Residues within the Polycationic Region of cGMP Phosphodiesterase γ Subunit Crucial for the Interaction with Transducin α Subunit

IDENTIFICATION BY ENDOGENOUS ADP-RIBOSYLATION AND SITE-DIRECTED MUTAGENESIS

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Interaction between the γ subunit (Pγ) of cGMP phosphodiesterase and the α subunit (Tα) of transducin is a key step for the regulation of cGMP phosphodiesterase in retinal rod outer segments. Here we have utilized a combination of specific modification by an endogenous enzyme and site-directed mutagenesis of the Pγ polycationic region to identify residues required for the interaction with Tα. Pγ, free or complexed with the αβ subunit (Pαβ) of cGMP phosphodiesterase, was specifically radiolabeled by prewashed rod membranes in the presence of [adenylate-32P]NAD. Identification of ADP-ribose in the radiolabeled Pγ and radiolabeling of arginine-replaced mutant forms of Pγ indicate that both arginine 33 and arginine 36 are similarly ADP-ribosylated by endogenous ADP-ribosyltransferase, but only one arginine is modified at a time. Pγ complexed with Tα (both GTP- and GDP-bound forms) was not AD-ribosylated; however, agmatine, which cannot interact with Tα, was AD-ribosylated in the presence of Tα, suggesting that a Pγ domain containing these arginines is masked by Tα. A Pγ mutant (R33,36K), as well as wild type Pγ, inhibited both GTP hydrolysis of Tα and GTP binding to Tα. Moreover, GTP-bound Tα activated Pαβ that had been inhibited by R33,36K. However, another Pγ mutant (R33,36L) could not inhibit these Tα functions. In addition, GTP-bound Tα could not activate Pαβ inhibited by R33,36L. These results indicate that a Pγ domain containing these arginines is required for its interaction with Tα, but not with Pαβ, and that positive charges in these arginines are crucial for the interaction.

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1 The abbreviations used are: PDE, cGMP phosphodiesterase; Pαβ, catalytic subunits of PDE; Pγ, γ subunit of PDE; ROS, rod outer segments; Tα and Tγ, subunits of transducin, retinal G-protein; GDPβS, guanyl-5′-yl thiophosphate; GTPγS, guanosine 5′-O-(3-thiotriphosphate); Gpp(NH)p, guanyl-5′-yl β,γ-imidotriphosphate; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol.
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### TABLE I

| Mutants        | Oligonucleotides |
|----------------|------------------|
| R11K           | A: 5'-GCGCGATTCTTTTGCAGCG-3' |
| R15K           | B: 5'-GCGCGAAATGATTGAGGGG-3' |
| R24E           | C: 5'-CCGTCACTCCGCCCAAAAGGCCC-3' |
| R24K           | D: 5'-CCGTACCTCCCAAAAGGCCCC-3' |
| R33K           | E: 5'-CGAATATTCGAAACACACGAGGG-3' |
| R36K           | F: 5'-CGGAAAGAAACAGCGTGAGGG-3' |
| R11,15K        | G: 5'-GGGCGCCACCGAATGATTGAGGGG-3' |
| R33,36L        | H: 5'-ATTGGAATGCTGACTGACTGCTGGAA-3' |
| R33,36K        | I: 5'-TTAAGCAAGAACACACAGCAGCGTCAGAG-3' |
| R15,24         | Oligo B: + oligo D |
| R11,15,23,36K  | Oligo G: + oligo I |
| R11,15,24,33,36K | Oligo D: + oligo G + oligo I |

* Underlined letters indicate mutation sites.

boxy-terminal region of Py corresponding to residue 46–87 as the sites for the interaction (a) (19–22). Mutational analysis of Py has also shown that the carboxy-terminal residues and several positive charged residues in the polycationic region are also involved in the inhibition of cGMP hydrolysis (23, 24). Without impairing interaction with Poβ, a frameshift mutation of Py has also revealed that the carboxy-terminal residues are involved in the cGMP hydrolysis inhibition (15). In addition, the polycationic region and a site near the carboxy terminus in Py have been suggested as sites required for the interaction (c) (19, 23, 25–27). However, little is known about Py domains involved in the interactions (b) and (d). The frameshif mutation of Py has suggested that the amino-terminal residues are involved in the stimulation of cGMP binding to noncatalytic sites on Poβ (15). However, a To interaction site on Py, which is required for the Py release to reduce the affinity of Poβ noncatalytic sites to cGMP, has not been identified.

In this study we have focused on identification of specific residues in the Py polycationic region for following reasons. (i) The polycationic region has been suggested to be involved in the interaction with both Poβ and GTP-To for the regulation of cGMP hydrolysis. Identification of amino acid residues in the polycationic region seems to be crucial to reveal the mechanism for the Py release by GTP-To. However, residues required for these interactions have not been identified. (ii) We found that the specific arginines in the Py polycationic region were ADP-ribosylated by an endogenous enzyme. Thus, protein-protein interactions in which the polycationic region is involved may be monitored by tracing the Py ADP-ribosylation under physiological conditions. (iii) We also found that the Py ADP-ribosylation was regulated by the interaction between Py and To. Therefore, the ADP-ribosylation can be a useful tool to learn the interaction between Py and To. We describe that both arginine 33 and arginine 36 in the Py, free or complexed with Poβ, are ADP-ribosylated by endogenous arginine-ADP-ribosyltransferase. The Py ADP-ribosylation is inhibited when Py is complexed with To (both GTP- and GDP-bound forms), suggesting that the domain including these arginines is not exposed to ADP-ribosyltransferase when Py is complexed with To. Then, using forms of Py mutated in these residues, we confirm that the domain is involved in the interaction with To. Moreover, we find that positive charges in these arginine are important for the interaction with To.

