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Purification of Bovine Soluble Guanylate Cyclase and ADP-Ribosylation on Its Small Subunit by Bacterial Toxins[1]

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Soluble guanylate cyclase (sGC) consisting of two different subunits (α: M_r=74,000, β: M_r=69,000) was purified more than 12,000-fold in terms of specific activity from the supernatant of bovine lung homogenates and characterized. The heme content determined with the pyridine hemochromogen method and Bradford's protein assay was 0.8 heme per dimer. Cholera, pertussis, and botulinum C3 toxins modified exclusively the β-subunit of sGC, yielding the ADP-ribose-bound compound with 1:1 stoichiometry, and V_{max} for the cyclase reaction was increased 10 times by this modification. When the ADP-ribosylation of sGC was performed simultaneously with two or three bacterial toxins which have distinct amino acid specificities, the resultant enzyme had only one ADP-ribose, and the activity was the same as that of the enzyme modified with one toxin. When NO was incorporated into the reaction mixture containing the ADP-ribosylated sGC, the cyclase activity noticeably increased by approximately the same amount as that seen for the unmodified enzyme. Such effects were not seen with CO. When ADP-ribosylated sGC was incubated with Mn^{2+}, the enzyme activity was synergistically increased. The heme-deleted sGC was also ADP-ribosylated by bacterial toxins and its activity was raised. These findings suggest that sGC has an ADP-ribosylation site near the GTP binding site, like other GTP-binding proteins, and that the β-subunit regulates the activity.

Key words: ADP-ribosylation, bacterial toxins, small subunit, soluble guanylate cyclase.

The mammalian GTP-binding proteins are usually composed of three different subunits. The α-subunit of the heterotrimer provides the site for binding and hydrolyzing GTP and its activity is regulated by association with and dissociation from the β/γ-dimer (*1*). The α-subunits of the trimeric GTP-binding proteins are ADP-ribosylated by cholera and pertussis toxins and mammalian ADP-ribosyltransferases (*2-6*). The toxin-catalyzed ADP-ribosylation reduces the GTPase activity and thus prolongs the active duration of the enzyme (*7*). On the other hand, soluble guanylate cyclase (sGC) is composed of two different subunits (α and β). The amino acid sequences of the two subunits are alike except for the terminal part (*8*). This enzyme contains one or two protoheme(s) per heterodimer (*9, 10*) and its activity is drastically increased by the addition of nitric oxide (NO) or an NO-producing reagent. The NO-induced enhancement of activity requires the presence of heme (*11, 12*). It is assumed that NO binds to the heme iron and causes some structural changes (*9, 10*) and in fact, we have observed the Fe-NO and N-O stretching Raman bands for the NO-bound form of the present enzyme (*13*). It has been reported that the heme-deleted sGC can be reconstituted with NO-heme (*14*). Since sGC is a GTP-binding protein and may undergo modifications by bacterial toxins, like other GTP-binding proteins, we tried to modify sGC with ADP-ribose from NAD. Here, we report the purification procedure of sGC from bovine lung, some biochemical properties of this preparation, and modification of sGC with ADP-ribose by bacterial toxins.

MATERIALS AND METHODS

Materials—Fresh bovine lungs within 2 h after slaughter were used for every preparation. GTP (Seikagaku), [^{3}H]-cyclic GMP assay kit (Amersham), [α-^{32}P]-GTP, and [*adenylate*-^{32}P]NAD (New England Nuclear), sodium nitroprusside (Sigma), and 3-(2-hydroxy-1-methyl-2-nitrosohydrazino)-*N*-methyl-1-propanamine (NOC-7) (Dohjin Chemicals) were used as purchased. Other reagents were of analytical grade.

Purification of Soluble Guanylate Cyclase—The purification procedure of sGC was slightly modified from the original method of Stone and Marletta (*9*). About 4 kg of bovine lungs (two animals), washed with cold saline, were chopped and minced after removal of the large trachea, and homogenized in an equal volume of buffer (25 mM triethanolamine-HCl, 50 mM NaCl, 5 mM dithiothreitol, 1 mM EDTA, 1 mM benzamidine, 1 μg/ml leupeptin, and pepstatin A, pH 7.4) using a food processor. The homogenate was centrifuged at 10,000×g for 20 min first and its supernatant was further centrifuged at 100,000×g for 30

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min. The supernatant from the high-speed centrifuge was put into a DEAE-Fast Flow column (about 1,000 ml, Pharmacia) equilibrated with the homogenizing buffer. The column was washed with the homogenizing buffer several times, adsorbates were eluted with a linear gradient of NaCl from 0.05 to 1.05 M, and a fraction containing NO-sensitive guanylate cyclase activity was collected.

