Activation of Retinal Guanylyl Cyclase-1 by Ca\(^{2+}\)-binding Proteins Involves Its Dimerization*

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Retinal guanylyl cyclase-1 (retGC-1), a key enzyme in phototransduction, is activated by guanylyl cyclase-activating proteins (GCAPs) if [Ca\(^{2+}\)] is less than 300 nM. The activation is believed to be essential for the recovery of photoreceptors to the dark state; however, the molecular mechanism of the activation is unknown. Here, we report that dimerization of retGC-1 is involved in its activation by GCAPs. The GC activity and the formation of a 210-kDa cross-linked product of retGC-1 were monitored in bovine rod outer segment homogenates, GCAPs-free bovine rod outer segment membranes and recombinant bovine retGC-1 expressed in COS-7 cells. In addition to recombinant bovine GCAPs, constitutively active mutants of GCAPs that activate retGC-1 in a [Ca\(^{2+}\)]-independent manner and bovine brain S100b that activates retGC-1 in the presence of 10 \(\mu\)M [Ca\(^{2+}\)] were used to investigate whether these activations take place through a similar mechanism, and whether [Ca\(^{2+}\)] is directly involved in the dimerization. We found that a monomeric form of retGC-1 (~110 kDa) was mainly observed whenever GC activity was at basal or low levels. However, the 210-kDa product was increased whenever the GC activity was stimulated by any Ca\(^{2+}\)-binding proteins used. We also found that [Ca\(^{2+}\)] did not directly regulate the formation of the 210-kDa product. The 210-kDa product was detected in a purified GC preparation and did not contain GCAPs even when the formation of the 210-kDa product was stimulated by GCAPs. These data strongly suggest that the 210-kDa cross-linked product is a homodimer of retGC-1. We conclude that inactive retGC-1 is predominantly a monomeric form, and that dimerization of retGC-1 may be an essential step for its activation by active forms of GCAPs.

In outer segments of vertebrate retinal photoreceptors, rhodopsin absorbs a photon which in turn triggers GTP-dependent activation of cGMP phosphodiesterase. The activated phosphodiesterase hydrolyzes cGMP. The resulting decrease in cytoplasmic [cGMP] leads to reduction in the activity of cGMP-gated cation channels and hyperpolarization of plasma membranes (1–4). Restoration of the dark membrane potential requires recovery of the dark level of cytoplasmic [cGMP]. Therefore, GC-1, the enzyme that converts GTP to cGMP, has a crucial role in visual transduction. A retinal membrane GC has been purified from frog, toad, and bovine photoreceptor outer segments (5, 6) and identified as a ~110 kDa protein. Isozymes of the GC have also been shown biochemically (5, 7). Subsequently two forms of membrane GC (retGC-1, ROS-GC1 or GC-E, and retGC-2, ROS-GC2 or GC-F) were cloned from human, bovine, and rat retinal cDNA libraries (8–12). The structure of these retGCs indicates that the enzyme is a member of the peptide-regulated, membrane-bound GC family, although the retGC is not activated by known peptides. Four functional domains in retGCs have been predicted: a N-terminal extracellular domain, a transmembrane domain, an intracellular protein-kinase-like domain, and a C-terminal catalytic domain. Immunocytochemistry has shown that retGC-1 is localized primarily in cone outer segments and to a lesser extent in rod outer segments (13–15). RetGC-1 is also detected in the plexiform layers of retina (13, 15), leading to speculation that the enzyme is not unique in photoreceptor outer segments. RetGC-2 is in photoreceptors (10); however, detailed localization of the enzyme in the retina has not been demonstrated. RetGC-1 appears to be more abundant than retGC-2 in the retina (10), and only retGC-1 may contribute to the pool of cGMP essential to support phototransduction in cone photoreceptors (16).

