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Purified soluble guanylyl cyclase expressed in a baculovirus/Sf9 system: stimulation by YC-1, nitric oxide, and carbon monoxide

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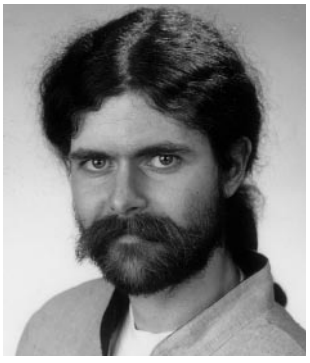

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Abstract Soluble guanylyl cyclase (sGC) is the main receptor for nitric oxide, a messenger molecule with multiple clinical implications. Understanding the activation of sGC is an important step for establishing new therapeutic principles. We have now overexpressed sGC in a baculovirus/Sf9 system optimized for high protein yields to facilitate spectral and kinetic studies of the activation mechanisms of this enzyme. It was expressed in a batch fermenter using a defined mixture of viruses encoding the α_1 and β_1 subunits of the rat lung enzyme. The expressed enzyme was purified from the cytosolic fraction by anion exchange chromatography, hydroxyapatite chromatography, and size exclusion chromatography. By use of this new method 2.5 l culture yielded about 1 mg of apparently homogeneous sGC with a content of about one heme per heterodimer without the need of a heme reconstitution step. The enzyme did not contain stoichiometric amounts of copper. The basal activities of the purified enzyme were 153 and 1259 nmol min^{-1} mg^{-1} in the presence of Mg^{2+} and Mn^{2+}, respectively. The nitric oxide releasing agent 2-(*N*,*N*-diethylamino)-diazenolate-2-oxide (DEA/NO) stimulated the enzyme 160-fold with Mg^{2+}, whereas the NO-independent activator 3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole (YC-1) induced an increase in the activity of 101-fold at a concentration of 300 µM. The combination of DEA/NO (10 µM) and YC-1 (100 µM) elicited a dose-dependent synergistic stimulation with a maximum of a 792-fold increase over the basal activity in the presence of Mg^{2+}, resulting in a specific activity of 121 µmol min^{-1} mg^{-1}. The synergistic stimulation of DEA/NO and YC-1 was attenuated by the sGC inhibitor 1*H*-(1,2,4)oxadiazole(4,3-a)quinoxalin-1-one (ODQ) (10 µM) by 94%. In a different experimental setup a saturated carbon monoxide solution in the absence of ambient oxygen or NO stimulated the enzyme 15-fold in the absence and 1260-fold in the presence of YC-1 compared to an argon control. The heme spectra of the enzyme showed a shift of the Soret peak from 432 to 399 and 424 nm in the presence of DEA/NO or carbon monoxide, respectively. The heme spectra were not affected by YC-1 in the absence or in the presence of DEA/NO or of carbon monoxide, which

reflects the fact that YC-1 does not interact directly with the heme group of the enzyme. In summary, this study shows that our expression/purification procedure is suitable for producing large amounts of highly pure sGC which contains one heme per heterodimer without a reconstitution step. The activator experiments show that in a synergistic stimulation with YC-1 sGC can be activated maximally both by nitric oxide and by carbon monoxide and that YC-1 does not directly act via heme. The described method should help to facilitate the investigation of the new therapeutic principle of NO-independent guanylyl cyclase activators.

Key words Guanylate cyclase (isolation and purification) · YC-1 · Nitric oxide (pharmacology) · Heme (physiology) · Recombinant proteins (biosynthesis)

Abbreviations *DEA/NO* 2-(*N*,*N*-Diethylamino)-diazenolate-2-oxide · *DMSO* Dimethyl sulfoxide · *FCS* Fetal calf serum · *ODQ* 1*H*-(1,2,4)Oxadiazole(4,3-a)quinoxalin-1-one · *sGC* Soluble guanylyl cyclase · *SNP* Sodium nitroprusside · *YC-1* 3-(5'-Hydroxymethyl-2'-furyl)-1-benzylindazole

Introduction

The NO/cGMP signal transduction pathway can be found in virtually any mammalian cell and plays a key role in a variety of physiological processes such as vasodilation, antiaggregation, antiproliferation, and neuronal signaling as well as in a variety of disorders of these functions [1]. Soluble guanylyl cyclase (sGC) is a highly regulated enzyme within this pathway and, together with particulate guanylyl cyclases, belongs to a family of nucleotide cyclizing enzymes [2]. sGC catalyzes the formation of the second messenger cGMP from its substrate GTP. The enzyme can be highly activated by nitric oxide [3] and by the recently described NO-independent activator 3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole (YC-1) [4], and to a lesser extent by carbon monoxide [5] and by fatty acids [6].

Several sGC isoforms from various species and various tissues have been sequenced and/or purified. Gerzer et al. [7] purified bovine lung sGC and showed that the enzyme is a dimer and contains stoichiometric amounts of heme and copper [8]. Kamisaki et al. [9] purified the corresponding enzyme from rat lung and determined the subunit sizes as 82 kDa for the α_1 and 70 kDa for the β_1 subunit. Later these [10] and other subunits (see review [11]) were cloned and sequenced.

The stimulation of the enzyme by NO and CO is mediated via the heme moiety. The activators form an adduct with the iron center and elicit a conformational change which leads to an increased catalytic activity. The binding of NO and CO to the heme group can be visualized in heme spectra, which show a shift of the absorption maximum from 432 nm to lower wavelengths [8].

