by peptide 3′–18′. Peptide 2′–13′ also did not inhibit binding of antibody rho 1D4 to rhodopsin. In contrast, both peptide 2′–13′ and peptide 3′–18′ were effective inhibitors of antibody rho 3A6, indicating that the two C-terminal amino acids are not required for binding of this antibody. Peptide 3′–18′ in which the Glu in position 8′ was substituted by a Gln residue, however, was a poor inhibitor for rho 3A6 (Figure 4). Antibody rho 1C5, which bound only to the longest peptides, was inhibited effectively by both peptide 3′–18′ and peptide 3′–18′ (Glu$^{8'}$ → Gln$^{8'}$).

Rhodopsin as a Competitive Inhibitor. The effect of solubilization of disk membranes with Triton X-100 and CHAPS detergents and the effect of bleaching of rhodopsin on the binding of these antibodies to rhodopsin were also examined in competition assays. In general, little, if any, effect of either of these treatments was observed with the carboxyl-terminal antibodies rho 1D4, rho 3C2, and rho 3A6. A relatively small decrease in competition (less than a factor of 5) for antibody rho 1C5 was observed when bleached rhodopsin in disk membranes was solubilized in Triton X-100 (Table I).

In contrast, the binding of antibody rho 2B2 directed against a determinant in the N-terminal two-thirds of the rhodopsin polypeptide was greatly affected by solubilization and bleaching of rhodopsin (Table I). Unbleached or bleached disks did not inhibit rho 2B2 binding; unbleached Triton X-100 solubilized disks required over 50 times higher concentration than bleached Triton X-100 solubilized rhodopsin to reach 50% inhibition. Triton X-100 solubilized disks proved to be a more effective inhibitor than CHAPS-solubilized disks. A similar effect of bleaching and detergent solubilization has been previously observed for the rho 4A2 monoclonal antibody (Molday et al., 1983).

Cross-Reactivity of Monoclonal Antibodies. The extent of binding of anti-bovine rhodopsin antibodies to rhodopsins from a variety of different vertebrate species was determined by using indirect solid-phase RIA. A known amount of each

Table II: Cross-Reactivity of Anti-Bovine Rhodopsin Antibodies[a]

ROS	rho 1D4	rho 3C2	rho 3A6	rho 1C5	rho 4B4	rho 2B2	rho 4A2
bovine (cow)	50420 (1.0)	10756 (1.0)	26784 (1.0)	39032 (1.0)	22144 (1.0)	7944 (1.0)	35094 (1.0)
porcine (pig)	44134 (0.88)	10372 (0.96)	5588 (0.21)	24074 (0.62)	20918 (0.94)	6952 (0.88)	30436 (0.87)
canine (dog)	35860 (0.71)	8038 (0.75)	166 (0.01)	3946 (0.10)	15632 (0.71)	5268 (0.66)	20172 (0.57)
feline (cat)	37192 (0.74)	8378 (0.78)	652 (0.02)	7232 (0.19)	16050 (0.72)	4968 (0.63)	23360 (0.67)
rat	39890 (0.79)	7342 (0.68)	576 (0.02)	18186 (0.47)	18474 (0.83)	7024 (0.88)	30452 (0.87)
rabbit	32792 (0.65)	7886 (0.73)	134 (0.005)	3522 (0.09)	15404 (0.70)	100 (0.01)	18626 (0.53)
catfish	4826 (0.10)	26 (0.002)	1938 (0.07)	6126 (0.16)	3030 (0.14)	−112	448 (0.01)

[a] Rhodopsin (6.25 μg) solubilized with Triton X-100 was added to each well and dried at 55 °C. Twenty-five microliters of hybridoma culture supernatant was added followed by 25 μL of ^{125}I-labeled goat anti-mouse Ig. Values are dpm bound with background (^{125}I-labeled goat anti-mouse Ig added to wells not treated with culture supernatant, 800 dpm) subtracted out. Values in parentheses are normalized with respect to bovine rhodopsin. Corrections for amount of rhodopsin bound per well were not made.

rhodopsin was solubilized in Triton X-100 and dried onto microtiter wells. Hybridoma supernatant was added to the wells followed by ^{125}I-labeled goat anti-mouse Ig. Results are given in Table II.

A high degree of cross-reactivity of the anti-C-terminal antibodies rho 1D4 and rho 3C2 was observed for all mammalian rhodopsins tested including pig, dog, cat, rat, and rabbit rhodopsin. Antibody rho 1C5 generally showed a lower degree of cross-reactivity particularly to rabbit, dog, and cat rhodopsins. Antibody rho 3A6 showed little, if any, cross-reactivity to the various rhodopsins with the exception of pig rhodopsin.

The rho 4A2 and rho 2B2 antibodies, which are directed against sites in the N-terminal region, showed a similar cross-reactivity profile as that for the anti-C-terminal antibodies rho 1D4 and rho 3C2. An exception, however, is the absence of binding of the rho 2B2 antibody to rabbit rhodopsin.