The reduction of cGMP-gated channel activity by lowering cytoplasmic [cGMP] blocks Na\(^{+}\) and Ca\(^{2+}\) influx, and allows a Na\(^{+}\)/Ca\(^{2+}\), K\(^{+}\) exchanger to decrease cytoplasmic [Ca\(^{2+}\)] (4, 17). When [Ca\(^{2+}\)] is low, retGC is stimulated (18). In contrast to other membrane-bound GCs that are regulated by binding of peptides to their extracellular domain (19, 20), this Ca\(^{2+}\)-sensitive stimulation of retGC is mediated by at least two calmodulin-like Ca\(^{2+}\)-binding proteins termed GCAPs, 1 and 2 (14, 21–23). GCAPs interact with the intracellular domain of the enzyme (24, 25). When free [Ca\(^{2+}\)] is less than ~300 nM, retGC-1 is activated by GCAPs. RetGC-2 has also been reported to be stimulated by GCAP-2 under similar [Ca\(^{2+}\)] (10, 12, 26). In addition, GCAPs appear to inhibit the basal GC activity in the presence of the higher [Ca\(^{2+}\)] (more than ~500 nM) (27, 28). Thus, the regulatory mechanism of retGC is completely different from that of peptide-regulated GCs. GCAP-1 is

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1 The abbreviations used are: GC, guanylyl cyclase; retGC, retinal guanylyl cyclase; GCAP, guanylyl cyclase-activating protein; ROS, rod outer segments; GCAP-1m, a constitutively active mutant of GCAP-1; Y99C; GCAP-2m, a constitutively active mutant of GCAP-2, E80Q/E160Q/D158N; BS\(^{3}\), bis(sulfosuccinimidyl) suberate; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride.

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mainly detected in cone outer segments, in particular, in disc membrane regions (29–31). GCAP-1 is also observed in rod outer segments, but the content is much lower than that in cone outer segments. Less GCAP-1 is also found in synaptic regions and inner segments of cones. GCAP-2 is predominantly observed in outer and inner segments of rods and cones (23, 29–31). Synaptic regions are also labeled by a GCAP-2 antibody. It has also been reported that Ca<sup>2+/-</sup>-binding proteins of the S100 family, especially S100b, activate retGC-1 in the presence of high [Ca<sup>2+/-</sup>] (32, 33). Half-maximal activation was observed at about 40–50 μM [Ca<sup>2+/-</sup>] (33). Therefore, it is believed that S100 proteins are not involved in phototransduction. Additional mechanisms and factors may also be involved in the regulation of retGCs, including phosphorylation (34, 35), ATP binding (36–38), actin binding (39), an inhibitor (guanyln cyclase-inhibitory protein) of GCAP-activated retGC in amphibian retina (40), and inhibition of retGC-1 by RGS9 (41).

Characterizations of membrane-bound GC and adenylyl cyclase have suggested that the mechanisms for the expression of these enzymatic activities are closely related (42, 43), and that at least two cyclase catalytic consensus domains may be required for the activity of these cyclases. Membrane adenylyl cyclase contains two putative catalytic domains that when separately expressed have no activity (44). Peptide-regulated GCs have also been proposed to exist as dimeric or oligomeric forms even in the absence of ligands (45–47). A recent study has also suggested that retGCs form homodimers in photoreceptor outer segments (48). However, it remains unknown whether dimerization or oligomerization of retGCs is related to the regulation of retGC activity. This question is important especially because the activity of retGCs is regulated so differently from peptide-regulated GCs and the regulatory mechanism of retGCs by GCAPs may not be identical to that of peptide-regulated GCs. Thus, we investigated the relationship between activation of retGC-1 by GCAPs and dimerization of the enzyme. In addition to GCAPs, we used constitutively active mutants of GCAPs and S100b to show that dimerization of retGC-1 is related to its activation, not to Ca<sup>2+/-</sup>-binding proteins or [Ca<sup>2+/-</sup>]. Dimerization of retGC-1 was monitored using a cross-linker under the conditions similar to those used for the measurement of the GC activity. Our results suggest that GCAPs activate retGC-1 by enhancing its dimerization, and that transition of the active form of retGC-1 to its inactive form is caused by either partial or complete dissociation of the dimeric form.

**EXPERIMENTAL PROCEDURES**

**Materials**—Dark-adapted frozen bovine retinas were purchased from Dr. Yee-Kin Ho (University of Illinois, Department of Biochemistry, Chicago). Other materials were purchased from the following sources: Sephacryl S-200 HR from Pharmacia Biotech Inc.; [α-32P]GTP and [3H]GMP from NEN Life Science Products Inc.; GMP and GTP from Roche Molecular Biochemicals; AG 1-X2 resin from Bio-Rad; alumina N-Super I from ICN; creatine phosphokinase, phosphocreatine, phenylmethylsulfonyl fluoride, leupeptin, pepstatin A, 1-methyl-3-isobutylxanthine, n-dodecyl-β-D-maltoside, S100b, GTP-agarose resin, and high molecular weight markers for SDS-PAGE from Sigma; Ultra Super Signal substrate, PVDF membranes, BS3, 3,3′-dithiobis(sulfonecyanidyl) propionate), and disuccinimidyl suberate from Pierce. Antibodies against retGC-1 (13), GCAPs (23, 31), and RGS9 (41) were prepared as described.