The mechanism of activation by YC-1 is still under investigation. Since the first description of YC-1 as an inhibitor of platelet aggregation mediated by an intracellular increase in cGMP [4, 12] we and other groups have been studying the mechanism of action of YC-1 on the isolated enzyme and in the cardiovascular system [13, 14]. With purified enzyme from bovine lung it was shown that the activation of the sGC by both NO and CO is enhanced by YC-1 in an overadditive manner. So far YC-1 has been shown to be a heme-dependent but NO-independent sGC activator which potentiates the responses of NO donors, CO, and protoporphyrin IX [15]. Due to its effects on the vasculature, on platelet aggregation [16] and adhesion [17], and on vascular smooth muscle cell proliferation [18], YC-1 has proved be a new and promising pharmacological principle which may lead to a potential therapeutic advantage over nitrovasodilators in a variety of applications, including cardiovascular diseases [11].

Bovine lung has long been the common source of purified sGC. However, the purification methods are usually tedious and time consuming [8, 19]. Cyclases from other tissues or from other species, for example, from humans, are difficult to obtain as the tissue amounts are usually limited. This limitation can be overcome by switching to an overexpression system which can be used to express a variety of different proteins. Therefore, the aim of this study was to upscale the baculovirus/Sf9 expression system and to develop a purification procedure which yields milligram quantities of purified sGC, thus facilitating further biochemical studies into the activation mechanisms and crystallization experiments. Special attention was paid to the heme content of the expressed and of the purified enzyme to obtain a preparation which best resembles enzymes purified from animal tissues. This enzyme was characterized using various types of activators such as YC-1, NO, and CO, their synergistic effects and the blocking effects of 1*H*-(1,2,4)oxadiazole(4,3-a)quinoxalin-1-one (ODQ).

Materials and methods

Sf9 cell culture

Suspension cultures of *Spodoptera frugiperda* (Sf9) cells were grown in flasks or in roller bottles at 28°C. SF900II medium was supplemented with 10% fetal calf serum (FCS).

Baculovirus stocks

A mixture of baculoviruses encoding the α_1 and β_1 subunits of sGC was prepared as described previously [20]. The viruses were separated by a plaque assay, and two separate virus stocks for the α_1 and β_1 subunits were produced.

Baculovirus/Sf9 expression system

A 3-l batch bioreactor stirred with a silicone tubing impeller was used for large-scale protein expression and for the production of virus stock. The bioreactor was filled with 2.5 l SF900II culture medium containing 10% FCS at 28°C and was inoculated with Sf9 cultures to give a count of 2×10^5 cells/ml. The headspace was flushed with air at a constant rate, and the impeller tubing was flushed with air and oxygen to keep the oxygen partial pressure in the culture

constantly at 50% of the ambient air. The cells were grown to 2×10^6 cells/ml, which still is in the exponential phase of growth, and were then infected with a mixture of the virus stocks for the α_1 and β_1 subunits for protein production or with a single virus stock for virus amplification. We had previously shown that a multiplicity of infection of 4–6 pfu/cell results in a high level of expression [20]; for reasons of economy a multiplicity of infection 4 pfu/cell was used routinely. In the case of protein production the cells were harvested 88 h after infection and stored at –85°C. For virus amplification the cells were pelleted by centrifugation 120 h after infection, and the supernatant was stored at 4°C for later use as virus stock.

Purification procedure

All purification steps were performed at 4°C. The cell pellets from one bioreactor run were thawed on ice and were homogenized with a Dounce homogenizer in 3 vol 20 mM bis-Tris buffer (pH 6.7) containing 1 mM EDTA, 5 mM dithiothreitol, 90 mM NaCl, 10% (v/v) glycerol, 1 µg/ml pepstatin A, 10 µg/ml phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, 10 µg/ml leupeptin, and 16 µg/ml benzamidine. The homogenate was centrifuged at 50,000 *g* for 30 min, and 120 ml supernatant was collected. All chromatographic steps were performed on a FPLC system (Pharmacia, Freiburg, Germany). The protease inhibitors benzamidine (1 mM) and phenylmethylsulfonyl fluoride (10 µg/ml) were used in all chromatographic steps. The supernatant was immediately applied to a Macroprep Q column (BioRad, Munich, Germany, 12×5 cm) at 3 ml/min. Ion exchange buffer A contained 20 mM bis-Tris (pH 6.7), 5 mM glutathione, 0.1 mM EDTA, and 5% (v/v) glycerol. Ion exchange buffer B was prepared by adding 1 M NaCl to buffer A. The column was washed at 9% B until OD_{280} was stable. A linear gradient from 9% B to 21% B for 625 ml was used to elute sGC. The sGC-containing fractions were pooled by determining the OD_{432}/protein content ratio as a measure for the specific heme content.

Previous experiments had shown that the yellow peak as measured at 432 nm exactly matched the peak of sodium nitroprusside (SNP) stimulated sGC activity. The pooled fractions (120 ml) were applied immediately to a ceramic hydroxyapatite column (BioRad, 8×1.6 cm) at 1.5 ml/min. Hydroxyapatite buffer A contained 10 mM potassium phosphate (pH 6.4), 5 mM glutathione, and 250 mM NaCl. Hydroxyapatite buffer B was the same except that it contained 500 mM potassium phosphate. The column was then washed with 9% B until OD_{280} was stable. The enzyme was eluted with a linear gradient running from 9% B to 30% B for 250 ml. The sGC-containing fractions (20 ml) were again pooled as described above and concentrated in centrifugal devices with a 50-kDa cutoff (Pall Filtron, Dreieich, Germany) to 2 ml. The enzyme was then loaded on a Superdex 200 column (Pharmacia, 60×2.6 cm) and eluted with a 20 mM triethanolamine HCl buffer (pH 7.5) containing 0.1 mM EDTA, 250 mM NaCl, 5% (v/v) glycerol, and 5 mM dithiothreitol. The fractions with the highest OD_{432}/protein content ratio were pooled and concentrated as described above to a protein content of about 0.5 mg/ml. The purified enzyme was stored at 4°C, and all subsequent analyses were completed within 5 days.