The rho 4B4 antibody binds to all mammalian rhodopsins tested. [It appears to be directed against the F_1–F_2 linking region since it has been shown to bind to trypsin-treated rhodopsin but not to Pronase-digested rhodopsin (MacKenzie & Molday, 1982) or *S. aureus* V8 protease digested rhodopsin (unpublished results).] The cross-reactivity of antibody rho 4B4 also closely parallels that for antibodies rho 1D4 and rho 3C2.

The binding of these antibodies to catfish rhodopsin was also investigated. Generally, these antibodies showed little cross-reactivity with catfish rhodopsin under the conditions used in these assays. In a separate experiment, the binding of the various antibodies to frog rhodopsin was investigated. As previously shown (Molday et al., 1983), rho 4A2 antibody bound almost as well to frog rhodopsin as to bovine rhodopsin. The rho 4B4 antibody also showed a high degree of cross-reactivity to frog rhodopsin. The binding of the rho 1D4 antibody to frog rhodopsin, however, was only two-thirds as great as that for bovine rhodopsin. This lower degree of binding may reflect, in part, the inability of the rho 1D4 antibody to bind to the lower molecular weight species of frog rhodopsin detected by $NaDodSo_4$ gel electrophoresis (Molday & Molday, 1979; Molday & MacKenzie, 1983). The C-terminal antibodies rho 3A6 and 1C5 and the N-terminal antibody rho 2B2 did not cross-react with frog rhodopsin under the conditions used.

Discussion

Previous studies have indicated that limited proteolytic digestion of rhodopsin in disk membranes can be used to determine the approximate location of the binding sites for anti-rhodopsin monoclonal antibodies. Thus, for example, the rho 1D4 antibody has been shown to bind to the carboxyl-terminal region of rhodopsin since this antibody binds to rhodopsin and to the nine amino acid C-terminal tryptic peptide of rhodopsin but not to the large membrane-bound fragment of trypsin-treated rhodopsin (Molday & MacKenzie, 1983).

In the present study, this same procedure was used to identify three new monoclonal antibodies (rho 3C2, rho 3A6, and rho 1C5) against the C-terminus of bovine rhodopsin and one antibody (rho 2B2) against a site along the N-terminal two-thirds of rhodopsin. More precise localization of the peptide binding sites for the C terminal specific anti-rhodopsin antibodies was obtained by measuring the ability of synthetic peptide analogues to compete with rhodopsin for antibody binding.

Two antibodies (rho 1D4 and rho 3C2) were observed to have similar, although not identical, reactivity to peptides of various length (Table I). The rho 1D4, studied in most detail, requires the C-terminal Ala amino acid and a C-terminal peptide length of greater than six amino acids since its binding to rhodopsin is inhibited with peptides 1′–8′ and longer but not 1′–4′, 1′–6′, 2′–13′, or 3′–18′.

The rho 3A6 binds further in from the C terminus since peptides of 1′–12′ and longer serve as competitive inhibitors for binding. The C-terminal amino acids are not required for binding since the 3′–18′ peptide is almost as effective as the 1′–18′ peptide in binding to this antibody. In fact, the C-terminal seven amino acids are not essential for binding since antibody rho 3A6 binds to *S. aureus* treated rhodopsin and its F_2 fragment lacking the seven amino acid C-terminus (Figure 1). The glutamic acid residue in the 8′-position, is required, however, since substitution of this residue with glutamine effectively abolishes binding. These results indicate that the 8′–12′ peptide segment of rhodopsin is required for binding.

Rho 1C5 binds to a region of rhodopsin even futher removed from the C-terminus since the 1′–18′ peptide but not the 1′–16′ peptide binds to this antibody. It appears that $Thr^{9'}$ also is required since rho 1C5 does not bind to trypsin-digested rhodopsin but does bind to *S. aureus* digested rhodopsin. Substitution of $Glu^{8'}$ with $Gln^{8'}$, however, has no effect.

On the basis of these results, it is possible to suggest binding sites for the anti-C-terminal monoclonal antibodies. As shown in Figure 5, the primary peptide determinants appear to be 1′–8′ (or possibly 1′–7′) for the rho 1D4 antibody, 8′–12′ for rho 3A6, and 9′–18′ (or 9′–17′) for rho 1C5. Amino acid residues outside these segments also appear to contribute since enhanced binding is observed with increasing peptide length. It is not known if these amino acid residues serve as additional binding determinants or if they are important in maintaining the peptide in a preferred conformation for binding. The importance of other factors such as conformation in binding of these antibodies is also apparent from the results that rhodopsin serves as a better antigen than any of the C-terminal peptides tested. This is to be expected since rhodopsin and not the synthetic peptide was used in the immunization protocol. It would be of interest to compare the binding of these

