Lectin Binding and Enzymatic Deglycosylation of the cGMP-gated Channel from Bovine Rod Photoreceptors*

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In order to investigate the lectin-binding properties of the photoreceptor cGMP-gated channel, solubilized and purified channel protein was incubated with immobilized lectins followed by reconstitution of unbound proteins. Of the lectins tested, only concanavalin A (ConA) was able to specifically sediment channel activity. A 240-kDa protein, which copurifies with the 63-kDa channel protein but does not bind ConA, was also found to be sedimented by the ConA-affinity matrix, thereby implicating that it is associated with the channel complex. Treatment of the purified channel protein with the enzyme glycopeptidase F in the presence of the denaturing detergent sodium dodecyl sulfate resulted in a rapid reduction of the apparent molecular mass by 1.90 kDa, and the abolition of ConA-binding. No intermediate molecular weight species were observed, suggesting that the channel protein is N-glycosylated at one site only. Under non-denaturing conditions, the kinetics of deglycosylation were distinctly two-phased: 50-60% deglycosylation was achieved rapidly; however, prolonged incubation was required to arrive at complete deglycosylation. Reconstitution experiments showed that deglycosylation had no significant effect on the kinetics of channel protein activation by cGMP.

Illumination results in the hyperpolarization of vertebrate photoreceptors by indirectly closing the cation-specific channels present in the plasma membrane of the photoreceptor outer segment (1). It is now known that cGMP keeps these "light-dependent" channels open in darkness by directly and cooperatively binding to a membrane receptor, probably the channel protein itself (2, 3). Light presumably initiates channel closure by reducing the cytosolic concentration of cGMP through the activation of a specific phosphodiesterase via a well characterized enzymatic cascade (4, 5).

In previous studies (6–8), we have succeeded in purifying and functionally reconstituting the cGMP-gated channel protein from bovine rod outer segment membranes. The evidence supporting our report (7) that a 63-kDa protein constitutes the cGMP-gated channel includes (i) channel density calculations after reconstitution (7), (ii) incorporation of the purified channel protein into planar lipid bilayers and demonstration of its ability to mediate cGMP-gated conductances identical to those of the channel protein in situ (8), (iii) direct photoaffinity labelling studies using radioactive cGMP which have shown specific labelling of proteins with molecular masses of 58 kDa (9) and 66 kDa (10), respectively similar to our reported value (7), and (iv) specific immunoprecipitation of channel activity by a monoclonal antibody directed against the 63-kDa protein (11). Current investigations are now being directed at characterizing the physicochemical properties of the channel protein and defining its topological organization in the rod outer segment plasma membrane.

Of the vertebrate membrane channel proteins so far purified, all have been demonstrated to be glycoproteins. In some cases, such as the voltage-dependent sodium channel (12–14) and calcium channel (15), carbohydrate has been reported to contribute about 30% of the total molecular weight of the polypeptide. The role of glycosylation in channel function is unknown and remains a subject of active investigation. In this report, we have undertaken the characterization of the glycoprotein properties of the vertebrate rod photoreceptor cGMP-gated channel and investigated the importance of glycosylation for channel function.

EXPERIMENTAL PROCEDURES

Materials—ConA—alkaline phosphatase conjugate was supplied by Medac (Hamburg, Federal Republic of Germany) and glycopeptidase F (peptide-N-glycosidase F) by Boehringer Mannheim (F.R.G.). Electrophoresis reagents and CHAPS were from Serva (Heidelberg, F.R.G.). Electrophoresis molecular mass standards were myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase b (97 kDa), bovine serum albumin (66 kDa), and ovalbumin (45 kDa) and were from Bio-Rad (München, F.R.G.). All other reagents and chemicals were of analytical grade or better.

Purification and Reconstitution of the cGMP-gated Channel from Bovine ROS—Channel protein purification from stripped ROS membranes was achieved by anion-exchange chromatography on DEAE-Fractogel TSK followed by affinity chromatography on AF Red-Fractogel TSK as described previously (7, 16). All chromatography procedures were carried out at 4 °C in the presence of the following protease inhibitors: 0.1 mM disopropyl fluorophosphate, aprotinin 2 mg liter−1, and leupeptin 2 mg liter−1.