Guanylyl cyclase assay

Enzyme activity was measured by the formation of [^{32}P]cGMP from α-[^{32}P]GTP modified according to Gerzer [8]. The modifications included using GTP, Mn^{2+}/Mg^{2+}, and cGMP at concentrations of 200 µM, 3 mM, and 1 mM, respectively. Enzyme concentrations were chosen carefully to achieve a substrate turnover of less than 10%, thus avoiding substrate or cofactor depletion. The characterization of the purified enzyme was performed at a protein concentration of 0.01 µg/ml. For enzyme characterization the specific activity of sGC was expressed as the multiple stimulation vs. specific basal activity. The specific basal activity of the enzyme was determined as 153±40 nmol min^{-1} mg^{-1} with Mg^{2+} and as 1259±253 nmol min^{-1} mg^{-1} with Mn^{2+} as a cofactor. The highest dimethyl sulfoxide (DMSO) concentration in the assay was 1% (v/v) and did not elicit any effect per se on cGMP production.

In a separate set of experiments sGC was stimulated with CO. These experiments were performed in septum flasks which contained the incubation mixture as described above. The headspace of the flasks was flushed with argon and then gassed with either CO or argon for 10 min under constant motion to achieve a saturated solution, corresponding to 890 µM CO. The reaction was started by adding GTP and at regular intervals samples were withdrawn with a syringe and immediately quenched by addition of zinc acetate and sodium carbonate. The increase in cGMP with the incubation time was linear for at least 15 min.

Spectroscopic studies

UV/Vis spectra were recorded from 350 to 650 nm on a DU 640 spectrophotometer (Beckman, Munich, Germany). NO was introduced via an aqueous solution of DEA/NO. The enzyme solution was saturated with CO by flushing the headspace of a septum flask containing 100 µl enzyme solution with a stream of CO for 10 min under constant motion. A 100 mM stock solution of YC-1 in DMSO was prepared and added in a final concentration of 10 µM, resulting in a final DMSO concentration of 0.1% (v/v). Previous experiments had shown that this DMSO concentration does not alter the properties of the enzyme. Pyridine-hemochrome spectra were recorded as described [21]. In brief, pyridine and NaOH were added to the sample at final concentrations of 25% (v/v) and 60 mM, respectively, and a spectrum was recorded from 350 to 650 nm. Reduction with dithionite did not alter the sGC spectra and therefore was not performed in these experiments. The heme content was calculated from a standard curve obtained with reduced myoglobin.

Protein determination

Protein content was determined according to the Bradford method [22] using bovine serum albumin as the standard. The protein contents of highly purified fractions were confirmed by calculating the protein concentration from optical densities using the formula [23]:

$$c=1.55\ (A_{280}-A_{320})-0.76\ (A_{260}-A_{320})$$

Determination of copper content

The copper content was determined by electrothermal atom absorption spectroscopy using bovine liver standard as a reference.

Analytical polyacrylamide gel electrophoresis

Both native and sodium dodecyl sulfate polyacrylamide gel electrophoreses were performed on a PhastSystem (Pharmacia, Freiburg, Germany) with precast gels and a silver staining method slightly modified from the manufacturer's protocols.

Statistical analysis

Unless otherwise indicated, the results represent means±SEM from at least three independent experiments performed in duplicate. Statistical analysis was performed either by analysis of variance followed by the Bonferoni test for comparison of means or by the *t* test as appropriate. The level of $P<0.05$ was considered statistically significant.

Materials and substances

α-[^{32}P]-GTP was obtained from NEN-DuPont (Dreieich, Germany). All cell culture media and supplements were from Life Technologies (Eggenstein, Germany). CO (4.7, >99.997%) and argon were obtained from Messer Griesheim (Krefeld, Germany). YC-1 was synthesized at Bayer by C. Robyr as described [24] and was used as a 10 mM stock solution in DMSO. ODQ was obtained from Tocris

Cookson (Bristol, U.K.) and 10 mM stock solutions were prepared in DMSO. DEA/NO was obtained from Alexis (San Diego, USA) and diluted freshly to each experiment on ice in 0.01 M NaOH. All other biochemicals were of the highest purity available and were purchased from Merck (Darmstadt, Germany), Boehringer Mannheim (Mannheim, Germany), and Sigma (Deisenhofen, Germany).

Results

Expression

For the overexpression of sGC a polyhedrin-promotor based baculovirus/Sf9 system was used. We had shown previously that the expression level 72 h after infection is higher than that after 24 h or 48 h [20]. To monitor the time-dependent expression of sGC activity more closely, aliquots of cells were harvested from 64 to 112 h after infection, and a cytosolic preparation was analyzed for sGC activity. sGC activity in the absence of a NO donor decreased slightly with incubation time, while SNP-stimulated activity showed a broad optimum at around 88 h after infection (not shown). This maximum coincided with the beginning of a sharp decrease in cell viability, indicating that the protein synthesis has ceased. Therefore in all further bioreactor runs cells were harvested at 88 h after infection. On optimizing an equal expression of the two subunits of sGC no defined optimum for the ratio of the two viruses was found, although the experiments showed that the α-virus had to be added in at least twice the concentration of the β-virus to obtain both a high total activity and a high stimulation. Therefore in all subsequent experiments a ratio of 3:1 (α:β) was used which is close to the ratio of 2.5:1 recently published by Gupta et al. [25].

The SF900II medium is designed for serum-free culture of insect cells. Addition of 10% FCS increased significantly the stimulation by 100 µM SNP of the sGC activity in the cytosolic fractions, indicating a higher heme incorporation (*t* test, $P<0.01$, Table 1). Total basal activity tended to be lower and total SNP-stimulated activity to be higher when using FCS, but the differences were not statistically significant. Therefore FCS was added to the medium in all further experiments. Addition of up to 4 µg/ml hemin to the culture medium supplemented with 10% FCS during expression did not further increase the stimulation of the sGC activity in the cytosols (data not shown).