**Preparation of retGC and GCAPs**—Bovine ROS was prepared from dark-adapted frozen retinas as described (49). Bleached ROS membranes from 20 retinas were suspended in 3 ml of Buffer A (10 mM HEPES (pH 7.5), 5 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 5 μM leupeptin, 5 μM pepstatin A, and 100 μM CaCl<sub>2</sub>), homogenized by passing through a No. 21 needle 10 times and centrifuged (200,000 × g, 4 °C, 15 min) (x 7). The membranes were further washed (3 times) with Buffer B (10 mM HEPES (pH 7.5), 5 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 5 μM leupeptin, and 5 μM pepstatin A), suspended in 3 ml of Buffer B, frozen with liquid nitrogen, and stored at −80 °C. The washed membranes are termed GCAPs-free ROS membranes. Purified retGC from bovine ROS was prepared using a GTP-agarose column (5). The purity of the preparation was greater than 95%. The purified retGC was stored in liquid nitrogen until used (up to months). Preparation of recombinant bovine retGC-1 expressed in E. coli (28) and constitutively active mutants of GCAP-1 (Y99C) (28) and GCAP-2 (E80Q/E160Q/D158N) (27) has been described.

**GC Activity Assay**—GC activity was measured as described (5). Our preliminary studies indicated that 5 μg of a protein of a ROS homogenate had high GC activity with negligible hydrolysis of cGMP under assay conditions. Thus, 5 μg of protein of ROS homogenate or GCAPs-free ROS membranes was used for all studies. Bovine ROS membranes (5 μg of protein) were incubated with 200 μl of Buffer C (50 mM HEPES (pH 7.5), 1 mM GTP, 1 mM cGMP, 2 mM 1-methyl-3-isobutylxanthine, 5 mM MgCl<sub>2</sub>, 15 mM phosphocreatine, 50 μg/ml creatine phosphokinase, ~5 μCi of [α-32P]GTP, and ~0.5 μCi of [3H]GMP). The reaction was initiated by addition of GTP and cGMP. Following incubation (37 °C for 10 min), the reaction was terminated by adding 40 μl of 1 N HCl and treated for 2 min. [32P]GTP and [3H]GMP were immediately separated by SDS-PAGE (5–20% acrylamide gradient). After electrophoresis, proteins were blotted to PVDF membranes and cross-linked products of retGC-1 were detected with a retGC-1-specific antibody (13) and chemiluminescent autoradiography using ULTRA Super Signal substrate. The bands of cross-linked products were scanned by Paragon 1200A3 Pro Scanner and the relative density (mm<sup>2</sup> OD) was calculated by Molecular Analyst Software (Bio-Rad). It should be emphasized that the molecular mass of the 210-kDa cross-linked product, but 1 mM cGMP did not. The cross-linking reaction was terminated by addition of SDS-sample buffer and boiling for 5 min. The cross-linked products were immediately separated by SDS-PAGE (5–20% acrylamide gradient). After elec...
Detection of a retGC-1 Dimer with Molecular Mass \( \approx 210 \) kDa—Dimerization or oligomerization of peptide-regulated GCs have been monitored using several methods including cross-linking (45), measurement of molecular weight (46), and the yeast two-hybrid system (47). In this study, to obtain the exact relationship between dimerization of retGC-1 and its activity, we attempted to fix dimeric (or oligomeric) forms of retGC-1 by cross-linking under a condition similar to these used for measurement of GC activity. One hundred % indicates the amounts of the 210-kDa product formed in the presence of 60 \( \mu \)g BS\(^3\). Purified retGC (\( \approx 0.5 \) \( \mu \)g) was also incubated with or without 50 \( \mu \)M BS\(^3\) under the same conditions. The lower inset shows the profile of cross-linked products of retGC-1 in the purified preparation. A \( \approx 110\)-kDa retGC-1 is a monomeric form. Resi