Reconstitution of the purified channel protein into calcium-containing asolectin liposomes was performed using a dialysis procedure (6). Briefly, a concentrated phospholipid suspension was added to channel protein extracts to give a final phospholipid concentration of 10 mg ml−1 and a CHAPS concentration of 10 mM. The suspension was dialyzed for 36 h against 5 changes of buffer containing 2 mM CaCl2, followed by 12 h of dialysis against calcium-free buffer. After

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1 The abbreviations used are: ConA, concanavalin A; ROS, rod outer segment; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonate; Heps, N-2-hydroxyethylpipеразин-N’-2-ethanesulfonic acid; SDS, sodium dodecyl sulfate; WGA, wheat germ agglutinin.

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the final dialysis step, the liposomes contained 10 mM HEPES-KOH, pH 7.4, 0.1 M KCl, and 0.1 mM dithiothreitol (all concentrations inside and outside), and 2 mM CaCl2 (inside only). The cGMP-activated efflux of calcium from liposomes was monitored spectrophoto metrically on an Aminco DW-2000 operating in the dual wavelength mode (650–730 nm) using the calcium-sensitive dye arsenazo III at a concentration of 50 μM as has been described previously (6, 7, 17).

Lectin Binding and Enzymatic Deglycosylation—Lectin binding of the channel protein was investigated by incubating purified channel extracts with lectin affinity matrices in 10 mM HEPES-KOH, pH 7.4, 0.15 M KCl, 1 mM dithiothreitol, 10 mM CaCl2, 0.8% (w/v) CHAPS, 0.16% (w/v) asolectin, and protease inhibitors at 4 °C with rapid shaking. After 1 h, incubations were centrifuged in a bench-top centrifuge and nonsedimented material was analyzed by electrophoresis and functional reconstitution.

The binding of ConA to electrotransferred channel protein was studied using ConA-alkaline phosphatase conjugate. Electroblots were blocked with 1% bovine serum albumin and then incubated with lectin conjugate in 10 mM Tris-HCl, pH 8.0, 0.15 M KCl, 1 mM dithiothreitol, 10 mM CaCl2, 0.8% (w/v) CHAPS, and 0.16% (w/v) asolectin, and protease inhibitors and, where appropriate, additives such as SDS. Incubations were terminated by addition of SDS electrophoresis sample buffer followed by freezing at −70 °C. The degree of deglycosylation was determined by estimating the abundance of the low molecular weight channel protein species relative to that of the unmodified protein using SDS-polyacrylamide gel electrophoresis and densitometry.

Electrophoresis and Electrotransfer—SDS electrophoresis was carried out using the method of Laemmli (18) on a "Mighty Small" apparatus from Hoefer. Samples were denatured in 0.05 M Tris-HCl, pH 8.9, 150 mM sucrose, 3% SDS, and 1% β-mercaptoethanol (final concentrations) before electrophoresis. The stacking and separation gels had acrylamide concentrations of 4 and 9%, respectively, with an acrylamide: Bisacrylamide ratio of 30:0.6 (w/w). Gels were stained with Coomassie Blue R-250 after electrophoresis. For the evaluation of the kinetics of channel protein deglycosylation, gels were rinsed in water and scanned using a Hoefer GS-300 scanning densitometer. Scans were integrated and quantified on an IBM personal computer using the GS-800 data system (Hoefer). Electrophoresed protein was checked by staining with Aurodye (Janssen).

RESULTS

Lectin-binding Properties of the cGMP-gated Channel—We incubated the purified channel protein with various affinity matrices in order to determine its lectin-binding properties. If the channel protein binds to a particular lectin, it should become immobilized during incubation, and reconstitution of the nonprecipitated material after centrifugation should show no channel activity. As can be seen from Fig. 1, of the lectins tested only ConA was effective in binding and sedimenting the channel protein. WGA also appeared to exhibit a slight binding capacity; however, we were unable to demonstrate a significant specific WGA binding after polyacrylamide electrophoresis and electrotransfer (see below for ConA), even at high channel protein concentrations. We investigated the binding of Ricin communis agglutinin lectin to the cGMP-gated channel since it has been previously reported (19, 20) that bovine ROS membranes contain plasma membrane-specific receptors for this lectin. Our results, however, indicate that the cGMP-gated channel is not one of them. Lens culinaris and Pisum sativum lectin matrices were also found to be incapable of binding channel protein (results not shown).

The binding of ConA to the channel protein was confirmed by electrotransfer followed by incubation with ConA-alkaline phosphatase. As can be seen in Fig. 2 (lane c), a 63-kDa polypeptide is the only protein in the purified extract capable of binding concanavalin A. Given that ConA-Sepharose 4B
specifically binds the channel protein (Fig. 1), this result clearly provides further support for our previous report (7) that the 63-kDa protein does indeed constitute the channel polypeptide.