Table 1 Effects of the addition of FCS on the expression of sGC

	Total basal activity (nmol min^{-1})	Total stimulated activity (nmol min^{-1})	Stimulation (-fold)
Without FCS (n=3)	27,580±7120	125,090±17,310	4.8±0.58
With FCS (n=12)	19,840±2590	188,470±24,720	9.7±0.63

Cells were harvested from fermenter runs, and cytosolic fractions were assayed for sGC activity under basal and stimulated (100 µM SNP) conditions with Mn^{2+} as a cofactor. The stimulation was significantly higher when FCS was used (unpaired *t* test, $P<0.01$); however, total activities did not differ significantly

Purification

In all chromatographic steps a single yellow peak appeared during elution. In the ion-exchange step sGC activity appeared in two peaks. SNP-stimulated sGC was present only in the first peak (>10-fold stimulation by 10 µM SNP), which matched the yellow peak, while the second peak contained mainly the heme-free enzyme (<2-fold stimulation), as published previously [26]. Only the heme-containing form was carried over to the next step. In the hydroxyapatite and size exclusion steps, basal and SNP-stimulated sGC eluted as a single peak. Table 2 summarizes a representative purification of sGC. The heme-containing enzyme was enriched more than 160-fold with a recovery of 9.1%. Native polyacrylamide gel electrophoresis showed a single band, while that with sodium dodecyl sulfate on gradient and homogeneous gels separated the two subunits (Fig. 1) and displayed apparent homogeneity.

Determination of heme and copper content

The pyridine-hemochrome spectra showed the α-, β-, and Soret-bands characteristic of the ferroprotoporphyrin IX-pyridine-hemochrome. Using reduced myoglobin spectra as a standard (ε=95.7 mM^{-1} cm^{-1}), the heme content of the

Table 2 Purification of sGC overexpressed in a baculovirus/Sf9 system

		Specific activity (nmol min^{-1} mg^{-1})		Total activity (nmol min^{-1})		Purification (-fold)	Recovery (%)
Step	Protein (mg)	–DEA/NO	+DEA/NO	–DEA/NO	+DEA/NO	+DEA/NO	+DEA/NO
Cytosol	1613	16.2	109.3	26,192	17,6198	1	100
Ion exchange	41.0	131.6	2,335	5,396	95,727	21.4	54.3
Hydroxyapatite	5.2	493.3	7,267	2,581	38,020	66.5	21.6
Size exclusion	0.9	1127	18,081	995.4	15,969	165.5	9.1

Activities were measured in the presence or absence of DEA/NO (100 µM) and with Mn^{2+} as a cofactor. The data shown are representative for four independent purifications

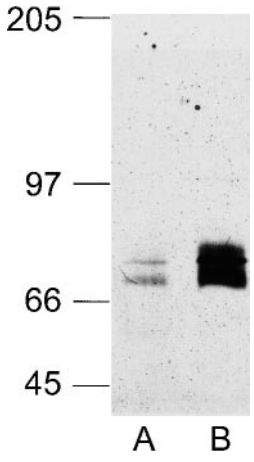

Fig. 1 Silver-stained SDS gel of purified sGC. A 7.5% homogeneous gel was loaded with 3.9 ng (**A**) and 7.8 ng (**B**) protein. The sizes of the marker proteins are indicated in kDa

preparation shown in Table 2 and Fig. 1 was calculated as 1.02 mol heme per mol heterodimer. Three similar preparations showed between 0.91 and 0.95 mol heme per mol heterodimer, thus indicating a stoichiometry of one heme per heterodimer. The copper content was measured in two preparations (13.0±0.6/13.4±1.0 and 3.4±0.5/4.3±0.4 ng/ml for the corresponding buffer controls). Based on protein concentrations of 0.59 and 0.55 mg/ml, respectively, the mean copper content was calculated as 0.04 mol Cu per mol heterodimer suggesting a nonstoichiometric contamination.

Comparison of recombinant sGC with native sGC

To compare purified recombinant enzyme to sGC purified from bovine lung [8] we prepared dose-response curves of the NO donor SNP. Figure 2 shows that the specific activity at the maximum of the SNP dose-response curve with Mg^{2+} as a cofactor was less than 50% of that of the SNP dose-response curve in the presence of Mn^{2+}. As the purified bovine lung enzyme shows the same or even more stimulated activity with Mg^{2+} than with Mn^{2+} at the maximum of the SNP dose-response curves [8], we prepared crude sGC containing fractions from rat lung and bovine lung by performing the homogenization and ion-exchange steps of the method described in [8], using the same buffers as with the recombinant enzyme. However, these preparations were found to closely resemble the corresponding purified enzymes, indicating that the use of an expression system was not the cause of the observed difference.