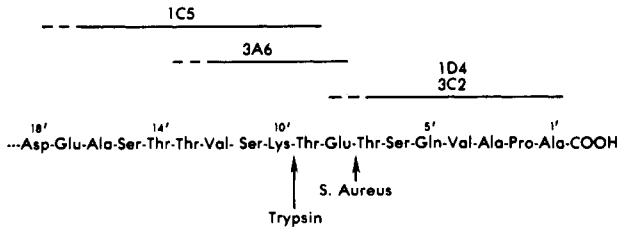

FIGURE 5: Carboxyl-terminal sequence of bovine rhodopsin showing the location of the peptide binding sites for the various anti-rhodopsin monoclonal antibodies on the basis of synthetic peptide inhibition studies and immunoblot analysis of protease-digested rhodopsin. The dashed lines indicate residues that may be required for antibody binding or alternatively greatly enhance binding. Other factors, such as peptide conformation, which also may contribute to antibody–antigen interaction, are not considered in this diagram.

monoclonal antibodies with monoclonal antibodies obtained from hybridoma cells generated from mice immunized with synthetic peptides.

Competitive binding studies designed to determine if bleaching and detergent solubilization had any effect on antibody binding indicated that little, if any, difference in antibody binding was observed for antibodies directed against the C-terminal region of rhodopsin. This indicates that the 1′–18′ carboxyl-terminal segment of bleached and unbleached rhodopsin is highly accessible both in disk membranes and in solubilized state. In contrast, the rho 2B2 antibody like the rho 4A2 antibody (Molday & MacKenzie, 1983) binds more strongly to bleached, solubilized rhodopsin. This would appear to indicate that the antigenic sites for these antibodies are largely inaccessible unless rhodopsin is bleached and solubilized in relatively strong detergent.

Cross-reactivity studies on rhodopsin from various mammalian species indicate that the C-terminal end of rhodopsin is highly conserved as shown in solid-phase binding studies with the antibodies rho 1D4 and rho 3C2 (Table II). The C-terminus of frog rhodopsin also is highly cross-reactive, possibly indicating that this region is an important functional domain of rhodopsin. A lower degree of cross-reactivity is observed for the rho 3A6 and rho 1C5 antibodies, which bind to peptide sequences further in from the C-terminus. It is not known whether this low degree of cross-reactivity is due to a change in one or several amino acids.

Antibodies that bind to other regions of rhodopsin are also highly cross-reactive to mammalian rhodopsins and frog rhodopsins. In particular, the binding site of the rho 4B4 antibody is highly conserved. This site appears to be in a segment of rhodopsin that is accessible on the cytoplasmic side of disk membranes (MacKenzie & Molday, 1982) and that links the F_1 and F_2 fragments of rhodopsin obtained by thermolysin or *S. aureus* digestion of membrane-bound rhodopsin. This is based on the finding that this antibody binds to trypsin-digested rhodopsin but not to the F_1 or F_2 fragments generated by *Staphylococcus griesus* protease or *S. aureus* V8 protease. Furthermore, preliminary results indicate that a synthetic peptide of this F_1–F_2 linking region can inhibit binding of the rho 4B4 antibody to rhodopsin. It has been suggested by Kühn & Hargrave (1981) on the basis of limited proteolysis studies that this F_1–F_2 linking region may be required for G protein binding and activation of its GTPase activity. Conservation of rho 4B4 reactivity for the various species tested supports the view that this segment of rhodopsin represents an important functional domain of rhodopsin. Antibodies rho 4A2 and rho 2B2 also show a high degree of cross-reactivity. These antibodies bind to the F_1 fragment containing the N-terminal segment of rhodopsin. Recent studies with peptide fragments of bovine rhodopsin suggest that their binding sites are close to the N-terminus. The functional role of this region of rhodopsin, however, is unknown at the present time.

In conclusion, the immunoreactivities of a series of monoclonal antibodies toward bleached and unbleached rhodopsin, proteolytic fragments of rhodopsin, and synthetic peptides have been investigated. These results have provided new insight into the accessibility of various segments of rhodopsin, in particular the C-terminal segment, conservation of specific segments of rhodopsin between species, and localization of antibody binding determinants. This information is essential for further studies directed toward using these antibodies as probes to identify and characterize the functional domains of rhodopsin. Studies are now in progress to determine the effect of C terminal specific antibody binding on light-dependent phosphorylation of rhodopsin and activation of G protein (transducin).

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Registry No. Peptide 1′–4′, 82376-78-1; peptide 1′–6′, 90579-12-7; peptide 1′–8′, 90579-13-8; peptide 1′–10′, 92900-74-8; peptide 1′–12′, 82376-76-9; peptide 1′–14′, 92900-75-9; peptide 1′–18′, 90579-11-6; peptide 2′–13′, 92900-76-0; peptide 3′–18′, 90579-14-9; peptide 3′–18′ ($Glu^{8'} \rightarrow Gln^{8'}$), 90579-15-0.

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