Ammonium sulfate was added to the collected fraction to a final concentration of 23% saturation and the resulting precipitate was removed by centrifugation at 15,000 × *g* for 20 min. Ammonium sulfate was further added to the supernatant to give a final concentration of 41% saturation. The precipitate was collected by centrifugation and was suspended in a small volume of buffer A (25 mM triethanolamine-HCl, 5 mM dithiothreitol, 1 mM benzamidine, 1 μg/ml leupeptin, and pepstatin A, pH 7.4). The suspension was dialyzed against buffer A overnight. After addition of $MnCl_2$ to 4 mM, the dialysate was centrifuged at 15,000 × *g* for 20 min, and the supernatant was applied to a GTP-Agarose column (about 100 ml) which had been equilibrated with buffer A containing 4 mM $MnCl_2$. It was eluted with a linear gradient of 4 mM $MnCl_2$ to 6 mM ATP. The fraction containing NO-sensitive GC activity was concentrated in an Amicon-50 concentrator and dialyzed against buffer A. The dialysate was applied to a hydroxyapatite column (30 ml, Seikagaku Kogyo), which had been equilibrated with buffer A, and eluted with a linear gradient of phosphate buffer from 0 to 200 mM. The fraction that contained NO-sensitive GC activity was concentrated with an Amicon-50 concentrator and further applied to a gel-filtration column (Superdex 200 pg, Pharmacia) that had been equilibrated with 25 mM triethanolamine-HCl (pH 7.4) containing 5 mM dithiothreitol. The fraction containing sGC was concentrated again with the Amicon-50. Glycerol was added to the concentrated sGC preparation at a final concentration of 20%. When the sGC preparation was kept in a freezer at 77 K, the activity of sGC was stable for 3 weeks.

Enzyme Assay—The sample was incubated with 50 mM Tris-HCl (pH 7.4) containing 1 mM theophylline, 1 mM dithiothreitol, 1 mM GTP, and 1 mM Mg^{2+} (or Mn^{2+}) in the presence or absence of 0.1 mM NOC-7 or NO gas at 37°C for 10 min. NO gas was passed through 1 M KOH solution to remove other nitrogen oxides before being passed into the enzyme solution. In the case of crude samples, 15 mM creatine phosphate and 0.2 mg/ml of creatine phosphokinase were added to the incubation mixture. The enzyme activity under the conditions mentioned above was constant for at least 15 min, and the amount of products increased linearly. After incubation, the reaction was stopped by addition of 9 volumes of ethanol, and the solution was centrifuged. The supernatant was dried in an evaporator and the dried residue was dissolved in 50 mM Tris-HCl (pH 7.4) containing 1 mM EDTA. The amount of cGMP present in this solution was measured with a [^{3}H]cyclic GMP immunoassay system (Amersham, TRK500) or by a high-pressure liquid chromatography method as follows.

[α-^{32}P]GTP was substituted for the cold GTP in the incubation mixture and the reaction was stopped by the addition of 5% perchloric acid. After centrifugation, the pH of the supernatant was adjusted to 4.0 with KOH. The resulting precipitate was removed by centrifugation. The supernatant was injected into a high-pressure column (C18 Inertsil, 4.6 × 150 mm) which had been equilibrated with 20 mM sodium phosphate buffer (pH 3.5) containing 5 mM tetra-*n*-butylammonium bromide, at a flow rate of 1.0 ml/min. The adsorbates were eluted with a linear gradient of acetonitrile from 0 to 60%. The effluent was monitored with a UV-detector at 260 nm and a β-ray detector (A-150, Packard Japan). The cGMP retained in the column was eluted 8.0 min after the sample injection.

In ADP-ribosylation, the purified sGC (12 μg/ml) was incubated with botulinum C3 toxin, cholera toxin, or pertussis toxin (10 ng/ml), together with 3 mM GTP, 2 mM dithiothreitol, and 0.1 mM [*adenylate*-^{32}P]NAD in 10 mM Tris-HCl (pH 8.0 or 7.4) containing 50 mM NaCl, at 37°C for 5 min. The ADP-ribosylation level was measured by the reported method (*15*). To quantify the amount of ADP-ribosylation, a portion of the reaction mixture was mixed with 5% trichloroacetic acid and centrifuged. The precipitate was washed twice with 5% trichloroacetic acid and subsequently with ether, suspended in 50 mM Tris-HCl (pH 7.5) containing 2 mM $MgCl_2$ and 1 mM $CaCl_2$ and incubated with venom phosphodiesterase (5 μg/ml, Sigma) at 35°C for 1 h. Proteins in the reaction mixture were removed by addition of 10% perchloric acid after the incubation, and the resultant radiolabeled product was analyzed by high-pressure liquid chromatography as reported previously (*4*).