We further analyzed the results of the lectin affinity precipitation experiments by submitting the material which did not sediment after lectin treatment to SDS electrophoresis (Fig. 2, lanes e–h). Interestingly, we found that a 240-kDa component which copurifies in variable amounts with the channel protein and does not appear to be necessary for its activity (7) can be sedimented by ConA-Sepharose 4B even though it does not bind this lectin. We take this to indicate that this polypeptide is intimately associated with the only ConA-binding protein present in the extract, the 63-kDa cGMP-gated channel. The identity of this large molecular weight protein is unknown; however, it may be spectrin, a cytoskeletal protein known to exist in bovine rod outer segment (ROS) (34); a-methylglucoside gave similar results to the lectin-precipitated protein was unable to render it soluble. This eliminates the possibility of using ConA affinity chromatography as a step in protocols directed at the purification of the channel protein.

Deglycosylation of the cGMP-gated Channel with Glycopeptidase F—To investigate further the nature and degree of deglycosylation of the channel protein, we exposed the purified protein to glycopeptidase F (21–23), an enzyme which cleaves between the asparagine residue and the first N-acetylglucosamine of N-glycans. As can be seen in Fig. 3 (lanes a and b), treatment with glycopeptidase F resulted in the formation of a lower molecular weight channel species. Electrophoresis of glycosylated and deglycosylated extracts at seven different acrylamide concentrations between 7 and 14% revealed that the mean loss of molecular weight due to deglycosylation was 1.90 (S.D. 0.21). In Fig. 3, lanes c and d, it can be seen that removal of this 1.90-kDa fragment also results in the abolition of ConA binding capacity. This confirms that the changes in molecular weight is due to the removal of carbohydrate residues and not, for example, due to protease activity. The absence of intermediate molecular weight species during deglycosylation and the subsequent loss of lectin binding capacity suggests that the channel protein is N-glycosylated at one site only.

We further examined the conditions of deglycosylation by glycopeptidase F in order to gain a greater understanding of the accessibility of the ConA-binding glycan to enzymatic removal. In Fig. 4, the deglycosylation of the channel protein at different concentrations of the denaturing detergent sodium dodecyl sulfate is shown. The rate of enzymatic deglycosylation was found to increase dramatically with increasing SDS concentrations. This observation has also been made for the deglycosylation of proteins by another endoglycosidase, endoglycosidase H (24), and presumably reflects increased accessibility of the glycan to the enzyme upon detergent denaturation of the channel protein.

Of particular interest were the kinetics of channel protein deglycosylation under nondenaturing conditions (i.e., without SDS). As can be seen in Fig. 5, the activity of glycopeptidase F on the channel protein showed two clearly distinct phases: deglycosylation of about 50–60% of the channel protein was achieved extremely rapidly; however, prolonged incubation was required to arrive at complete deglycosylation. The fact that this phenomenon was observed at different enzyme concentrations suggests that inhibition of the enzyme by its products (e.g., released glycan) is not responsible. The most probable explanation is that the channel complex exists as an oligomer of the 63-kDa subunit and is arranged in such a way that half of the glycan residues are more exposed than the other half (see the “Discussion” for a more detailed consideration of this point). The addition of saturating concentrations of cGMP to the incubation medium was not found to influence the kinetics of deglycosylation in any way.

The Effects of Deglycosylation on Channel Protein Activity—The importance of glycosylation for channel protein function
FIG. 4. SDS increases the rate of channel protein deglycosylation by glycopeptidase F. Purified channel extract (12.5 µg of 63-kDa protein ml⁻¹) was incubated with glycopeptidase F (2 units ml⁻¹) in the presence of different concentrations of SDS. At different time points, aliquots were removed from the incubation and frozen at -70°C until analysis by SDS electrophoresis. At each SDS concentration the arrows indicate the relative electrophoretic mobilities of the glycosylated (upper arrow) and deglycosylated (lower arrow) channel forms.

% SDS
- 0.1 0.3 1.0
0 5 10 15 30 45 60 90

FIG. 5. Enzymatic deglycosylation of the cGMP-gated channel is biphasic. Purified channel extract (4.8 µg of 63-kDa protein ml⁻¹) was incubated at 21°C with glycopeptidase F at the following enzyme concentrations: 1.5 ( ● ), 3.0 ( ● ), 4.5 ( ● ), and 6.0 ( ● ) units ml⁻¹. During incubation, aliquots were removed and analyzed by SDS electrophoresis and densitometry to determine the extent of deglycosylation.