**RESULTS**

**FIG. 1.** Formation of the 210-kDa retGC-1 cross-linked product in a bovine ROS homogenate. With various [BS\(^3\)], bovine ROS homogenate (50 \( \mu \)g) was incubated (0 \( \degree \)C, 30 min) in 50 \( \mu \)l of Buffer D containing 1 mM EGTA. The cross-linking reaction was quenched by the addition of SDS sample buffer and boiling for 5 min. The cross-linked protein products were immediately separated by SDS-PAGE, transferred to a PVDF membrane, and detected by Western immunoblotting using rabbit antibodies specific to these proteins and a chemiluminescent substrate. There is no chemiluminescent band between 55 and 110 kDa. 

RetGC-1 has been shown to be regulated by proteins, such as GCAPs (14, 21–23) and RGS9 (41), in photoreceptor outer segments. It is possible that the 210-kDa cross-linked product of retGC-1 in ROS homogenates (Fig. 1) is a complex of retGC-1 with these retGC regulators. As shown in Fig. 2, the ROS homogenate contained GCAPs; however, GCAPs were not detected in the 210-kDa cross-linked product although under these conditions the formation of the 210-kDa cross-linked product was stimulated by GCAPs, as described below. In addition, the ROS homogenate also contained RGS9; however, RGS9 was not present in the 210-kDa product. These observations support our conclusion that the 210-kDa cross-linked product is a homodimer of retGC-1, but not a monomeric form of retGC-1 cross-linked with other proteins, such as GCAPs and RGS9.

**FIG. 2.** Cross-linked products of retGC-1 and its regulators in a bovine ROS homogenate. Bovine ROS homogenate (50 \( \mu \)g of protein) was incubated in 50 \( \mu \)l of Buffer D containing 1 mM EGTA in the presence or absence of 50 \( \mu \)M BS\(^3\) (0 \( \degree \)C and 30 min). The cross-linking reaction was quenched by the addition of SDS sample buffer and boiling for 5 min. The cross-linked products were separated by SDS-PAGE and transferred to PVDF membranes. The cross-linked products of retGC-1, RGS9, and GCAPs were detected by Western immunoblotting analysis using rabbit antibodies specific to these proteins and a chemiluminescent substrate. To avoid a larger figure, parts of gels are shown. There is no chemiluminescent band between 55 and 110 kDa.
argument but also to indicate that \([\text{Ca}^{2+}]\) does not directly regulate the formation of the 210-kDa product. The GC activity of these membranes was low even when \([\text{Ca}^{2+}]\) was low (Fig. 4A), indicating that functional amounts of GCAPs had been washed out from membranes. Addition of GCAP-1 increased GC activity of membranes if \([\text{Ca}^{2+}]\) was low; however, the GC activation was drastically diminished when \([\text{Ca}^{2+}]\) was increased (Fig. 4A). On the other hand, even without \([\text{Ca}^{2+}]\), the 210-kDa product in GCAPs-free membranes was reduced to ~35% of that found in the ROS homogenate (Fig. 4, B and C).

Addition of GCAP-1 recovered the 210-kDa product to ~85% of that found in the ROS homogenate if \([\text{Ca}^{2+}]\) was low; however, the recovery was not detected in the presence of more than 500 nM \([\text{Ca}^{2+}]\) (Fig. 4A). Under these conditions, the amount of the 210-kDa product decreased slightly if \([\text{Ca}^{2+}]\) was increased (Fig. 4, B and C). These observations indicate that the 210-kDa cross-linked product of retGC-1 is less when the retGC activity is basal, and...
activity. With or without 2 mM Ca²⁺ the 210-kDa product formation. We note that, when [Ca²⁺] was more than 500 nM, GCAP-1 did not inhibit the basal activity of retGC in membranes (Fig. 4A), although amounts of the 210-kDa product were reduced to ~20% of the basal level (Fig. 4, B and C). We speculate that the ability of GCAP-1 to inhibit retGC basal activity is weak. Alternatively, the final concentration of GCAP-1 in the cross-linking mixture may be higher than that in the mixture for the assay of enzymatic activity because larger amounts of membranes were used for the cross-linking reaction; that is, the residual amounts of GCAP-1 in membranes may be much higher than that for the enzyme assay. We also note that the cross-linked product(s) of retGC-1 with >400-kDa molecular mass was not detected under these conditions.