Other Procedures—To prepare heme-deleted sGC, the pH of the fraction exhibiting GC activity in the effluents from the GTP-Agarose chromatography was adjusted to 5.8 with 0.5 M sodium acetate (pH 5.0) and the solution was kept standing for 1 h in the cold. The resulting precipitates were washed twice with 0.5 M sodium acetate (pH 5.8) by centrifugation at 85,000 × *g* for 20 min. The washed precipitates were dissolved in a small amount of 25 mM triethanolamine (pH 7.4) containing 5 mM dithiothreitol and subjected to the enzyme assay.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (*16*). The gel was stained with Coomassie Brilliant Blue or silver. The gel was dried, and placed on an X-ray film. Protein assay was performed by the method of Bradford (*17*).

The heme content of the purified enzyme was determined by the pyridine hemochromogen method (*18*); 300 μl of the enzyme solution was mixed with 100 μl of pyridine and 4.5 μl of 8 M NaOH (*19*) and then reduced with sodium dithionite under an N_2 atmosphere. Its absorption spectrum between 500 and 600 nm was measured within 2 min after reduction and the absorbance difference between 540 and 556 nm was determined. The same procedure was applied to myoglobin solutions of various concentrations and a calibration line for absorbance *vs.* heme content under the present solution conditions was established.

RESULTS

Purification of Soluble Guanylate Cyclase—When the supernatant of bovine lung homogenates was subjected to ion-exchange chromatography on a DEAE-Sepharose column, a peak in which GC activity was sensitive to NO was obtained. After concentration of the peak fraction, the dialysate was subjected to affinity chromatography on a GTP-Agarose column. Although a large amount of proteins

was not always adsorbed by this resin, a single peak exhibiting NO-sensitive GC activity was obtained. This fraction was concentrated and the dialysate was applied to a hydroxyapatite column. A single peak having NO-sensitive GC activity was obtained and it was further subjected to gel-filtration. The changes of specific activity of sGC at each purification process are summarized in Table I. In terms of specific activity, sGC was purified more than 12,000-fold as compared with the lung extract.

For four different preparations, the purified sGC gave $K_m = 44 \pm 8\ \mu M$ for GTP and $V_{max} = 77 \pm 10$ nmol/min/mg protein in the presence of Mg^{2+}; these values are regarded as presenting the basal activity of this preparation. Gel filtration on a TSK 3000SW column gave the molecular weight of *ca.* 140,000 for the purified sGC. As shown in the inset of Fig. 1, SDS-PAGE experiments demonstrated that the purified sGC is a heterodimer consisting of two subunits with $M_r = 74,000$ and $M_r = 69,000$. When NO was incorporated into the purified sGC preparation, the Soret band (431 nm) of the enzyme was shifted to 399 nm, as depicted in Fig. 1.

The amount of heme contained in the purified sGC was determined by means of the pyridine hemochromogen method based on a calibration line constructed with myoglobin. The protein content of the enzyme solution, determined by using Bradford's method, and the molecular weight determined by SDS-PAGE, yielded a value of the heme content of the present sGC of 0.8 heme/dimer. This is an uncorrected value, and its validity depends on the validity of the protein content thus determined.

Effect of NO, Mn^{2+}, and ADP-Ribosylation on sGC Activity—When Mn^{2+} was substituted for Mg^{2+}, the sGC activity was increased about 10-fold from the basal level. The K_m for GTP did not change ($44 \pm 8\ \mu M$ for four different preparations), but V_{max} was increased by ~10-fold (710 ± 55 nmol/min/mg protein). When NO gas was introduced into the reaction mixture, the amount of the product (cGMP) was drastically increased (more than 150-fold) compared with that under the basal conditions. In fact, V_{max} of sGC was increased by 150-fold ($11,500 \pm 900$ nmol/min/mg protein) and K_m for GTP was decreased to about one half ($22 \pm 4\ \mu M$) after incorporation of NO gas. After ADP-ribosylation by each toxin, the amount of cGMP was increased 10-fold in comparison with the unmodified enzyme. The addition of NAD alone did not change the sGC

TABLE I. **Purification of sGC from bovine lung.** Assay condition: with Mg^{2+} as the required divalent metal.

Fraction	Total protein (mg)	Total activity (nmol/min)	Specific activity (nmol/min/mg)	Purification (fold)
Supernatant	69,000	414	0.006	1
DEAE-Sepharose	5,200	1,870	0.36	60
Ammonium sulfate	3,100	1,800	0.58	193
GTP-Agarose	100	950	9.5	1,583
Hydroxyapatite	16	464	29.0	4,833
Superdex 200 pg	3.2	240	75.0	12,500