### EXPERIMENTAL PROCEDURES

**Materials**—Mono Q (5 × 50 mm), Pep RPC HR5/5 (5 × 50 mm), TSK G2000SW (7.5 × 300 mm), DEAE-Sephacel, SP-Sephacel Fast Flow, and Blue Sepharose CL-6B were purchased from Pharmacia Biotech Inc. AG 1-X2 resin was obtained from Bio-Rad. Other materials were purchased from the following sources: [adenylate-32P]NAD, [3H]cGMP, [32P]GTP, and [35S]GTPyS from DuPont NEN; cGMP, GTP, GDP, GDPyS, Gpp(NH)p, and GTPyS from Boehringer Mannheim; nucodio- cin, snake venom phosphodiesterase, arginine methyl ester, PMSF, agmatine, and NAD-agarose from Sigma. Phosphatidylinositol-specific phospholipase C (from Bacillus thuringiensis) was obtained from ICN, and 1 unit was defined as the supplier described. Suppliers of materials for molecular biological experiments are described in these sections.

**Preparation of ROS Membranes**—Under dim red light, ROS were prepared from dark-adapted bullfrogs (Rana catesbiana or Rana grylio) by sucrose flotation in Buffer A. Various amounts of ROS membranes were washed in this study, we used a similar method. Bleached ROS membranes from 20 frogs were suspended in 3 ml of Buffer B and passed through a no. 21 needle seven times. Membranes and soluble fractions were separated by centrifugation (200,000 × g, 4°C, 15 min). ROS membranes were washed seven times in the same way, and these ROS membranes were termed prewashed ROS membranes. The prewashed ROS membranes were washed seven more times with 3 ml of Buffer C and seven times with 3 ml of Buffer C containing 400 μM GTP. To (more than 90%) and Py (about 50%) were released from these membranes. These membranes contain Py-less (active) PDE and were termed as Pγ-insensitive ROS membranes. Residual Py in these membranes is not sensitive to GTP-To (12), suggesting that the membrane preparation contains a distinct subset of Py which is not released by GTP-To. This subset of Py was termed GTP-To-insensitive Py. When Py-depleted ROS membranes were used as a source for ADP-ribose transferase, these membranes were washed twice with 2 ml of Buffer C to remove residual GTP. When the Py-depleted membranes were washed an additional seven times with 3 ml of Buffer D, T4 lysozyme (more than 90%) was released. These membranes were termed Pyγ- and transducin-depleted ROS membranes. The mixture without membranes was washed seven more times with 3 ml of Buffer C and seven times with 3 ml of Buffer C containing 400 μM GTP. Pyγ (more than 90%) and Pyγ was not detected in these membranes. This suggests that GTP-To-insensitive Py cannot be ADP-ribose transferred. Urea-treated ROS membranes were prepared as described (13).

**Site-directed Mutagenesis of Py**—All DNA manipulations were carried out using standard procedures (28). Full-length bovine Py cDNA (29), which was ligated into the EcoRI-HindIII-digested plasmid pALTER-1 (Promega), was used in the mutagenesis steps. All mutagenic oligonucleotide primers used (Table I) were purchased from Dainagene Inc. (Malvern, PA). Mutagenesis was carried out using Altered Site Site-Directed Mutagenesis Kit (Promega). Mutant clones were identified by in situ hybridization with 32P-labeled mutagenic oligonucleotides. The mutations were confirmed by double-stranded DNA sequencing using the fmol of DNA sequencing System (Promega). Two oligonucleotides: Up-5'-GCCAACCTGCATAGACAGCTGGAG-3' and Down-5'GGGGCGGTCAGCTCATGATGAGAGCTGG-3' were used in a polymerase chain reaction to introduce NdeI and BamHI sites at the ends of Py genes. The NdeI-BamHI fragment was cloned into the NdeI-BamHI
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Preparation of ADP-ribosylated Pγ—Although the amounts of components in each reaction mixture were slightly different (see each figure legend), ADP-ribosylated Pγ was prepared by incubation of purified Pγ, Pγ- and transducin-depleted ROS membranes, and [adenylate-32P]NAD. ADP-ribosylated Pγ was isolated using a Pep RPC column (see Experimental Methods) at 5–7 M NaCl. In the case of the Pep RPC column, the same volume of 0.2 M formic acid was added into the sample to terminate the reaction, and the mixture was heated for 5 min at 80 °C. Then, the sample was centrifuged (345,000 × g, 30 min, 4 °C). The process of the Pep γ extraction by formic acid was repeated two times. The pooled supernatant was applied to a Pep RPC HR5/5 column that had been equilibrated with 0.1% trifluoroacetic acid. Elution of Pγ was carried out with an acetone/1 M NaCl gradient (0–60%) containing 0.1% trifluoroacetic acid, as shown in Fig. 2. The flow rate was 1 ml/min, and the fraction volume was 0.5 ml. Following measurement of both radioactivity and Pγ activity in each fraction, the active fractions were dried, suspended in water, and kept at −80 °C. In the case of SDS-gel electrophoresis, samples were heated with SDS-sample buffer to terminate the reaction. The gel was stained in 20% methanol containing 0.2% (v/v) Coomassie Blue and 0.5% (v/v) acetic acid (20 min) and destained with 30% (v/v) methanol (1 h). The visualized Pγ was excised from the gel.

Identification of ADP-ribose in the [adenylate-32P]NAD-ribosylated Pγ—To identify ADP-ribose in the ribosylated Pγ, the ribosylated frog Pγ was incubated with glycine/NaOH buffer (pH 10.0) or treated with the snake venom phosphodiesterase, as shown in centrifugation of these reaction mixtures (345,000 × g, 30 min, 4°C). Supernatants were applied with ADP-ribose (100 μM) or AMP (100 μM) to a Mono Q column that had been equilibrated with 10 mM sodium phosphate (pH 6.0). After washing the column, radioactive compounds were eluted. Chromatographic conditions were: A, 10 mM sodium phosphate (pH 6.0); and B, 10 mM sodium phosphate (pH 6.0) and 0.2 M NaCl; 0–60% B in 16 ml on a linear gradient. The flow rate was 1 ml/min, and the fraction volume was 0.5 ml.