GCAP-2 and its constitutively active mutant, E80Q/E160Q/D158N, showed similar effects on the GC activity and the formation of the 210-kDa cross-linked product of retGC-1 in the GCAPs-free ROS membranes (Fig. 5). In addition, both the GC activity and the formation of the 210-kDa product were reduced to less than these basal levels if [Ca²⁺] was higher than 1 μM. These observations clearly indicate the relationship between the GC activation and the formation of the 210-kDa cross-linked product of retGC-1 in ROS membranes.

Effects of GCAPs on GC activity and formation of the 210-kDa cross-linked product of retGC-1 in the GCAPs-free ROS membranes (Fig. 5). The cross-linking reaction (30 min, 0 °C) was carried out with 50 μl of Buffer D containing various [Ca²⁺]. The cross-linking reaction of ROS homogenate (50 μg) was also performed with or without BS3. The cross-linked products were immediately isolated by SDS-PAGE, transferred to PVDF membrane, and detected by Western immunoblotting analysis using a chemiluminescent substrate using a retGC-1-specific antibody. The 210-kDa cross-linked product in membranes (Fig. 4, A) and relative amounts of the 210-kDa product (C) were scanned and the relative density was shown in C. One hundred % indicates the amounts of the 210-kDa product formed immediately without Ca²⁺ in the ROS homogenate. ROS, ROS homogenate; Ca-W-Me, GCAPs-free ROS membranes; R+, ROS homogenate without BS3; R, ROS homogenate with BS3; C, GCAPs-free ROS membranes with BS3; + GCAP-2, GCAPs-free ROS membranes and GCAP-2 in the presence of BS3; + GCAP-2m, GCAPs-free ROS membranes and GCAP-2m in the presence of BS3.

that the formation of the 210-kDa product is stimulated when the GC activity was stimulated by GCAP-1 or its mutant. Moreover, [Ca²⁺] is not directly involved in the regulation of the 210-kDa product formation. We note that, when [Ca²⁺] was more than 500 nM, GCAP-1 did not inhibit the basal activity of retGC in membranes (Fig. 4A), although amounts of the 210-kDa product were reduced to ~20% of the basal level (Fig. 4, B and C). We speculate that the ability of GCAP-1 to inhibit retGC basal activity is weak. Alternatively, the final concentration of GCAP-1 in the cross-linking mixture may be higher than that in the mixture for the assay of enzymatic activity because larger amounts of membranes were used for the cross-linking reaction; that is, the residual amounts of GCAP-1 in membranes may be much higher than that for the enzyme assay. We also note that the cross-linked product of retGC-1 with >400-kDa molecular mass was not detected under these conditions.

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was virtually unaffected by the high [Ca^{2+}]. These results indicate that active forms of GCAPs affect the retGC-1 activity in these membranes in a manner similar to that observed in photoreceptor membranes. Under these conditions, when GCAPs were added to membranes and Ca^{2+} was not present, the formation of the 210-kDa product was not observed; however, the 210-kDa product was not detected in the presence of 1.5 μM Ca^{2+} (Fig. 6A). When constitutively active mutants of GCAPs were added to membranes, the formation of the 210-kDa product was detected and the formation was not sensitive to Ca^{2+}. These results indicate the clear relationship between the activation of retGC-1 by GCAPs and the formation of the 210-kDa cross-linked product of retGC-1. Moreover, these observations demonstrate that Ca^{2+} is not directly involved in the formation of the 210-kDa product. We note that the retGC-1 complex(s) with molecular mass >400 kDa was constantly detected in these membranes even without BS3, and that formation of the complex was not related to the GC activity.

The Effect of S100b on the GC Activity and the Formation of the 210-kDa Cross-linked Product of retGC-1—The GC activity in ROS membranes is activated by the S100 family proteins in a [Ca^{2+}]-dependent manner. The half-maximal activation was observed to be ~40 μM [Ca^{2+}]. Under the same conditions, the amounts of the 210-kDa product were increased when [Ca^{2+}] was increased (Fig. 7A). We note that without S100b such high [Ca^{2+}] stimulated neither the GC activity nor the formation of the 210-kDa product (data not shown). These results indicate that the clear relationship exists between the activation of retGC by S100b and the formation of the 210-kDa product of retGC-1. Since GCAPs also stimulate the formation of the 210-kDa product in the presence of low [Ca^{2+}] (Figs. 4–6), these observations imply that the 210-kDa product is formed whenever retGC is activated by any activator, and that Ca^{2+} is only involved in the regulation of activators.