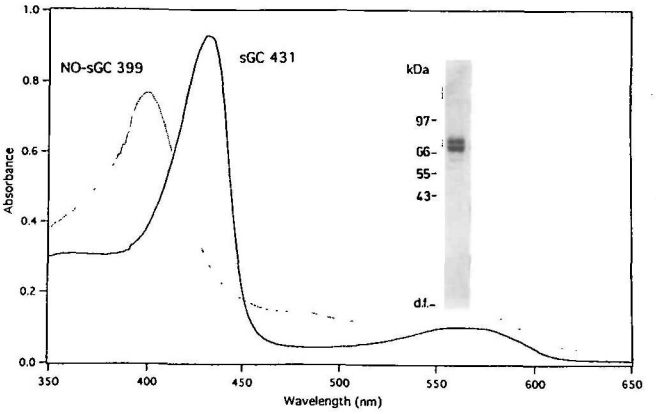

Fig. 1. **Spectral properties and SDS-PAGE of purified sGC.** Solid line: as purified; dotted line: after introduction of NO gas. The inserted photographs show the SDS-PAGE of purified sGC. The gel was stained with silver. Molecular markers: 97,000 (phosphorylase *b*), 66,000 (bovine serum albumin), 55,000 (glutamate dehydrogenase), and 43,000 (ovalbumin). d.f.: dye front.

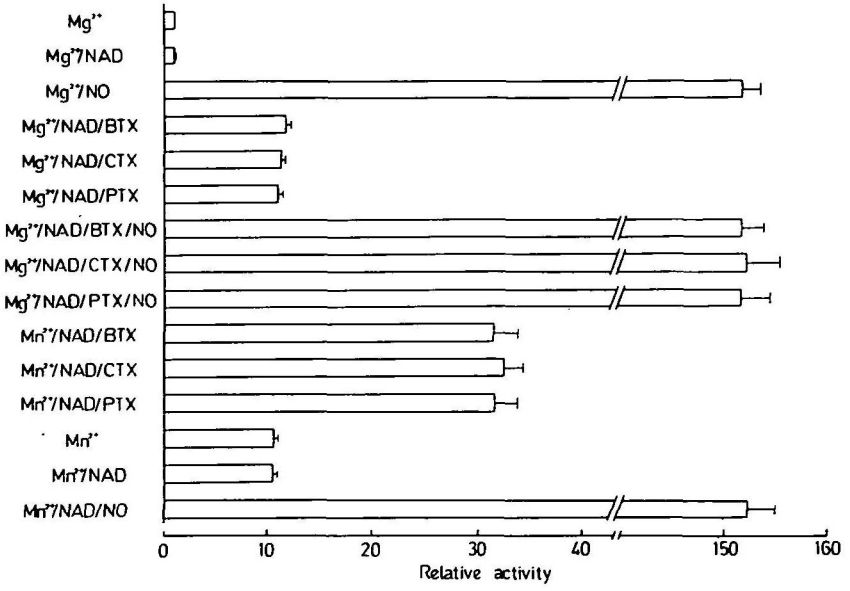

Fig. 2. **Effects of NO, Mn^{2+}, and ADP-ribosylation on sGC activity.** Basically, the purified sGC (0.2 μg/ml) was incubated in 50 mM Tris-HCl (pH 7.4), 3 mM GTP, 5 mM Mg^{2+} (or Mn^{2+}), and 1 mM dithiothreitol in a total volume of 100 μl, for 10 min at 37°C. NO gas was bubbled into the incubation mixture before addition of GTP. In ADP-ribosylation, bacterial toxin (10 ng/ml) was added in the basic medium plus 0.1 mM NAD for 10 min at 37°C. BTX: botulinum C3 toxin, CTX: cholera toxin, PTX: pertussis toxin. Horizontal bars indicate ±SD of the means of three experiments.

activity, but after the addition of NAD and each toxin, the sGC activity increased 10-fold over the basal activity. When the enzyme was incubated with NAD, one of the toxins, and Mn^{2+}, the amount of cGMP produced by sGC was over 30-fold compared with the basal condition. The effect of NO on the sGC activity was to prevent ADP-ribosylation-induced activity. These results are graphically illustrated in Fig. 2.

To confirm that sGC was ADP-ribosylated, the purified sGC was incubated with [^{32}P]NAD and bacterial toxin, and subsequently analyzed by means of SDS-PAGE and autoradiography. The results are shown in Fig. 3. Although no radioactivity was noted for the proteins in the absence of toxin, strong radioactivity was found on the small subunit (β-subunit) of sGC in the presence of each toxin. Furthermore, radiolabeled 5'-AMP was found in high-pressure liquid chromatography of the reaction mixture after the modified sGC was incubated with phosphodiesterase (data not shown).

When the purified sGC was incubated simultaneously with two or three bacterial toxins, the modified enzyme contained only ADP-ribose, and the enzymatic activity increased as much as when the enzyme was ADP-ribosylated by one toxin. The results are illustrated in Fig. 4. After ADP-ribosylation by each bacterial toxin, K_m of sGC was unaltered ($44 \pm 8\ \mu M$), but V_{max} in the presence of Mg^{2+} was increased 10-fold (783 ± 32 nmol/min/mg protein for five different preparations) compared with that of the unmodified enzyme.