Measurement of Molecular Ion Mass of ADP-ribosylated Pγ—Fifty μl of ADP-ribosylated recombinant bovine Pγ (0.2 mg/ml) was injected into a high performance liquid chromatography system that consisted of Ultrafast Microprotein Analyzer model 600 (Microm BioResources, Auburn, CA) equipped with a Reliasil C18 reverse phase column (5 μm particle size, 300 A pore size, 1.0 × 150 mm) with a flow rate 40 μl/min. Chromatographic conditions were: A, 0.1% trifluoroacetic acid, 2% acetonitrile, H2O; B, 0.07% trifluoroacetic acid, 90% acetonitrile, H2O, 0.5–56% B in 25 min on linear gradient. The flow was monitored at 215 nm, and the eluate was introduced directly into an API-III triple quadrupole mass spectrometer (Perkin-Elmer Sciex, Thornhill, Ontario, Canada) equipped with an electrospray atmospheric pressure ionization source. The tuning and calibration were done using polypropylene glycol. The mass spectrometer was set to scan in a positive ion mode at orifice potential of 90 V from m/z = 500–2,200 with a mass step of 0.4 Da. The mass data were analyzed by MacSpec 3.22 (Perkin-Elmer Sciex).

Analytical Methods—Activities of PDE and Pγ were assayed as described (12). GTPase activity of Tα and GTPγS binding to Tα were measured as described (18). Immunological detection of Pγ was carried out as described (34). SDS-polyacrylamide gel electrophoresis was performed as described (30). When [adenylate-32P]NAD was present in the electrophoresis, the gel was cut above the dye front to remove free radioactive NAD for the reduction of background in autoradiography. Therefore, we do not show the dye front in each picture of gel. Protein concentrations were assayed with bovine serum albumin as standard (35). The amount of Pγ was assayed by densitometric scanning (12). To calculate the Pγ concentration, 9,625 and 9,669 were used as molecular weights of frog (36) and recombinant bovine (see Fig. 4) Pγ, respectively, although Pγ was detected as a 13,000 band in SDS-gels. It should be emphasized that all experiments were carried out more than two times, and the results were similar. Data shown are representative of these experiments.

RESULTS

Arginine Residue (Arg-33 or Arg-36) in Pγ Is ADP-ribosylated by Endogenous Arginine-ADP-ribosyltransferase—In the presence of [adenylate-32P]NAD a 39-kDa protein in prewashed ROS membranes was radiolabeled with pertussis toxin (Fig. 1). However, the radiolabeling almost disappeared if ROS membranes were washed with a buffer containing GTP (data not shown). These data indicate that Tα in prewashed ROS...
membranes is ADP-ribosylated by pertussis toxin, as described previously (31, 37, 38). In the absence of pertussis toxin, Tα ADP-ribosylation was not detected, indicating that endogenous Tα ADP-ribosylation (39, 40) is negligible under our conditions. Under the same conditions, a 13-kDa protein was also radiolabeled, approximately 23% of Tα protein was not detected (data not shown). These data indicate that under the same conditions, transducin-depleted membranes, the radiolabeling of the 13-kDa protein was not detected (data not shown). These data indicate that endogenous Tα and exogenous Tα are radiolabeled by an enzyme(s) in prewashed ROS membranes in the presence of adenylate-32P]NAD. Following densitometric scanning of the protein band and measurement of its radioactivity, we estimate that approximately 50% of endogenous Tα protein was radiolabeled if one amino acid in Tα was radiolabeled.

To confirm that the radiolabeled 13-kDa protein is Tα, the radiolabeling was conducted using purified Tα and Tα- and transducin-depleted membranes in the presence of adenylate-32P]NAD. Then, the 13-kDa protein was isolated by using a reverse phase column (Fig. 2A). In the column chromatography, both radioactivity and PDE inhibitory activity were detected in the same fractions (Fig. 2B). Analysis of the radioactive fractions by SDS-gel electrophoresis and autoradiography of the gel indicate that the 13-kDa protein was isolated with a purity of more than 95% in these fractions and that the radioactivity was incorporated into the 13-kDa protein (Fig. 2C). Without Tα, the 13-kDa protein was not observed in the column chromatography (data not shown). Without Tα- and transducin-depleted membranes, the radiolabeling of the 13-kDa protein was not detected (data not shown). These data indicate that Tα is radiolabeled by a membrane-bound enzyme(s) in the presence of adenylate-32P]NAD. If one amino acid in Tα was radiolabeled, approximately 23% of Tα was radiolabeled in the reconstituted system. Under these conditions Tα is roughly estimated as a mixture of free Tα (95%) and Tα complexed with Paβ (5%) if all of the Paβ in the membranes is occupied by exogenous Tα. Thus, these data indicate that free Tα is radiolabeled. We also note that Tα complexed with Paβ is radiolabeled. Under the conditions shown in Fig. 1, Tα appears to be complexed with Paβ for because (i) PDE activity in the ROS membranes was low, and (ii) addition of GTP or GTP-γS stimulated PDE activity. We also radiolabeled bovine Paβ2 using partially purified frog ADP-ribosyltransferase after separation of Paβ2 from Paβγ and Paβ. We found that ~20% of Tα in the complex was radiolabeled (data not shown). Thus, we conclude that Tα, free or complexed with Paβ, is radiolabeled by a membrane-bound enzyme(s).