Molecular Mass of Various GC Preparations from Bovine ROS Measured by Gel Filtration—The purified retGC has been shown to behave as a very large molecular mass complex or an aggregated form (5). We confirmed that a large portion of retGC purified from ROS membranes appeared to be aggregated (Fig. 8A). This observation is also supported by the presence of substantial amounts of the oligomeric form(s) of retGC-1 with molecular mass >400-kDa in the purified GC even without a cross-linker (Fig. 1). Since the activity of purified retGC is basal (5) and the amounts of the large molecular mass complex(es) was not proportional to the retGC activity (data not shown), the large molecular mass complex should not be related to the activation of retGC. In order to estimate the formation mechanism of the large molecular mass complex, we measured the molecular mass of the retGC solubilized freshly from photoreceptor membranes. As shown in Fig. 8B, the enzyme solubilized freshly was eluted in a peak corresponding to a molecule with a Stoke’s radius of 48.9 Å and an estimated molecular mass of ~150 kDa. The size of the retGC is consistent with that of a monomeric form of retGC with detergent bound and/or that of elongated retGC. However, when the same retGC preparation was stored overnight and applied to the column, the retGC was eluted in fractions similar to that of the purified retGC (Fig. 8B). Aparicio and Applebury (54) also reported similar data. These results suggest that retGC can be present as a monomeric form in a fresh preparation, and that
the storage of retGC preparations makes the enzyme aggregated. These observations imply that the aggregated form of the purified retGC may be formed during its purification.

**DISCUSSION**

In retinal photoreceptor cells, retGC is known to be activated by GCAPs when [Ca$^{2+}$] is less than 300 nM. The activation is believed to be essential for the recovery of photoreceptors to the dark state, although the molecular mechanism of the activation is unknown. In this study, we have shown that retGC-1 exists in a monomeric form (~110 kDa) whenever GC activity is at basal or low level, and the 210-kDa cross-linked product is increased whenever the GC activity is stimulated. We have also indicated that the 210-kDa cross-linked product is a homodimer of retGC-1, and the [Ca$^{2+}$] directly regulates neither the GC activity nor formation of the 210-kDa cross-linked product. Thus, we conclude that dimerization of retGC-1 is involved in its activation by active forms of GCAPs, as summarized in Fig. 9. To reach these conclusions, we monitored the GC activity and formation of the 210-kDa cross-linked product of retGC-1 under similar conditions. Moreover, we used constitutively active mutants of GCAPs and S100b, not only to strengthen our argument but also to indicate that [Ca$^{2+}$] does not directly regulate the formation of the 210-kDa product. In addition to retGC-1 expressed in COS cells, ROS homogenates and GCAPs-free ROS membranes were used. Then, the total GC activity in these membranes was compared with the amounts of cross-linked products detected by a retGC-1-specific antibody. Although these ROS membranes contain two retGCs (retGC-1 and -2) (8–12, 48), our results show a clear relationship between retGC activity and the 210-kDa cross-linked product of retGC-1. It is possible that retGC-2 is also dimerized when activated by GCAPs because retGC-2 is similar to retGC-1 in its activation by GCAPs (8–12). We could not use retGC-2 expressed in COS cells to investigate this possibility because of low activity. Alternatively, the GC activity measured may be mainly contributed by retGC-1 because retGC-2 may be less abundant (10).