Effect of ADP-Ribosylation to Heme-Deleted sGC on Its Activity—The fraction containing sGC in the effluent from GTP-Agarose chromatography showed strong absorbance at 431 nm, but when the preparation was treated with sodium acetate, the Soret band apparently disappeared, indicating the formation of the heme-deleted sGC (data not

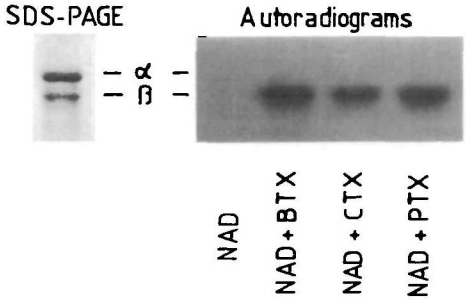

Fig. 3. **Autoradiography of sGC after ADP-ribosylation.** The purified sGC (12 μg/ml) was incubated with bacterial toxin (60 ng/ml) in 10 mM Tris-HCl (pH 8.0) containing 1 mM GTP, 1 mM $MgCl_2$, 1 mM dithiothreitol, and 0.1 mM [^{32}P]NAD for 15 min at 37°C.

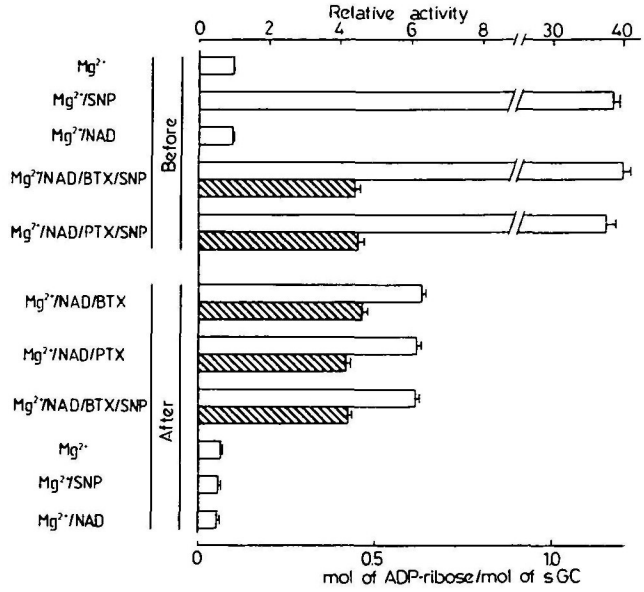

Fig. 5. **Effects of ADP-ribosylation on the partially purified heme-deficient sGC.** The partially purified material was treated with 0.5 M sodium acetate. The final acid treatment fraction had a specific activity of 11.2 ± 2.1 nmol/min/mg (three preparations) in the presence of $MgCl_2$. ADP-ribosylation was done as in the case of Fig. 4. SNP: 1 mM sodium nitroprusside was added just before incubation. BTX: botulinum C3 toxin, PTX: pertussis toxin. White columns: Relative activities compared with that in the presence of $MgCl_2$. Shaded columns: ADP-ribosylation level. Before: Before treatment with sodium acetate. After: After treatment with sodium acetate. Horizontal bars indicate $\pm$SD of the mean of three experiments.

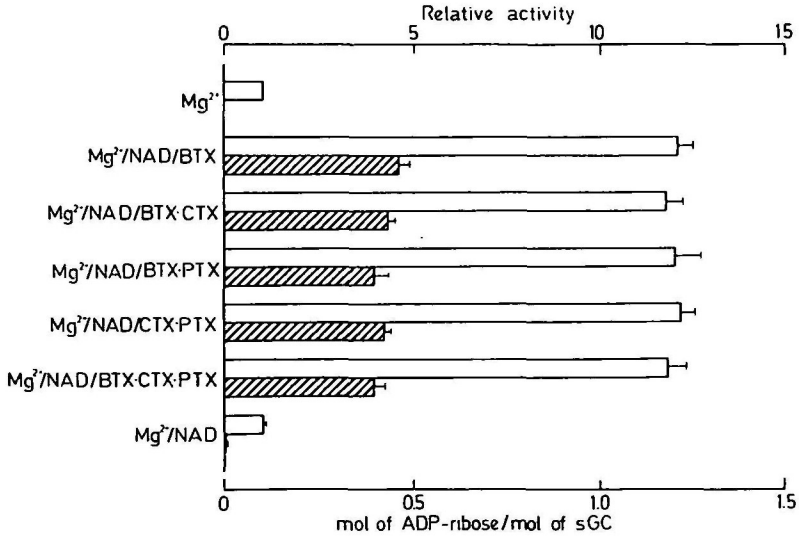

Fig. 4. **The effects of simultaneous ADP-ribosylation by two or three toxins.** The purified sGC (0.2 μg/ml) was incubated with two or three bacterial toxins (each 10 ng/ml) in 10 mM Tris-HCl (pH 8.0), 5.0 mM $MgCl_2$, 1 mM [^{32}P]NAD or nonradiolabeled NAD, 0.1 mM GTP, and 0.1 mM dithiothreitol in the total volume of 80 μl, for 5 min at 37°C and was further incubated for 10 min after the addition of 10 μl of 50 mM Tris-HCl (pH 7.4) and of 2.9 mM GTP. The ADP-ribosylation rate was assayed by the method of Hung and Robinson (*15*). White columns: Relative activities compared with that in the presence of $MgCl_2$. Shaded columns: ADP-ribosylation levels. The meaning of the horizontal bars is the same as in Fig. 2.