Radiolabeled Tα was treated under the following conditions: (i) incubation in a glycine/NaOH buffer (pH 10), and (ii) incubation with snake venom phosphodiesterase. These treatments have been used for the identification of ADP-ribose in the ADP-ribosylated α subunit of G-protein (41–43). The radioac-

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**Fig. 1. Pertussis toxin-independent radiolabeling of Tα by [adenylate-32P]NAD.** After prewashed ROS membranes were divided into eight portions, each portion (145 µg of protein) was incubated (30 min, 0 °C) with or without frog Py (0.6 µg) in 50 µl of Buffer G containing 16 µM GDP. Radiolabeling was initiated by the addition of NAD (final 10 µM, ~4 µCi) with or without pertussis toxin (11 µg). Radiolabeling was conducted for 20 min at 33 °C, and the reaction was terminated by the addition of 30 µl of SDS-sample buffer and heating at 80 °C for 5 min. Py radiolabeling was analyzed by SDS-gel electrophoresis and autoradiography. PT, pertussis toxin. ROS Me, prewashed ROS membranes. Arrows A and B indicate molecular weights of 39,000 and 13,000, respectively.

**Fig. 2. Isolation of [adenylate-32P]NAD-radiolabeled Tα.** Purified frog Py (10 µg) was incubated with Tα- and transducin-depleted ROS membranes (276 µg of protein) in 180 µl of concentrated (×1.25) Buffer G for 30 min at 0 °C. Radiolabeling of Tα was performed by the addition of 20 µl of 500 µM NAD (~25 µCi) at 33 °C. After a 1-h incubation, an additional 20 µl of 500 µM NAD (~25 µCi) was applied to the reaction mixture, and the mixture was further incubated for 1 h. The reaction was terminated by the addition of 200 µl of 0.2 M formic acid and heating at 80 °C for 5 min. The supernatant was collected by centrifugation (345,000 × g, 30 min, 4 °C) and applied to a Pep RPC column. Tα was eluted with an acetonitrile gradient as described. Panel A, profile of absorbance at 280 nm. The arrow indicates the position of Tα eluted when purified frog Py was applied to the column. Panel B, radioactivity (●) and PDE inhibitory activity (○) of each fraction. Each fraction (20 µl) was used to measure its 32P radioactivity. After drying each fraction (20 µl), its PDE inhibitory activity was measured using Py-depleted ROS membranes. Panel C, purity of radiolabeled Tα. Lane A, M₃ standards: a, 94,000; b, 68,000; c, 43,000; d, 30,000; e, 20,000; f, 14,000. Lane B, radiolabeled Tα (1 µg) visualized by Coomassie Blue. Lane C, autoradiography of radiolabeled Tα.

2 A. Yamazaki, unpublished method.
After the addition of Buffer H (590 μl) was not inhibited by preincubation of Pγ and transducin-depleted ROS membranes (85 μg of protein) in 50 μl of Buffer G for 60 min at 33 °C. The reaction was terminated by the addition of SDS-sample buffer (30 μl) and heating for 5 min at 80 °C. Following SDS-gel electrophoresis of the sample, the gel was rapidly stained and destained as described. After visualization, Pγ was excised from the gel and cut into two pieces, and these two gel pieces were immersed overnight in 2 ml of water at 0 °C and then in 600 μl of acetonitrile for 20 min at room temperature (twice). These two gel pieces were dried under reduced water at 0 °C and then in 600 μl of Buffer H, and then snake venom phosphodiesterase (5 μg) was added for the further swelling of the gel (10 min, room temperature). After the addition of Buffer H (590 μl) the gel piece was incubated at 37 °C for 3 h. Then, 400 μl of Buffer H was added to the mixture. The sample was spun (345,000 × g, 30 min, 4 °C), and the supernatant was analyzed by a Mono Q column. Panel A, chromatogram of mixture of NAD, AMP, and ADP-ribose. a, NAD; b, AMP; c, ADP-ribose. Panel B, Pγ treated with pH 10.0 buffer. The supernatant of Pγ treated with the pH 10.0 buffer (620 μl) was applied to the column with ADP-ribose (100 μl), and then the radioactivity of each fraction (200 μl) was measured. The yield of the radioactivity was 70%. Panel C, Pγ treated with phosphodiesterase. The supernatant of Pγ treated with phosphodiesterase (430 μl) was applied to the column with 100 μM AMP, and then the radioactivity of each fraction (200 μl) was measured. The yield of the radioactivity was 86%.

Fig. 3. Identification of ADP-ribose in Pγ radiolabeled with [adenylate-32P]NAD. Purified frog Pγ (4.0 μg) was incubated with [adenylate-32P]NAD (15 μM; ~4 μCi) and Pγ- and transducin-depleted ROS membranes (85 μg of protein) in 50 μl of Buffer G for 60 min at 33 °C. After visualization, Pγ was excised from the gel and cut into two pieces, and these two gel pieces were immersed overnight in 2 ml of water at 0 °C and then in 600 μl of acetonitrile for 20 min at room temperature (twice). These two gel pieces were dried under reduced pressure. One gel piece was incubated overnight in 50 mM glycine/NaOH (pH 10.0) at room temperature and spun (345,000 × g, 30 min, 4 °C). After the supernatant (275 μl) was adjusted to pH 7.0 with HCl, the volume of the sample was also adjusted to 1 ml with water and spun (345,000 × g, 30 min, 4 °C). The supernatant was analyzed by a Mono Q column as described. The other gel piece was swollen with 5 μl of Buffer H, and then snake venom phosphodiesterase (5 μg) suspended in 5 μl of Buffer H was added for the further swelling of the gel (10 min, room temperature). After the addition of Buffer H (590 μl) the gel piece was incubated at 37 °C for 3 h. Then, 400 μl of Buffer H was added to the mixture. The sample was spun (345,000 × g, 30 min, 4 °C), and the supernatant was analyzed by a Mono Q column. Panel A, chromatogram of mixture of NAD, AMP, and ADP-ribose. a, NAD; b, AMP; c, ADP-ribose. Panel B, Pγ treated with pH 10.0 buffer. The supernatant of Pγ treated with the pH 10.0 buffer (620 μl) was applied to the column with ADP-ribose (100 μl), and then the radioactivity of each fraction (200 μl) was measured. The yield of the radioactivity was 70%. Panel C, Pγ treated with phosphodiesterase. The supernatant of Pγ treated with phosphodiesterase (430 μl) was applied to the column with 100 μM AMP, and then the radioactivity of each fraction (200 μl) was measured. The yield of the radioactivity was 86%.