Characterization of sGC

To examine the characteristics of highly purified sGC from Sf9 cells we studied the effects of DEA/NO, YC-1, and CO on activation of the highly purified sGC and the blocking effects of ODQ. The specific basal activity of the enzyme in this set of experiments was determined as 153±40 nmol min^{-1} mg^{-1} with Mg^{2+} and 1259±253 nmol min^{-1} mg^{-1} with Mn^{2+}. Stimulation by the various activators or

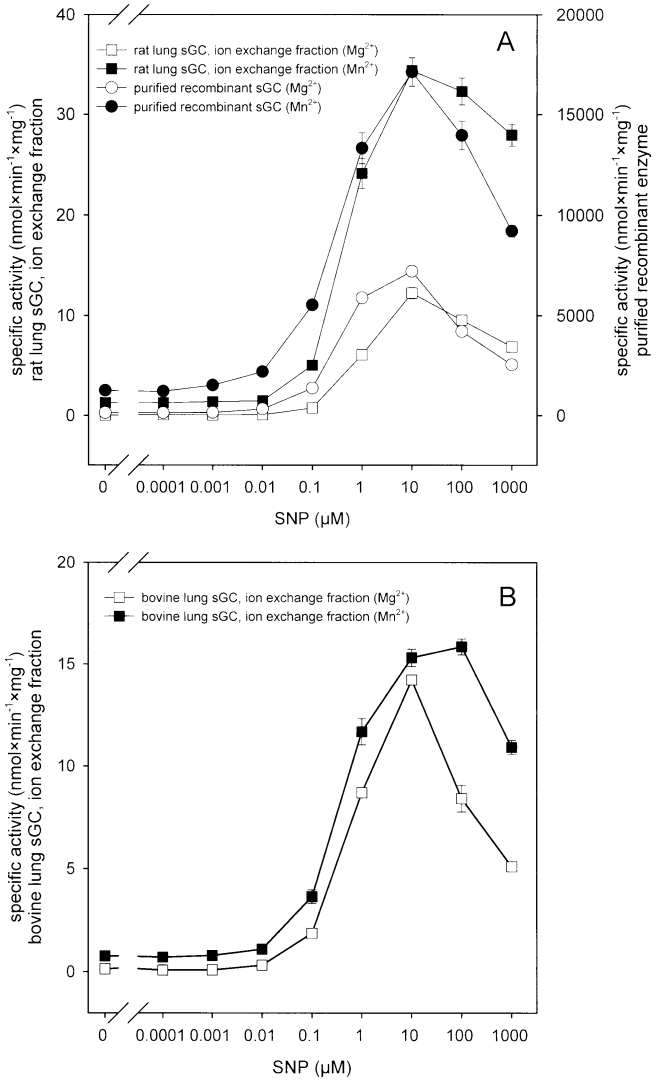

Fig. 2 Comparison of the SNP dose-response curves of the purified recombinant enzyme and partially purified sGC from rat lung (**A**) and bovine lung (**B**). Data are means±SEM from two independent experiments performed in triplicate

combinations of activators was expressed as multiples of stimulation vs. basal specific activity with regard to the physiological cofactor Mg^{2+}. DEA/NO showed a concentration-dependent activation of sGC with a maximal effect of 160±14-fold activation (100 µM DEA/NO) and an EC_{50} value of 0.5 µM. YC-1 activated sGC with a maximal effect of 101±5-fold (300 µM YC-1; Fig. 3). In combination studies (Fig. 4) YC-1 and DEA/NO showed synergistic effects over the whole range of the dose response curve, with a maximal activation of sGC by DEA/NO (10 µM) and YC-1 (100 µM) of 792±102-fold and a shift of EC_{50} values of DEA/NO and YC-1 by about one order of magnitude to lower concentrations. This level of activation seems to be maximal for purified sGC from Sf9 cells with a maximum specific activity of about 121 µmol min^{-1} mg^{-1}. To rule out cGMP elevating unspecific effects we investigated the blocking effects of ODQ, a potent and se-

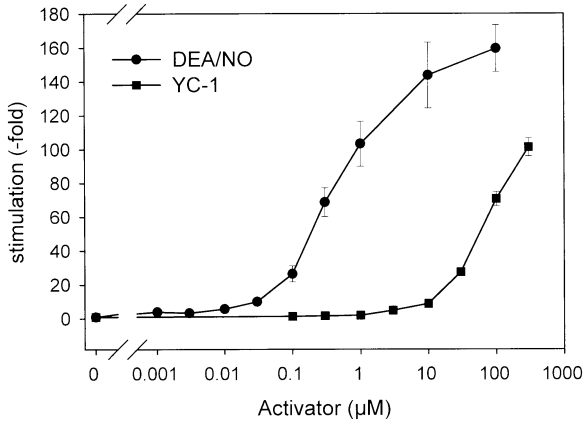

Fig. 3 Stimulation of purified sGC as functions of the concentrations of DEA/NO and of YC-1. Data are means±SEM from four (DEA/NO) or five (YC-1) independent experiments performed in duplicate