Incubation time (h)
0 1.0 2.0

Incubation time (mins)

FIG. 6. Temperature dependence of cGMP-gated channel protein deglycosylation by glycopeptidase F. Purified channel protein extract (5 µg of 63-kDa protein ml⁻¹) was incubated with glycopeptidase F (2.5 units ml⁻¹) at the following temperatures: 4°C ( ● ); 21°C ( ● ); 37°C ( ● ). At different time points aliquots were removed and analyzed by electrophoresis and densitometry to determine the extent of deglycosylation.

Incubation time (h)
0 1.0 2.0

has been a subject of intensive investigation. In this study we were interested in finding conditions of deglycosylation which would not result in denaturation of the channel protein. For this reason we investigated the temperature dependence of deglycosylation of the cGMP-gated channel by glycopeptidase F since it is only at low temperatures that the channel protein remains stable in its solubilized form (t₁/₂ 20 min at 37°C). As can be seen from Fig. 6, significant deglycosylation can also be achieved at temperatures as low as 4°C. Deglycosylation was found to proceed most rapidly at higher temperatures, probably not just because of greater enzyme activity but also because of thermal denaturation (resulting in greater glycan accessibility) of the channel protein.

The observation that glycopeptidase is significantly active at low temperature permitted the investigation of the effects of deglycosylation on cGMP-gated channel activity. Fig. 7 shows efflux signals from liposomes reconstituted with channel protein incubated for one hour at 4°C with or without glycopeptidase F (in this experiment, glycopeptidase F treatment resulted in 78% deglycosylation of the channel protein). In both cases an identical loss of activity of about 20% was observed (relative to a control sample which was directly reconstituted without incubation) which was concluded to be due to the inherent instability of the channel protein in detergent solution. From this experiment it is evident that deglycosylation did not reduce the number of functional channels (relative to the control incubation) i.e. glycopeptidase treatment does not result in any significant loss of channel activity, as assessed by reconstitution.

In Fig. 8, a typical experiment on the kinetics of cGMP activation of the channel protein in its glycosylated and deglycosylated forms is shown. We investigated activation kinetics of several preparations of channel protein deglycosylated in the range 70–85% with similar results. We were unable to investigate completely deglycosylated preparations since this required extensive incubation times which resulted in significant denaturation, even at 4°C. The separation of deglycosylated from glycosylated channel forms by ConA chromatography was also found to be unsatisfactory: even when high degrees (about 80%) of deglycosylation was achieved, the amount of channel-protein complex which did not bind to the ConA-Sepharose 4B matrix was insufficient to permit kinetic studies. As shown in Fig. 8, the kinetics of channel activation by cGMP are not affected by deglycosylation: the Kₐ value (27 µM) and cooperativity (n = 3.0 from Hill plots) were...
efflux rates reported for these parameters in the literature (15, 25). For 2 h at sylated channel protein. liposomes, 10 mg of phospholipid ml⁻¹. The kinetics of activation were determined by recording of calcium efflux rates after injection of calcium-sensitive dye arsenazo III. Purified channel extract (4 µg of channel protein ml⁻¹) was incubated for 1 h at 4 °C with (GPase t = 1.0h, yielding 78% deglycosylation) or without (Control t = 0h) glycopeptidase F (3.0 units ml⁻¹) followed by functional reconstitution (2 µg of channel protein and 10 µg of phospholipid ml⁻¹ liposomes). For comparison, channel protein directly reconstituted without incubation (Control t = 0) is shown. For flux measurements, 0.4 ml of liposomes was present in the cuvette and 150 µM cGMP was injected at the time point indicated by the arrow.

unchanged. These results lie within the range of values reported for these parameters in the literature (15, 25).

**Discussion**

The Nature of cGMP-gated Channel Protein Glycosylation—Our results are consistent with the notion that the cGMP-gated channel 63-kDa polypeptide is N-glycosylated at one site only. The evidence for this is that treatment with glycopeptidase F results in a reduction of molecular mass by 1.90 kDa without the formation of intermediate species. Furthermore, treatment with this enzyme also results in the abolition of the channel protein’s lectin binding capacity. We also attempted to chemically deglycosylate the channel protein using trifluoromethanesulfonic acid (26); however, this gave inconclusive results due to aggregation and denaturation of the channel protein. Alkaline treatment, reported to result in the removal of both N- and O-glycosylated residues (27) did not yield an even lower molecular weight species.