tive products were then fractionated using a Mono Q column. As shown in Fig. 3, the radioactivity was detected in ADP-ribose fractions when the radioactive Pγ was incubated in the glycine/NaOH buffer. In contrast, the radioactivity emerged in the AMP fractions when the Pγ was incubated with phosphodiesterase. No other radioactive peak emerged in any fractions. It should be emphasized that nonenzymatic binding of NAD to Pγ is not involved in the Pγ radiolabeling, since the radioactivity was not detected in NAD fractions (fractions 4 and 5). We also note that nonenzymatic binding of ADP-ribose to Pγ is excluded, since the radiolabeling of Pγ by [adenylate-32P]NAD was not inhibited by preincubation of Pγ with ADP-ribose (data not shown). These observations indicate that Pγ is ADP-ribosylated.

We also analyzed the radiolabeled Pγ by electrospray ionization mass spectrometry. Two different molecular ion masses were detected in the purified sample (Fig. 4). The estimated molecular ion mass of the major peak is 9,668.48 (standard deviation 0.97). The major peak is believed to be nonmodified Pγ, since (i) without radiolabeling, Pγ was detected as a single peak with the exact same molecular ion mass (data not shown); and (ii) the calculated molecular ion mass of the nonmodified Pγ is 9,670.28. The estimated molecular ion mass of the second peak (approximately 20% of the major peak) was 10,209.90 (standard deviation 1.08). The difference in the observed masses of these two peaks is 541.52. This value is in fair agreement with gain in molecular ion mass of G-protein α subunit by ADP-ribosylation (541.3). Taking into account the purity of radiolabeled Pγ and the level of radiolabeling (Fig. 2), we conclude that the second peak is ADP-ribosylated Pγ. These data indicate that a single ADP-ribose moiety is incorporated into Pγ. These observations also confirm that nonenzymatic binding of NAD to Pγ (663.4 increase in molecular ion mass) is excluded.

To identify an ADP-ribosylated amino acid, we treated the radiolabeled Pγ under different conditions. Neither low pH (HCl, 0.1 M) nor HgCl2 (10 mM) reduced the radioactivity from the Pγ (data not shown). In contrast, as shown in Fig. 3, the radiolabeled Pγ is sensitive to high pH (pH 10.0). The radioactivity was also decreased when the radioactive Pγ was incubated with hydroxylamine (Fig. 5A). Moreover, the Pγ radiolabeling was inhibited by novobiocin (Fig. 5Ba), an inhibitor of arginine-ADP-ribosyltransferase (42), and by L-cysteine methyl ester (Fig. 5Bb). These observations indicate that an arginine in Pγ is ADP-ribosylated.

Bovine Pγ contains five arginine residues: Arg-11, Arg-15, Arg-24, Arg-33, and Arg-36 (29). We attempted to isolate a peptide(s) containing an ADP-ribosyl arginine after proteolytic digestion of the radiolabeled Pγ, but we failed. This is probably because the ADP-ribose moiety was released from the radiolabeled Pγ during proteolytic digestion. The radioactive Pγ may be sensitive to longer incubation (room temperature, 18–24 h).
in these buffers (pH 8.0–8.5). In fact, after incubation of the radiolabeled Py under the same conditions (except without proteinase), a large portion of radioactivity was detected in the reaction mixture and incubated for various periods (33 °C). Following dilution of the samples (50 μl) with water to 500 μl and dialysis of the reaction mixture against water and SDS (0.1%), the reaction mixture was analyzed by SDS-gel electrophoresis and autoradiography.

Panel B, effects of inhibitors of ADP-ribosyltransferase on Py ADP-ribosylation. In the presence of the various concentrations of inhibitors, purified frog Py (0.26 μg) was incubated (30 min, 33 °C) with Py-depleted ROS membranes (80 μg of protein) in 50 μl of Buffer G. Py radiolabeling was initiated by the addition of [adenylate-32P]NAD (10 μM; ~0.6 μCi). To terminate the reaction, 30 μl of SDS-sample buffer was added, and the reaction mixture was heated at 80 °C for 5 min. Radiolabeled Py was isolated by SDS-gel electrophoresis and autoradiography. a, novobiocin; b, l-arginine methyl ester.

in these buffers (pH 8.0–8.5). In fact, after incubation of the radiolabeled Py under the same conditions (except without proteinase), a large portion of radioactivity was detected in the flow-through fractions in the reverse phase column chromatography. Therefore, to identify an arginine for ADP-ribosylation, we created mutant forms of Py in which an arginine was replaced by lysine. PDE inhibitory activities of these Py mutants, summarized in Table II, show that all mutants have inhibitory activities similar to that of wild type Py. These observations suggest that the mutation does not cause drastic change in the Py conformation required for the inhibition of cGMP hydrolysis. As shown in Fig. 6, these Py mutants were radiolabeled if each arginine was singly replaced by lysine (R11K, R15K, R24K, R33K, and R36K). However, the Py radiolabeling was abolished if both Arg-33 and Arg-36 are replaced by lysines (R11,15,24,33,36K, R11,15,33,36K, and R33,36K). In contrast, the Py radiolabeling was detected even if two other arginines are replaced by lysines (R11,15K and R15,24K). We also confirmed these data using a peptide corresponding to residues 30–39 of Py (FKQQRQTRQFK) and its mutant forms. The wild type peptide and mutant forms of the peptide (R33K and R36K) were similarly ADP-ribosylated by Py- and transducin-depleted membranes. However, a mutant form of the peptide (R33,36K) was not modified under the same conditions (data not shown). Together with data that one ADP-ribose is incorporated in the radiolabeled Py (Fig. 4), these results indicate that Arg-33 and Arg-36 in Py can be ADP-ribosylated; however, only one ADP-ribose is incorporated at a time into one of these two arginines. The time course of radiolabeling on both R33K and R36K mutants appeared similar (Fig. 6), suggesting that the possibility for the radiolabeling of Arg-33 and Arg-36 is similar under these conditions.