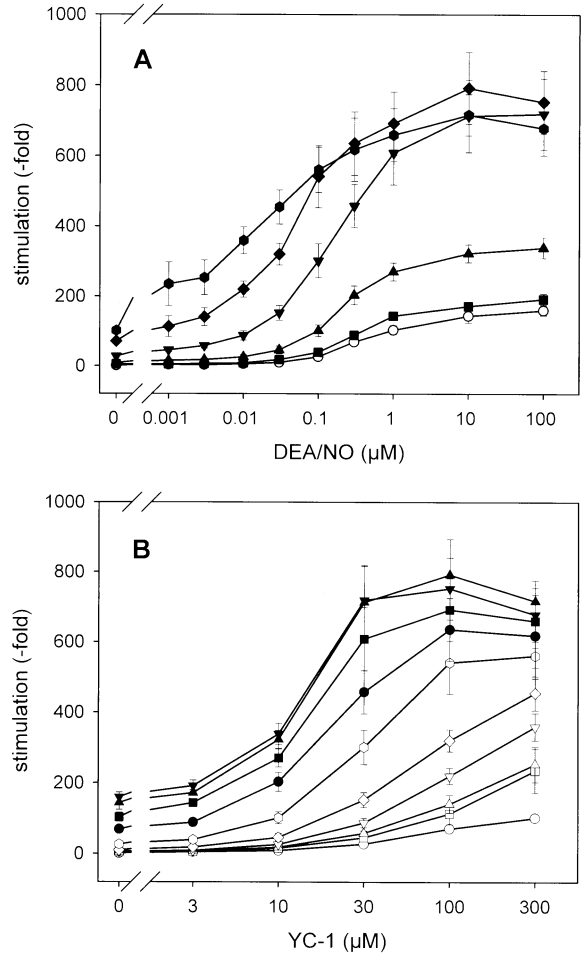

Fig. 4A,B Synergistic effects between DEA/NO and YC-1 induced sGC stimulation. **A** sGC activity was measured in the presence of increasing concentrations of DEA/NO in the absence (○) or presence of 3 (■), 10 (▲), 30 (▼), 100 (◆), or 300 (●) μM YC-1 with Mg^{2+}. **B** The same data shown as a function of the YC-1 concentration in the absence (○) or presence of 0.001 (❑), 0.003 (Δ), 0.01 (▽), 0.03 (◊), 0.1 (⬡), 0.3 (●), 1 (■), 10 (▲), 100 (▼) μM DEA/NO. Data are means±SEM from four independent experiments performed in duplicate.

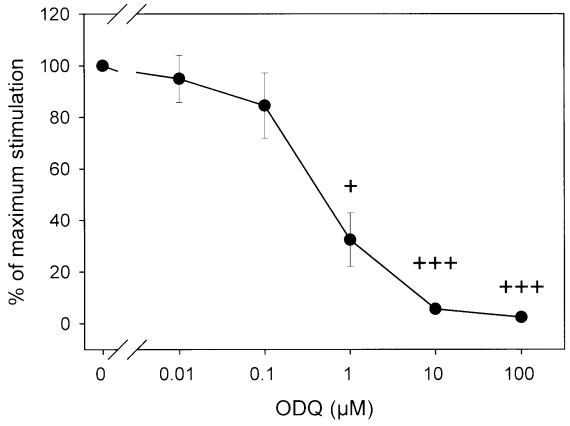

Fig. 5 Inhibition of synergistic effects on sGC of DEA/NO (0.1 μM) and YC-1 (100 μM) by ODQ. sGC activity was measured in the presence of increasing concentrations of ODQ in the presence of DEA/NO (0.1μM) and YC-1 (10 μM) with Mg^{2+} as a cofactor. sGC activity without ODQ was taken as 100%. Data are means±SEM from two independent experiments performed in duplicate. For comparison of increasing ODQ concentrations vs. unblocked sGC stimulation: $^{+}P<0.05$, $^{+++}P<0.001$

Table 3 Fatty acids fail to stimulate sGC

	3 μM	30 μM	300 μM
Linoleic acid	1.07±0.05	1.03±0.09	2.80±1.24
Arachidonic acid	0.83±0.04	0.73±0.02	0.81±0.02

Using Mg^{2+} as a cofactor, arachidonic acid and linoleic acid were added as stock solutions in DMSO. The multiples of stimulations±SEM were calculated from the basal specific activity of 153±40 nmol min^{-1} mg^{-1}. There was no significant effect of the fatty acids (analysis of variance)

Table 4 Stimulation of sGC with CO and YC-1

Condition	Specific activity (nmol min^{-1} $mg^{-1)}$
Basal	46.4± 11.2
+CO	683.5± 78.0
+YC-1 (3 μM)	43.7± 19.7
+YC-1 (100μM)	4,335±701.0
+CO+YC-1 (3 μM)	6,810±124.7
+CO+YC-1 (100μM)	58,633±867.5

The experiments were performed in septum flasks with permanent gas flow as described in the text with Mg^{2+} as a cofactor (n=3)

lective sGC inhibitor [27, 28] on DEA/NO and YC-1 stimulated enzyme activity. ODQ showed a concentration- dependent inhibition of sGC activation (IC_{50}=0.6 μM) to levels below the stimulation with either of the activators. Thus ODQ (10 μM) inhibited sGC stimulation of the combination of DEA/NO (0.1 μM) and YC-1 (10 μM) to 7% of the maximum stimulation (Fig. 5). The fatty acids arachidonic acid and linoleic acid at concentrations up to 300 μM did not significantly stimulate the enzyme (Table 3).

Table 5 Effects of sGC activators on Soret peak wavelengths

Condition	Soret peak wavelength (nm)	
	control	+YC-1 (10 µM)
Control	432.0	432.0
+DEA/NO (1 mM)	399.0	399.5
+CO (saturated)	424.0	424.0

These data are representative for three independent determinations. The solutions contained 210 µg/ml enzyme

In contrast to the experiments described above, activation with CO was determined under a constant gas stream. The samples were either equilibrated with CO before adding the substrate, resulting in a concentration of 890 µM CO or with argon as a control. As pure gases were used, all constituents of the ambient air which may affect sGC activity, such as oxygen and NO, were thus excluded from the incubation mixture. Due to these differences the basal activity was lower than that obtained in open tubes (Table 4). CO activated the enzyme 15-fold over the controls gassed with argon. The activation of the enzyme with CO and different concentrations of YC-1 resulted in an increase in stimulation up to 1260-fold, resulting in a specific activity of 58.6±0.9 µmol min^{-1} mg^{-1}. YC-1 at a concentration of 3 µM did not significantly increase sGC activity in these experiments. However, the activation of 3 µM YC-1 together with CO was significantly greater than the activation with CO alone.

Spectroscopic studies

NO and CO are heme-directed activators of sGC and as such affect the heme spectra of sGC. We investigated the effect of the sGC activators NO, CO, and YC-1 on the spectra of the enzyme (Fig. 6). NO and CO elicited the characteristic shift of the Soret peak to lower wavelengths (Table 5). YC-1 did not shift this peak, but sGC was significantly stimulated under this condition (see above). When we added YC-1 to a NO- or CO-stimulated enzyme, the Soret peaks were shifted maximally 0.5 nm to the right or remained unaffected, respectively.