shown). The basal activity of the heme-deleted enzyme decreased by 62–74% compared with that of ordinary sGC. After treatment with nitroprusside, the activity of the heme-deleted enzyme remained essentially unaltered. Although the addition of NAD alone to the heme-deleted enzyme did not cause any change of activity, further addition of pertussis or cholera toxin brought about an increase of the activity. These results are graphically represented in Fig. 5.

DISCUSSION

Blue-Sepharose was reported to be effective for purification of sGC from bovine lung (*10*). However, it was also reported that an artificial heme-deleted sGC is generated during DEAE-Sephacel and Blue-Sepharose column chromatography (*20*). In our preliminary experiments, the recovery rate of the enzymatic activity from the Blue-Sepharose effluent was very low. Therefore, we omitted the Blue-Sepharose column from the purification procedures, but instead incorporated a column of hydroxyapatite after the GTP-Agarose column. This worked satisfactorily. The sGC thus purified has a strong absorption at 431 nm, indicating that the present procedures prevent the formation of the heme-deleted enzyme.

Three motifs, GxxxxGKS, NxxD, and DxxG, are highly conserved among different GTP-binding proteins such as elongation factors and ras proteins (*21*). The amino acid sequence of bovine sGC deduced from the cDNA analysis (*22*) contain DxxG motif in the β-subunits. Therefore, GTP binding site of sGC has recognition sequence. Since GTP-binding proteins and sGC are similarly ADP-ribosylated, DxxG motif may have a specific relation with ADP-ribosylation.

The ADP-ribosylation of GTP-binding proteins by cholera, pertussis, and botulinum C3 toxins is specific to an arginine, cysteine, or asparagine residue, respectively, and it occurs on the α-subunit in the case of heterotrimeric GTP-binding proteins and *rho* gene product (*23–25*), to which GTP binds. The modification is specific to ADP-ribose from NAD. Although the binding site and the number of bound GTP are not known for sGC, the present data demonstrate that these toxins recognize the β-subunit (smaller) of sGC, performing ADP-ribosylation exclusively on the β-subunit despite the fact that similar sequences should be present in the α-subunit (larger). These results may suggest that the GTP binding site of sGC is located in its β-subunit.

The results shown in Fig. 4 indicate that the number of ADP-ribosylation sites of sGC is only one per heterodimer even when the three different kinds of toxins were used simultaneously for ADP-ribosylation. Presumably, the asparagine, arginine, and cysteine residues, which can be ADP-ribosylated specifically by a single bacterial toxin, are located close to each other, and when the first ADP-ribose is donated to one of them by a toxin, it would block the second one to be provided by another toxin.

When NO was introduced into the purified sGC preparation, the absorption maximum was shifted from 431 to 399 nm. This shift of the Soret band is indicative of the formation of the five-coordinate ferrous-nitrosyl-heme complex. Previously it was pointed out that enzyme-bound heme is required for the stimulation of sGC by NO (*10–12*) and that a marked increase of enzymatic activity, 100–200-fold in terms of V_{max}, is triggered by binding of NO to the heme iron (*10*). In fact, the present experiments have demonstrated that V_{max} increases by 150-fold, K_m for GTP decreases to one half, and the amount of produced cGMP increases more than 150-fold upon inclusion of NO in the enzyme solution. The Fe-NO and N-O stretching Raman bands were observed for the present preparation upon incorporation of NO (*13*). In contrast, ADP-ribosylation increased the amount of cGMP by 10-fold, and this increase occurred without heme as well; in this preparation, NO caused no increase in activity (Fig. 5). Therefore, the mechanism of activity enhancement by NO-binding seems to differ from that by ADP-ribosylation.

There are two different points of view with regard to the heme-binding site of sGC. Inspection of the published cDNA sequence of sGC suggests that the heme binding site exists in the central portion of each subunit, corresponding to residues 213–370 in the case of β-subunit of bovine sGC (*14*). On the other hand, a genetic mutation study suggests that His-105 in the β-subunit serves as the proximal His of general His-coordinated heme proteins (*26*). However, the corresponding His is not found in the α-subunit. Therefore, the latter conclusion is not compatible with the recent observation of a 1.5/1.0 heme/peptide stoichiometry (*10*), but is compatible with the value of this study. Since the effect of ADP-ribosylation on the activity is different from the case of NO-binding, the heme binding site seems to be different from the ADP-ribosylation site. It is reported that just one point mutation in the β-subunit completely inhibits the enzymatic activity (*27*). Accordingly, the β-subunit of sGC, which can be ADP-ribosylated, may play a critical role in regulation, although both α- and β-subunits contain many homologous sequences.