Effect of Ta on Py ADP-ribosylation—ADP-ribosylation of Py by partially purified ADP-ribosyltransferase was inhibited by both GTPγS- and GDP-bound forms of Ta (Fig. 7). ADP-ribosylation of Py by the enzyme solubilized from ROS membranes by n-dodecyl-β-maltoside was also inhibited by Ta (data not shown). We note that Py forms a complex with both GTPγS/Ta and GDP-Ta (12, 34). These observations suggest the following two possibilities: (i) after complex formation with Ta (both GTP- and GDP-bound forms), Py is not a substrate for ADP-ribosyltransferase; and/or (ii) ADP-ribosyltransferase is inhibited directly by Ta.

Agmatine is a simple arginine derivative often used as an artificial substrate for arginine-ADP-ribosyltransferase (33). ADP-ribosylation of agmatine by partially purified ADP-ribosyltransferase was carried out in the presence or absence of Ta (GTPγS- or GDP-bound forms). As shown in Fig. 8, agmatine was ADP-ribosylated; however, the ADP-ribosylation was not affected by Ta. Using a TSK-250 column (34), we confirmed that agmatine does not form a complex with Ta (data not shown). These observations indicate that ADP-ribosyltransferase is not inhibited by Ta. Therefore, we conclude that Py is no longer a substrate for ADP-ribosyltransferase after complex formation with Ta. The simplest explanation for these phenomena is that both Arg-33 and Arg-36 in Py are masked by Ta. Thus, a domain including these arginines is involved directly in the Py interaction with Ta. In contrast, Py complexed with Paβ is a substrate for ADP-ribosyltransferase, as described above. Thus, these arginines seem to be exposed to the enzyme when Py is complexed with Paβ, suggesting that these arginines are not directly involved in the Py interaction with Paβ.

Effect of Site-directed mutagenesis of Arg-33 and Arg-36 in Py on the interaction between Py and Ta—To confirm the role of Arg-33 and Arg-36 in Py in the interaction with Ta, both Arg-33 and Arg-36 were replaced by lysine or leucine. These mutants inhibited PDE activity similarly to wild type Py (Table II). These data support our conclusion that these arginines are not crucial for the interaction with Paβ to inhibit PDE activity. Then, GTase activity of Ta and GTPγS binding to Ta was measured in the presence of various amounts of these Py mutants. We have already shown that wild type Py inhibits both GTase activity of Ta and GTPγS binding to Ta under our conditions and that these phenomena are used as evidence for the interaction between Ta and Py (34, 44). As shown in Fig. 9A, the Py mutant R33,36K inhibited GTase activity; however, the Py mutant R33,36L did not inhibit GTase activity. Moreover, the R33,36K mutant inhibited GTPγS binding to Ta, but the R33,36L mutant did not inhibit GTPγS binding (Fig. 9B). Furthermore, GTPγS/Ta activated PDE that had been inhibited by the R33,36K mutant, but not PDE that had been
ADP-ribosylation of arginine-mutant Pγs. In the presence of Pγ- and transducin-depleted ROS membranes (320 μg), mutant Pγ (0.8 μg) were incubated at 33 °C in 250 μl of Buffer G. Pγ radiolabeling was initiated by the addition of [adenylate-32P]NAD (50 μM; ~2 μCi). Following incubation for various periods, an aliquot (50 μl) was mixed with 20 μl of SDS-sample buffer and heated at 80 °C for 5 min. After SDS-gel electrophoresis and autoradiography, the Pγ band was excised from gel, and its radioactivity was measured. Based on the radioactivity of wild type Pγ after a 60-min incubation, the radioactivity of each mutant was calculated. a, wild type Pγ; b, R11K; c, R15K; d, R11,15K; e, R33K; f, R36K; g, R33,36K; h, R24K; i, R11,15,33,36K; and j, R11,15,24,33,36K. A mutant R15,24K shows the same radiolabeling as R11,15K, however, the data are not shown because of the limited space.

Transducin-cGMP Phosphodiesterase Interaction

**Table II**

| Py and its mutant | Amino acid sequence of Py (10–37) | IC50a (nm) |
|-------------------|----------------------------------|-----------|
| Wild type         | IRATRVMGGPVTFRKGPKFQCRQOCRQ-     | 3.2 ± 1.0 |
| R11K              | K R R R R R R R R                | 2.6 ± 0.8 |
| R15K              | R K R R R R R R R                | 3.7 ± 1.0 |
| R24E              | R K R R R R R R R                | 3.2 ± 0.6 |
| R24K              | R R R R R R R R R                | 3.3 ± 0.9 |
| R33K              | R R R R R R R R R                | 4.4 ± 1.2 |
| R36K              | R R R R R R R R R                | 4.3 ± 1.2 |
| R11,15K           | K K R R R R R R R                | 3.3 ± 0.9 |
| R15,24K           | K K R R K K K K R                | 3.5 ± 1.1 |
| R33,36L           | R R R R K K K K L L              | 3.1 ± 0.8 |
| R33,36K           | R R R R K K R K K K              | 3.4 ± 0.7 |
| R11,15,33,36K     | K K R R K K K K K K              | 2.0 ± 0.8 |
| R11,15,24,33,36K  | K K K R K K K K K K              | 3.4 ± 1.0 |

a Concentration of Pγ and its mutants for 50% inhibition of Pγ-depleted PDE activity. Inhibitory effects of bovine recombinant Pγ and its mutants were investigated using Pγ-depleted ROS membranes and various amounts of Pγ. The specific inhibitory activity values (inhibition/mg of protein) and maximal inhibitory effect on Pαβ were similar for wild type and mutant Pγ (±5%).