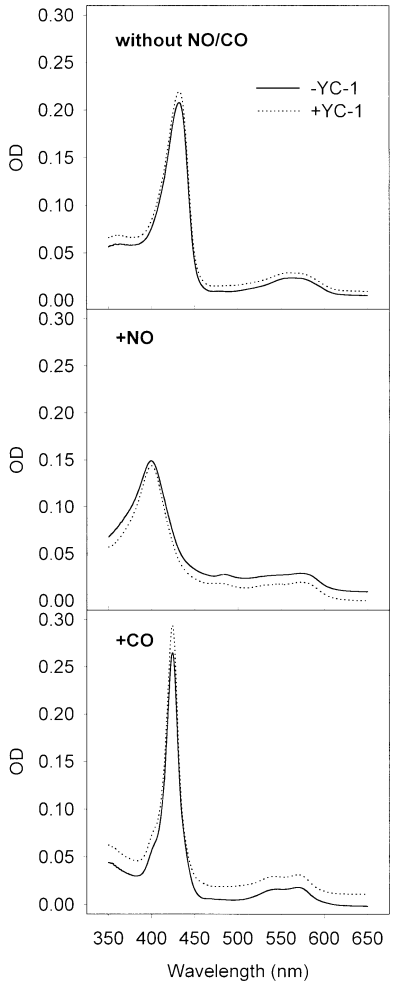

Fig. 6 Heme spectra of sGC under basal conditions and in the presence of activators. These data are representative for three independent determinations. The solutions contained 210 µg/ml enzyme

Discussion

In this study we describe the large-scale purification of a heme-containing sGC expressed in a baculovirus/Sf9 system. We used various types of activators and combinations of activators to obtain a full characterization of the recombinant enzyme that is suitable as the starting point for future development of new target structures by molecular drug design.

Two criteria must be considered in overexpressing sGC: the total amount of functional protein and the heme content. Buechler et al. [20] showed that the baculovirus/Sf9 system can be used to express milligram quantities of native, heme-containing sGC. Coexpression of the two subunits is mandatory for obtaining a catalytically active enzyme [29, 30]. Our expression system was optimized by infecting during the exponential growth of the Sf9 cells and by keeping the oxygen concentration constant in spite of the largely increased consumption during expression [31]. The ratio of the two viruses encoding the two subunits was chosen to result in a maximum stimulated total activity which indicates a high content of holoenzyme and a low content of unpaired subunits. The cells were harvested when the viability started to decrease, which occurred around 88 h after infection. In contrast to other proteins such as neuronal nitric oxide synthase [32] sGC thus seemed to be very stable in the Sf9 cells, which we had shown previously for up to 72 h [20].

The aim of this study was to develop an expression and purification procedure that is suitable for producing milligram amounts of highly pure heme-containing recombinant sGC without a heme-reconstitution step. The previously published baculovirus/Sf9 expression and purification

protocols for sGC show a large variation in the heme content and in the need to supplement heme precursors. sGC purified from bovine lung contains one heme per heterodimer without a reconstitution step [8], which is thought to be the native form of the enzyme in tissues. Gupta et al. [25] reported that the baculovirus/Sf9-expressed enzyme is heme free, but Buechler et al. [20] found that the recombinant enzyme contains heme only after supplementing the medium with hemin during expression. In contrast, Wedel et al. [33] purified an enzyme with a heme content of 0.3 without hemin supplementation, suggesting that at least a part of the expressed enzyme contained heme. In our system we found SNP-stimulated sGC in the cytosolic fractions of all bioreactor runs. The addition of hemin during the expression had no effect on the SNP-stimulated specific activity of the cytosolic fraction, suggesting that there was no lack of heme precursors. However, the heme content of expressed sGC in the cytosolic fraction seemed to be affected by FCS. The only major changes in our system compared to other published procedures are the use of a bioreactor with a defined oxygen partial pressure and the use of SF900II medium instead of TC-100 medium or TNM-FH medium. The composition of the SF900II medium, which was designed for serum-free culture, is proprietary to the manufacturer, but it is reasonable to assume that it is a much richer medium than other media which probably lack sufficient amounts of the heme precursors.

We used the pyridine-hemochromogen method to determine the heme content of our preparations. The protein contents as measured with the Bradford method, which were used for these calculations, were not corrected for amino acid composition as suggested by Stone and Marletta [19] because the values did not differ from the data obtained with the UV-photometric determination. The measured heme contents suggest a stoichiometry of one heme per heterodimer, which is in agreement with previous results for sGC purified from bovine lung from our group [7] and other groups [19]. In a later report Stone and Marletta [34] suggested a heme stoichiometry of 2 mol per heterodimer, based upon protein contents which were obtained by applying the above correction factor. However, when uncorrected data are compared, this preparation contained essentially the same amount of heme per heterodimer as our preparations. In two recent publications the same group unambiguously localized the heme-binding site to the N-terminal region of the β subunit with His-105 as the proximal ligand [35, 36]. As the α subunit does not possess a corresponding heme-binding domain with a conserved His residue [34], and only one type of heme is spectrally visible, the dispute over the heme stoichiometry seems to be settled in favor of the one heme per heterodimer hypothesis.

It is of major importance that our purification system yields a heme-containing enzyme without the need of a heme reconstitution step. The heme spectra are identical to the spectra obtained with sGC purified from animal tissues [7]. Therefore we believe that the heme in our preparations is exactly in the same environment as in the native enzyme. This is of utmost importance as differences in the properties between native heme-containing sGC and heme-reconstituted sGC have been reported. The Raman spectra have been shown to be different [37], and the stimulation of heme-depleted and reconstituted enzyme amounted to about 60% or less of that of the untreated control [38], suggesting that reconstitution steps may not result in a fully functional enzyme.