Peptide-regulated GCs have been proposed to be present as oligomeric forms even without activation by peptides (42–44). In this study, we have also shown that retGC-1 complexes with molecular mass >400 kDa was observed in several GC preparations. As summarized in Fig. 9, we believe that the high molecular mass complex(es) of retGC-1 is formed artificially from monomeric and/or dimeric forms of retGC-1 for the following reasons. (a) The detection of the high molecular mass complex was not constant in ROS membranes. For example, the complex was detected in ROS homogenates shown in Figs. 3, 5, and 7, but not in Figs. 2 and 4. In addition, these complexes were detected in the GCAP-free membranes shown in Fig. 5, but not in Fig. 4. Moreover, the presence or absence of these complexes appears not to be related to the GC activity. Thus, the high molecular mass complex may be formed during preparation of membranes. (b) We have shown that a retGC preparation becomes aggregated if stored, and that a purified retGC preparation behaves in a gel filtration column as the retGC preparation stored overnight (Fig. 8). Moreover, the purified preparation contains substantial amounts of the retGC complex with molecular mass >400 kDa (Fig. 1). These observations imply that the high molecular mass complex in the purified sample is self-aggregated by storage or purification procedures. (c) In recombinant retGC-1 expressed in COS-7 cells, the high molecular mass complex was detected without cross-linker (Fig. 6). The high molecular mass complex was also observed even when the GC activity was basal and the content of the complex was not changed even when the activity of retGC-1 was changed. The high molecular mass complex was also detected in retGC-2 expressed in COS cells (data not shown). These results suggest that the high molecular mass complex is not related to the GC activity. It is possible that the complex is a self-aggregated form and/or a form of misfolding retGC-1 during its expression.

Although it is difficult to rule out completely the possibility that the 210-kDa cross-linked product is a retGC-1 complexed with proteins other than retGC-1, we anticipate that the 210-kDa product is a dimer form of retGC-1 for the following reasons. (a) The molecular mass of the cross-linked product (~210 kDa) is similar to the calculated molecular mass (~220 kDa) of the retGC-1 dimer. (b) The 210-kDa product was detected in a preparation of purified retGC-1 (Fig. 1). This retGC preparation did not contain GCAPs and RGS9 (data not shown). Formation of the 210-kDa product in the purified retGC preparation was much less than that in ROS homogenates because the purified preparation did not contain any activator and its GC activity is low. Even if GCAPs are contaminated in the purified preparation, its GC activity should be basal because GCAPs do not function as retGC activators in the presence of a detergent (6). (c) The 210-kDa product was also detected in a preparation of recombinant retGC-1 expressed in COS-7 cells (Fig. 6). (d) The 210-kDa product is increased whenever the GC activity is stimulated (Figs. 1 and 3–7). These observations exclude the possibility that inhibitory regulators, such as guanylyl cyclase-inhibitory protein (40) and RGS9 (41), are complexed with retGC-1 to form the 210-kDa product. Using a RGS9-specific antibody (41) we have shown that RGS9 is not involved in the 210-kDa product (Fig. 2). (e) The 210-kDa product does not contain GCAPs even when the formation of the 210-kDa product was stimulated by GCAPs. Western blotting of the 210-kDa product using GCAP-specific antibodies has indicated that the 210-kDa product does not contain GCAPs under our conditions (Fig. 2). (f) The 210-kDa product in ROS homogenates was also observed by different cross-linkers, such as 3,3’-dithiodiobis(sulfosuccinimidyl propionate) and disuccinimidyl suberate, when the GC activity in the preparation was stimulated by lowering
GCAPs were not found in the ~210-kDa cross-linked product of retGC-1 when the dimerization of retGC-1 was stimulated by GCAPs (Fig. 2). However, it should be emphasized that this study does not exclude the possibility that the real retGC dimer contains Ca\textsuperscript{2+} activators if the formation of the retGC dimer is enhanced by Ca\textsuperscript{2+} activators. As depicted in Fig. 9, we rather believe that Ca\textsuperscript{2+} activators were contained in the retGC dimer before cross-linking reaction if the dimer formation was stimulated by Ca\textsuperscript{2+} activators. We believe that our conditions for the cross-linking reaction fix the interaction between retGCs, but not between retGC and GCAPs. A previous study has already shown that a high molecular mass cross-linked product contains retGC-1 and GCAP-1 (24). Previous studies have suggested that a different region of retGC-1 appears to be required for its interaction with each Ca\textsuperscript{2+} activator (24, 25, 55). Thus, it is possible that each Ca\textsuperscript{2+} activator forms a retGC-1 dimer in a different way, and that each retGC-1 dimer may be complexed with a different Ca\textsuperscript{2+} activator. A slight difference in the conformation of the retGC-1 dimers may be important for the fine regulation of free [cGMP] in photoreceptor outer segments.

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