![FIG. 6. ADP-ribosylation of arginine-mutant Py.](image)

**FIG. 6. ADP-ribosylation of arginine-mutant Py.** In the presence of Pγ- and transducin-depleted ROS membranes (320 μg), mutant Pγ (0.8 μg) were incubated at 33 °C in 250 μl of Buffer G. Pγ radiolabeling was initiated by the addition of [adenylate-32P]NAD (50 μM; ~2 μCi). Following incubation for various periods, an aliquot (50 μl) was mixed with 20 μl of SDS-sample buffer and heated at 80 °C for 5 min. After SDS-gel electrophoresis and autoradiography, the Pγ band was excised from gel, and its radioactivity was measured. Based on the radioactivity of wild type Pγ after a 60-min incubation, the radioactivity of each mutant was calculated. a, wild type Pγ; b, R11K; c, R15K; d, R11,15K; e, R33K; f, R36K; g, R33,36K; h, R24K; i, R11,15,33,36K; and j, R11,15,24,33,36K. A mutant R15,24K shows the same radiolabeling as R11,15K, however, the data are not shown because of the limited space.

![FIG. 7. Inhibitory effect of Ta on Py ADP-ribosylation.](image)

**FIG. 7. Inhibitory effect of Ta on Py ADP-ribosylation.** After partial purification of ADP-ribosyltransferase, ADP-ribosylation of Pγ was measured with recombinant bovine Pγ (0.5 μg) and the enzyme (2.0 μg) in the presence of GTPγS/Ta (12.5 μg) or GDP-Ta (12.5 μg) in 300 μl of Buffer G. Pγ ADP-ribosylation was initiated by the addition of NAD (50 μM; ~2 μCi). Following incubation for various periods (33 °C), the Pγ ADP-ribosylation in 50 μl of the reaction mixture was terminated by the addition of 20 μl of SDS-sample buffer. Samples were analyzed by SDS-gel electrophoresis and autoradiography. a, control; b, GTPγS-Ta; c, GDP-Ta.

Inhibited by the R33,36L mutant (Fig. 10). These data indicate that arginines 33 and 36 are involved in the Pγ interaction with Ta and that positive charges of these arginines are important for the interaction between Ta and Pγ. We note that another arginine in the polycationic region, Arg-24, is not involved in the interaction with Ta. A mutant form of Pγ, R24E, inhibited GTPase activity in the same manner as wild type Pγ (data not shown). This indicates that arginines 33 and 36 in the Pγ polycationic region have a special function for the interaction with Ta.

**DISCUSSION**

PDE, a key protein to regulate the level of cGMP in retinal photoreceptors, is composed of Pαβ and two Pγ subunits. Pγ has two roles in Pαβ regulation: inhibiting cGMP hydrolysis by Pαβ (12, 17) and stimulating cGMP binding to high affinity, noncatalytic sites on Pαβ (13, 14). We have recently indicated that an identical Pγ expresses these different functions by
Reactions were initiated by the addition of \([35S]GTP\) to an AG 1-X2 column (1 ml). \([32P]ADP-ribosylagmatine was applied to a membrane filter (Millipore, HA, pore size 0.45 m). GTPase activity was measured in 100 m l of Buffer G. ADP-ribosylation of agmatine (20 mM) was carried out with partially purified ribosyltransferase (2.0 \(\mu\)g) in 1,100 m l of Buffer G. ADP-ribosylation was initiated by the addition of \([adenylate-32P]NAD\) (50 \(\mu\)M; ~2 \(\mu\)Ci). Following incubation for various periods (33 °C), samples (100 m l) were applied to an AG 1-X2 column (1 ml). \([35S]ADP-ribosylagmatine was eluted with 5 ml of H\(_2\)O. ●, control; ○, GTP\(\gamma\)S-To; and ▲, GDP-To.

![Transducin-cGMP Phosphodiesterase Interaction](image.png)

**FIG. 8.** Effect of To on ADP-ribosylation of agmatine. ADP-ribosylation of agmatine (20 m\(\mu\)M) was carried out with partially purified ADP-ribosyltransferase (2.0 \(\mu\)g) in the presence or absence of GTP\(\gamma\)S-To (25 \(\mu\)g) or GDP-To (25 \(\mu\)g) in 1,100 m l of Buffer G. ADP-ribosylation was initiated by the addition of \([adenylate-32P]NAD\) (50 \(\mu\)M; ~2 \(\mu\)Ci). Following incubation for various periods (33 °C), samples (100 m l) were applied to an AG 1-X2 column (1 ml). \([35S]ADP-ribosylagmatine was eluted with 5 ml of H\(_2\)O. ●, control; ○, GTP\(\gamma\)S-To; and ▲, GDP-To.

**FIG. 9.** Effect of \(\gamma\) mutants on the GTPase activity of To and GTP\(\gamma\)S binding to To. Bovine recombinant \(\gamma\) and its mutants were used. ○, wild type \(\gamma\); ■, R33,36K; ▲, R33,36L. Panel A, GTPase activity. GTPase activity was measured in 100 m l of Buffer G containing To (1.0 \(\mu\)g), T\(\beta\) (0.5 \(\mu\)g), urea-treated frog ROS membranes (2.0 \(\mu\)g), and various amounts of \(\gamma\). GTP hydrolysis was initiated by the addition of \([\gamma\text{-}32P]GTP\) (1 \(\mu\)M; ~0.1 \(\mu\)Ci). Following incubation (30 min, 33 °C), the reaction was terminated by the addition of 500 m l of stop solution (6% charcoal, 10% trichloroacetic acid, and 5 m M NaH\(_2\)PO\(_4\)). After spinning (1,000 \(\times\) g, 10 min), radioactivity of the supernatant (100 m l) was measured. Panel B, GTP\(\gamma\)S binding. GTP\(\gamma\)S binding to To was measured in 100 m l of Buffer G containing To (0.8 \(\mu\)g), T\(\beta\) (0.33 \(\mu\)g), urea-treated ROS membranes (2.0 \(\mu\)g), and various amounts of \(\gamma\). Reactions were initiated by the addition of \([\gamma\text{-}32P]GTP\(\gamma\)S (1 \(\mu\)M; 0.06 \(\mu\)Ci). Following incubation (30 min, 0 °C, 80 \(\mu\)l of the reaction mixture was applied to a membrane filter (Millipore, HA, pore size 0.45 m) and washed with 4 ml of Buffer G (four times). Radioactivity bound was measured.