The purification procedure shown here has several advantages over previously known methods. All chromatographic steps are performed on an FPLC device and are highly reproducible. The procedure does not use affinity media such as GTP or ATP columns which have been reported to be of varying quality and thus make the procedures less reliable [34]. All media can be reused for at least several months without repacking. As during routine operation activity assays are not necessary between the chromatographic steps, the method is very fast (<30 h), which is a major advantage for the enzyme quality. Finally, the use of a size exclusion column as the last step facilitates the use of any buffer suitable for subsequent experiments without the need of buffer exchange steps with concomitant enzyme losses.

In contrast to our previous results with sGC purified from bovine lung [8], the expressed enzyme did not contain copper in stoichiometric amounts. Unfortunately, the copper contents of purified enzymes were not published by other authors, and further experiments will therefore have to show whether copper is a constituent of native sGC, and whether it has any physiological effect. However, since our pure enzyme has the highest specific activities after full stimulation published to date, we feel that copper is not required for a fully functional enzyme. This does not rule out a possible participation of a bound copper in the NO release from S-nitrosothiols.

To verify the activity of the enzyme preparation we characterized in this study the highly purified sGC using various types of sGC activators such as DEA/NO, YC-1, and CO. In addition, ODQ, a well-studied selective and potent inhibitor of sGC [27] was used to block the effects of the various activators on the highly purified enzyme. Beyond the direct effect of YC-1 on sGC, this compound has been reported to sensitize sGC for NO and CO activation on the purified enzyme, in vascular smooth muscle cells, and in precontracted aortic rings [13, 14]. Therefore we tested the purified enzyme for the synergistic effects of YC-1 and NO or YC-1 and CO. These studies were also performed to clarify whether the enzyme purified from a Sf9 cell system shows the same characteristics as purified native tissue enzymes. Indeed, characterization based upon DEA/NO, CO, YC-1, and ODQ shows that the purified enzyme does not differ in its characteristics from native enzymes. The basal specific activities of 153 nmol min^{-1} mg^{-1} with Mg^{2+} and 1259 nmol min^{-1} mg^{-1} with Mn^{2+} are among the highest of purified native bovine lung enzymes [7, 13, 14, 39, 40]. EC_{50} values of DEA/NO, YC-1, and ODQ do not differ from the described values in the literature [13, 39, 28]. Stimulation by YC-1 (101-fold) and CO (15-fold) was higher using our preparation than described

with recently published data concerning activation of purified enzymes from native tissues [13, 14, 19]. A stimulation of sGC at 792-fold by DEA/NO and YC-1 or at more than 1000-fold by CO and YC-1 had not been reported so far. By inhibition of the synergistic effect between DEA/NO and YC-1 by ODQ in a concentration-dependent manner by at least 93% we showed that stimulation by both activator types DEA/NO and YC-1 is blocked by ODQ. Therefore we also assured that the cGMP increase was mediated by activation of sGC. In conclusion, we obtained a highly purified enzyme with identical main characteristics as those of enzymes purified from tissues. However, it is more active in regard to stimulation by the various activator types of sGC, especially in regard to YC-1, and virtually insensitive to stimulation by fatty acids.

The maximum stimulated activity of the enzyme lies well in the range reported for the phosphorylated form of particulate guanylyl cyclase [41]. As the catalytic domains of soluble and particulate guanylyl cyclases show a considerable homology, one might speculate that these specific activities are close to the limit of this design of a catalytic site. Given the high basal activity and low stimulation of particulate guanylyl cyclases, this would mean that the catalytic sites of these enzymes are less deactivated by the lack of their activating ligand than is the soluble counterpart.

In contrast to substances such as SNP and DEA/NO, YC-1 does not stimulate sGC by releasing NO [13]. At concentrations which significantly increase enzyme activity YC-1 does not affect the heme spectrum of the enzyme in the presence or in the absence of a heme ligand. In agreement with the report of Friebe et al. [42], this suggests an allosteric regulation of the enzyme which does not involve a direct interaction with the heme moiety.

In the separate set of experiments using gas-tight vials and either CO or argon as the headspace gas we noted a marked difference in basal activities and in experiments in open vials exposed to the ambient air. The basal activities were reduced to 38% and 75% with Mg^{2+} and Mn^{2+}, respectively. Several reports have shown that sGC is subject to a redox regulation [43, 44, 45]. Recently it has been suggested that the NO content of the ambient air affects the basal activities of sGC [46]. By using highly purified gases we also excluded NO from the headspace which may be present in the air. We believe that the basal activities were lower than expected in our experiments due to the exclusion of oxygen and NO. Therefore the calculated stimulation factors are higher than expected and cannot be compared directly to data from assays in open tubes.

Interestingly, the SNP dose-response curves of the expressed rat lung enzyme shows different properties than in our previous data obtained from bovine lung sGC [7] regarding the effect of the required divalent metal. From the comparison of ion-exchange fractions and purified enzymes of the two species we conclude that a much lower specific activity under maximum SNP-stimulated conditions, when using the more physiological Mg^{2+} instead of Mn^{2+}, is typical for the rat lung sGC. These results indicate that there are kinetic differences between sGCs from different species. It should be further investigated whether such kinetic differences between sGC isozymes exist and can be exploited by the different types of activators.

In conclusion, our experiments show that the baculovirus/Sf9 system combined with our purification procedure is a useful method for producing highly purified, heme-containing and both NO and YC-1 stimulated sGC in quantities sufficient for a variety of biochemical investigations, including crystallization. Since the quality of our preparations appears to be superior to those in other published methods, we consider it an ideal tool for studies on the activation mechanism of the enzyme. In future studies we will use the enzyme in initial steps of molecular drug design to establish new compounds replacing or supplementing organic nitrates in therapeutic applications.

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