It is well known that basal sGC activity is 5–10-fold greater in the presence of Mn^{2+} than Mg^{2+} at comparable metal concentrations (Fig. 1). This difference can be ascribed to the differences in the substrate, that is, GTP-Mn and GTP-Mg. In the ADP-ribosylated enzyme, the activity is further raised in both cases. In this sense, the metal effect and ADP-ribosylation effect are additive. For the ADP-ribosylated sGC, the presence of Mn^{2+} gives rise to higher activity than the presence of Mg^{2+}, but the ratio is not so large as in the case of the unmodified enzymes (Fig. 1). This may mean that another metal ion is bound to the ADP moiety and its effect is opposite to that of GTP. In the presence of excess Mn^{2+}, all activators only moderately increase the V_{max} and fail to alter the K_m for GTP (*9*). Probably, the increase of V_{max} is owing to the complex formation of GTP with Mn, that is, owing to substrate activation. Since this effect seems to be influenced by ADP-ribosylation, the ADP-ribosylation site might be located in close proximity to the GTP-binding site. In the case of heterotrimeric GTP binding proteins, the subunit providing the GTP binding site is the same as that which is ADP-ribosylated. The ADP-ribosylation of sGC occurs only at the β-subunit. These facts suggest that the GTP binding site of sGC may be located in the β-subunit.

ADP-ribosyltransferase and ADP-ribosylhydrolase are widely distributed in animal tissues (*4, 5*). The adenylate cyclase activity is controlled by heterotrimeric GTP-binding proteins which have been modified with ADP-ribose (*28*). According to a recent report (*29*), endogenous carbon

monoxide (CO) production is high in cerebellar neurons and the generated CO may modulate the NO-cGMP signaling system. There is a suggestion that CO activates the sGC activity by coordinating to the heme iron, similar to the case of NO (*30*). However, the activity of the present sGC was scarcely affected by binding of CO. The heterotrimeric GTP-binding proteins are covalently modified by fatty acylation, isoprenylation, phosphorylation, and endogenous ADP-ribosylation (*31*). Therefore, it is highly likely that ADP-ribosylation of the β-subunit of sGC plays an important role in the regulation of sGC activity and thus in the cGMP signaling system, together with NO.