binding to different sites on \(\beta\) (16) and that different regions in \(\gamma\) are involved in these functions (15). Since these \(\gamma\) functions are expressed by interaction with \(\beta\) and interrupted by \(\gamma\) release with GTP\(\gamma\)To, the functional structure of \(\gamma\) required for these interactions should be clarified to understand the regulation of these \(\gamma\) functions. In this study we have shown that two arginines, 33 and 36, in the \(\gamma\) polycationic region are equally ADP-ribosylated by endogenous ADP-ribosyltransferase, but only one arginine is ADP-ribosylated at a time. We speculate that steric hindrance may contribute to ADP-ribosylation of one arginine. The ADP-ribosylation was detected when \(\gamma\) is complexed with \(\beta\). However, the ADP-ribosylation was inhibited when \(\gamma\) is complexed with To (GTP- and GDP-bound forms). These data imply that these arginines are masked when \(\gamma\) is complexed with To. Then, site-directed mutagenesis was applied to replace these arginines with lysines or leucines, and the effects of these \(\gamma\) mutants on To functions were measured. These experiments confirm that these arginines are crucial for the interaction with To. We have also shown that arginine 24, another arginine in the \(\gamma\) polycationic region, is not involved in the \(\gamma\) interaction with To.

Thus, it is concluded that the polycationic region in \(\gamma\) may be divided into at least two subdomains, and a subdomain containing arginines 33 and 36 appears to be involved in the interaction with To, but not in the interaction with \(\beta\).

As summarized in the Introduction, various methods have been applied to identify specific domains in \(\beta\). Proteolytic digestion of \(\gamma\) has also been applied to identify a specific domain in \(\gamma\) (25). Although these methods are potentially useful in identifying specific domains in proteins, they have also serious problems. For example, one cannot be sure that conformation of the peptide corresponding to the specific region is the same or similar to that of the region in the protein. Site-directed mutagenesis of the specific residues does not necessarily change only the conformation of the region in which these residues are involved. However, deletion of the specific residues does not necessarily delete functions of the specific residues. We have already shown that deletion of the carboxy-terminal residues of \(\gamma\) reduces not only its inhibitory activity of cGMP hydrolysis but also its ability to interact with \(\beta\) (15). Therefore, we seek a method to identify specific residues in the \(\gamma\) polycationic region under more physiological conditions. In this study we have used ADP-ribosylation to identify two arginines in the polycationic region. ADP-ribosylation was carried out by endogenous ADP-ribosyltransferase under physiological conditions. Then, we used peptides and site-directed mutagenesis to confirm data obtained by the ADP-ribosylation.

In a system reconstituted by exogenous \(\gamma\) and \(\gamma\) and
transducin-depleted ROS membranes, the maximum level of Py ADP-ribosylation is estimated about 20% by the measurement of Py ADP-ribosylation after SDS-gel electrophoresis (Fig. 2). Although we do not think that the conclusions in this study are affected by the low level of ADP-ribosylation, we try to specify the reasons for such a low level of ADP-ribosylation. One possible interpretation is that Py complexed with Pyβ may be a better substrate for ADP-ribosyltransferase, especially in membranes, because high Py ADP-ribosylation (about 50%) was detected in native membranes (Fig. 1). We note that all Py membranes, because high Py be a better substrate for ADP-ribosyltransferase, especially in membranes, might participate that an activator of ADP-ribosyltransferase may be present in these membranes because ADP-ribosyltransferase might be regulated by several proteins. Another possible interpretation is that the apparent incorporation of ADP-ribose to Py measured after SDS-gel electrophoresis may be underestimated. In this study, the pH values of the separating gel buffer and the running buffer are 8.8 and 8.4, respectively. SDS-gel electrophoresis was carried out without a cooling system. It is possible that these conditions accelerate the release of ADP-ribose from Py. This speculation is supported by the observation that ADP-ribose-arginine linkage was sensitive to in buffers (pH 8.0–8.5) as described above. Zolkiewska and Moss (43) also described possible breakdown of ADP-ribose-arginine linkage during electrophoresis.

We have shown previously that Py complexed with GTP-Tα is phosphorylated by a kinase; however, Py complexed with Pyβ is not a substrate for the kinase (36, 44). The phosphorylation of Py inverts the relative affinities of Py to GTP-Tα and to Pyβ, and the change in the relative affinities may function in the turnoff mechanism of GTP-Tα-activated PDE without GTP hydrolysis. In this study we have shown that Py complexed with Pyβ, but not with Tα, is ADP-ribosylated by arginine-ADP-ribosyltransferase in ROS membranes. We have utilized the Py ADP-ribosylation as a tool to identify arginines in the polycationic region which are involved in the interaction with Tα. However, the physiological significance of the Py ADP-ribosylation in phototransduction remains unsolved. We anticipate that Py ADP-ribosylation may control phototransduction through regulation of Py interaction with specific proteins involved in phototransduction. The information obtained in this study will also be useful to reveal the physiological significance of the Py ADP-ribosylation.

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