The magnitude of the molecular weight change after enzymatic deglycosylation suggests that the channel protein possesses an N-glycan of the high mannose type (28). The quasi-irreversible binding to ConA provides further evidence for this conclusion and would make the presence of a hybrid-type high mannose glycan unlikely (29). A minimal binding capacity for WGA may suggest the presence of N-acetylglucosamine or sialic acid (given the glycan size, polysialic acid is most unlikely) on a small fraction of channel proteins. The failure of the channel protein to bind to L. culinaris and P. sativum lectins would further suggest that the glycan is not fucosylated at the asparagine-linked N-acetylglucosamine (32). We attempted to characterize further the carbohydrate content of the channel protein using gas-liquid chromatography; however, the limited amount of material available and the low degree of glycosylation meant that the detection limit of the method was insufficient.

During incubation of the channel protein with glycopeptidase F under non-denaturing conditions we found that deglycosylation occurred in two distinctly different phases. 50–60% deglycosylation was achieved rapidly; however, prolonged incubation was necessary to achieve complete deglycosylation. This would appear to suggest that there exists two approximately equally abundant species of glycan differing in their accessibility to enzymatic cleavage. Two different species with differing sugar contents and subsequently different rates of deglycosylation would also explain this result; however, this would seem unlikely since denaturation with SDS (which would result in monomerization of the 63-kDa polypeptide) was found to result in rapid, single-phased deglycosylation by glycopeptidase F.

If indeed the biphasic deglycosylation is due to differing accessibility, then the cGMP-gated channel must (i) exist as a monomer with at least two different conformations, or (ii) exist as an oligomer of the 63-kDa protein where the subunits are arranged so that one-half is easily accessible to deglycosylation and the other half is not. If the latter were indeed the case, then the channel complex would contain either a dimer or tetramer (or greater) of the 63-kDa subunit; a trimer is unlikely since the transition point between the two phases occurs when about half (and not one-third or two-thirds) of the 63-kDa proteins is deglycosylated. A pentamer, where two-thirds of the glycans are accessible, would also be reasonably compatible with our results. On the basis of kinetic evidence, we have suggested elsewhere (16, 25) that the channel complex may be constituted by at least a tetramer of the 63-kDa subunit.

A 240-kDa Component Is Associated with the Channel Complex—In this study we were able to show that a 240-kDa protein, unable to bind ConA, could be quantitatively removed from solubilized extracts (in which the 63-kDa channel protein was the only ConA-binding protein present) by ConA-Sepharose 4B. We concluded that this high molecular weight component is directly associated with the cGMP-gated channel protein. In previous reports (7, 11, 16) we have noted that the 63-kDa channel protein consistently copurifies with a 240-kDa protein, although it is present in highly variable amounts and sometimes even absent. The identity of this
protein is as yet uncertain; however, it may be spectrin, a cytoskeletal protein, recently shown to exist in bovine rod outer segments (30). The fact that the 240-kDa protein does not bind ConA has an identical molecular mass, and shows a great variability in yield (which may be indicative of the high sensitivity to proteolysis known to be a property of spectrin) supports this suggestion. Indeed, spectrin has recently been shown to associate with another ion channel, the voltage-dependent sodium channel (31). The identification and further characterization of this 240-kDa protein will be one of the most important tasks for the further characterization of the cGMP-gated channel.

The Importance of Glycosylation for Channel Function—In this paper we have reported conditions for deglycosylation of the cGMP-gated channel under nondenaturing conditions. This allowed us to demonstrate that the properties of the enzymatically deglycosylated protein with regard to its activation by cGMP are identical to those of the untreated protein. This is not completely surprising however, since in contrast to all other known receptor-gated ion channels the cGMP-gated channel has the binding site for its activating ligand (cGMP) on the intracellular side of the plasma membrane. Since membrane proteins are generally assumed to be glycosylated extracellularly, this would mean that the glycan is located on the opposite side of the membrane from (and therefore unlikely to influence) the cGMP-binding sites. The presumed neutrality of the sugars composing the cGMP-gated channel glycan would also make it unlikely that they influence channel gating, as has been shown for the heavily sialated voltage-dependent sodium channel (32). Therefore, from the work presented here, we conclude that glycosylation is not of functional importance for the rod photoreceptor cGMP-gated channel although it may be important for the assembly or processing of the channel protein. The apparent uselessness of glycosylation for channel function may mean that the cGMP-gated channel could eventually be cloned and expressed in non-eukaryotic systems.

REFERENCES