REFERENCES

1. Sternweis, P.C. (1986) The purified alpha subunits of Go and Gi from bovine brain require beta gamma for association with phospholipid vesicles. *J. Biol. Chem.* **261**, 631-637
2. Angus, C.W., Van Meurs, K.P., Tsai, S.C., Adamik, R., Miedel, Y.C., Pan, T.Y.C., Kung, H.F., Moss, J., and Vaughan, M. (1986) Identification of the probable site of choleragen-catalyzed ADP-ribosylation in Goα-like protein based on cDNA sequence. *Proc. Natl. Acad. Sci. USA* **83**, 5813-5816
3. Hoshino, S., Kikkawa, S., Takahashi, K., Itoh, H., Kajiro, Y., Kawasaki, H., Suzuki, K., Katada, T., and Ui, M. (1990) Identification of sites for alkylation by *N*-ethylmaleimide and pertussis toxin-catalyzed ADP-ribosylation on GTP-binding proteins. *FEBS Lett.* **276**, 227-231
4. Matsuyama, S. and Tsuyama, S. (1991) Mono-ADP-ribosylation in brain: Purification and characterization of ADP-ribosyltransferases affecting actin from rat brain. *J. Neurochem.* **57**, 1380-1387
5. Fujita, H., Okamoto, H., and Tsuyama, S. (1995) ADP-ribosylation in adrenal glands: Purification and characterization of mono-ADP-ribosyltransferases and ADP-ribosylhydrolase affecting cytoskeletal actin. *Int. J. Biochem. Cell Biol.* **27**, 1065-1078
6. Tsuyama, S., Matsuyama, S., Fujita, H., Awa, T., and Takahashi, K.P. (1992) Mono-ADP-ribosylation of actin and GTP-binding protein in neurotransduction in rat brain in *Recent Advances in Cellular and Molecular Biology* (Wegmann, R.J. and Wegmann, M.A., eds.) Vol. 3, pp. 95-107, Peeters Press, Leuven
7. Ueda, T. and Hayaishi, O. (1985) ADP-ribosylation. *Annu. Rev. Biochem.* **54**, 73-100
8. Nakane, M., Arai, K., Saheki, S., Kuno, T., Buechler, W., and Murad, F. (1990) Molecular cloning and expression of cDNAs coding for soluble guanylate cyclase from rat lung. *J. Biol. Chem.* **265**, 16841-16845
9. Stone, J.R. and Marletta, M.A. (1994) Soluble guanylate cyclase from bovine lung: Activation with nitric oxide and carbon monoxide and spectral characterization of ferrous and ferric states. *Biochemistry* **33**, 5636-5640
10. Stone, J.R. and Marletta, M.A. (1995) Heme stoichiometry of heterodimeric soluble guanylate cyclase. *Biochemistry* **34**, 14668-14674
11. Ignarro, L.J., Wood, K.S., and Wolin, M.S. (1982) Activation of purified soluble guanylate cyclase by protoporphyrin IX. *Proc. Natl. Acad. Sci. USA* **79**, 2870-2873
12. Ignarro, L.J., Adams, J.B., Horwitz, P.M., and Wood, K.S. (1986) Activation of soluble guanylate cyclase by NO-hemoproteins involves NO-heme exchange. *J. Biol. Chem.* **261**, 4997-5002
13. Tomita, T., Ogura, T., Tsuyama, S., Imai, Y., and Kitagawa, T. (1997) Effects of GTP on bound nitric oxide of soluble guanylate cyclase probed by resonance Raman spectroscopy. *Biochemistry*, in press
14. Wolin, M.S., Wood, K.S., and Ignarro, L.J. (1982) Guanylate cyclase from bovine lung: A kinetic analysis of the regulation of the purified soluble enzyme by protoporphyrin IX, heme, and nitrosyl-heme. *J. Biol. Chem.* **257**, 13312-13320
15. Hung, K.-P. and Robinson, J.C. (1976) A rapid and sensitive assay method for protein kinase. *Anal. Biochem.* **72**, 593-599
16. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685
17. Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-254
18. de Duve, C. (1948) A spectrophotometric method for the simultaneous determination of myoglobin and haemoglobin in extracts of human muscle. *Acta Chem. Scand.* **2**, 264-289
19. White, K.A. and Marletta, M.A. (1992) Nitric oxide syntase is a cytochrome P-450 type hemoprotein. *Biochemistry* **31**, 6627-6631
20. Gerzer, R., Radany, N.E., and Garbers, D.L. (1982) The separation of the heme and apoheme forms of soluble guanylate cyclase. *Biochem. Biophys. Res. Commun.* **108**, 678-686
21. Jurnak, F. (1988) The three-dimensional structure of c-H-ras p21: implications for oncogene and G protein studies. *Trends Biochem. Sci.* **13**, 195-198
22. Koesling, D., Herz, J., Gausepohl, H., Niroomand, F., Hinsch, K.-D., Mulsh, A., Bohme, E., Schultz, G., and Frank, R. (1988) The primary structure of the larger subunit of soluble guanylyl cyclase from bovine lung. Homology between the two subunits of the enzyme. *FEBS Lett.* **239**, 29-34
23. Moss, J. and Vaughan, M. (1979) Activation of adenylate cyclase by choleragen. *Annu. Rev. Biochem.* **48**, 581-600
24. West, R.E. Jr., Moss, J., Vaughan, M., Liu, T., and Liu, T.-Y. (1985) Pertussis toxin-catalyzed ADP-ribosylation of transdusin cysteine 347 is the ADP-ribose acceptor site. *J. Biol. Chem.* **260**, 14428-14430
25. Sekine, A., Fujiwara, M., and Narumiya, S. (1989) Asparagine residue in the rho gene product is the modification site for botulinum ADP-ribosyltransferase. *J. Biol. Chem.* **264**, 8602-8605
26. Wedel, B., Humbert, P., Harteneck, C., Foerster, J., Malkewitz, J., Bohme, E., Schultz, G., and Koesling, D. (1994) Mutation of His-105 in the β_1 subunit yields a nitric oxide-insensitive form of soluble guanylyl cyclase. *Proc. Natl. Acad. Sci. USA* **91**, 2592-2596
27. Yuen, P.S.T., Doolittle, L.K., and Garbers, D.L. (1994) Dominant negative mutants of nitric oxide-sensitive guanylyl cyclase. *J. Biol. Chem.* **269**, 791-793
28. Gilman, A.G. (1987) G proteins: transducers of receptor-generated signals. *Annu. Rev. Biochem.* **56**, 615-649
29. Ingi, T., Cheng, J., and Ronnett, G.V. (1996) Carbon monoxide: An endogenous modulator of the nitric oxide-cyclic GMP signaling system. *Neuron* **16**, 835-842
30. Kharitonov, V.G., Sharma, V.S., Pilz, R., Magde, D., and Koesling, D. (1995) Basis of guanylate cyclase activation by carbon monoxide. *Proc. Natl. Acad. Sci. USA* **92**, 2568-2571
31. Pennington, S.R. (1994) GTP-binding proteins: Heterotrimeric G proteins. *Protein Profile* **1**, 208-215