A Functional Domain of the $\alpha_1$ Subunit of Soluble Guanylyl Cyclase Is Necessary for Activation of the Enzyme by Nitric Oxide and YC-1 but Is Not Involved in Heme Binding*

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Soluble guanylyl cyclase is a heterodimeric enzyme consisting of an $\alpha_1$ and a $\beta_1$ subunit and is an important target for endogenous nitric oxide and the guanylyl cyclase modulator YC-1. The activation of the enzyme by both substances is dependent on the presence of a prosthetic heme group. It has been unclear whether this prosthetic heme group is sandwiched between the $\alpha_1$ and $\beta_1$ subunits or whether it exclusively binds to the $\beta_1$ subunit. Here we analyze progressive amino-terminal deletion mutants of the human $\alpha_1$ subunit after co-expression with the human $\beta_1$ subunit in the baculovirus/Sf9 system. Spectral, biochemical, and pharmacological analysis shows that the first 259 amino acids of the $\alpha_1$ subunit can be deleted without loss of sensitivity to nitric oxide (NO) or YC-1 or loss of heme binding of the respective enzyme complex with the $\beta_1$ subunit. This is in contrast to previous data indicating that NO sensitivity and a functional heme binding site requires full-length amino termini of both subunits (11, 12). Deletion of 364 amino acids of the $\alpha_1$ subunit leads to an enzyme complex with preserved heme binding but loss of sensitivity to NO or YC-1 despite induction of the typical spectral shift by NO binding to the prosthetic heme group. We conclude that 1) the amino-terminal part of the $\alpha_1$ subunit is not involved in heme binding and 2) amino acids 259–364 of the $\alpha_1$ subunit represent an important functional domain for the transduction of the NO activation signal and likely represent the target for NO-sensitizing substances like YC-1.

Soluble guanylyl cyclase (sGC) is an important target for endogenous nitric oxide (NO), NO-releasing drugs like glyceryl trinitrate, and novel substances like YC-1 or BAY 41-2272 that sensitize the enzyme for activation by NO (1, 2). The enzyme has been purified from lung as a heterodimeric, heme-containing enzyme consisting of an $\alpha_1$ and a $\beta_1$ subunit. After cloning of the $\alpha_1$ and $\beta_1$ cDNAs, two other subunit cDNAs have been cloned by homology screening: the $\alpha_1$ subunit shows NO sensitivity and a functional heme binding site of sGC has been demonstrated on the protein level in human placenta by co-precipitation experiments (5). We recently isolated a $\beta_2$ cDNA variant from rat kidney that shows NO-sensitive enzyme activity after expression in Sf9 or HEK-293 cells in the absence of a second subunit, most likely as $\beta_2/\beta_2$ homodimer (6).

Most studies regarding the activation mechanism by binding of NO to the prosthetic heme group have concentrated on the $\alpha_1/\beta_1$ heterodimeric enzyme. Since the first purification of this enzyme isoform it has been assumed that the enzyme contains one prosthetic heme group per heterodimer (7). Before the cDNA sequences of the two subunits were identified, it was proposed that one subunit was regulatory and bound the heme and that the other subunit was catalytic (8). However, analysis of the cDNA sequences revealed that the two subunits show a high degree of homology both in their amino-terminal and their carboxyl-terminal halves (9, 10). While the carboxyl-terminal parts were assigned as being responsible for catalysis based on homology to the related adenylyl cyclases, it seemed plausible that both homologous amino-terminal regions of the $\alpha_1$ and $\beta_1$ subunits participate in binding of the prosthetic heme. This hypothesis was strengthened by findings using amino-terminal deletion mutants of the bovine $\alpha_1$ and $\beta_1$ subunits showing that NO sensitivity and a functional heme binding site of sGC requires full-length amino termini of both subunits (11, 12).

In the present study, we used deletion mutagenesis to identify functional regions that are responsible for NO-heme and YC-1-mediated activation of sGC. To our surprise we found that the deletion of the first 259 amino acids of the human $\alpha_1$ subunit leads to an enzyme with strong sensitivity toward the heme-dependent activators NO and YC-1. Deletion of 364 amino acids of the $\alpha_1$ subunit leads to an enzyme complex that is insensitive to the heme-dependent activators NO and YC-1 but shows preserved heme binding with the typical shift in the spectral analysis by NO binding to the prosthetic heme group. This indicates that the amino-terminal part of the $\alpha_1$ subunit is not involved in heme binding and that amino acids 259–364 of the $\alpha_1$ subunit represent an important functional domain for the transduction of the NO activation signal and likely represent the target for NO-sensitizing substances like YC-1.

EXPERIMENTAL PROCEDURES

Materials—3-(5’-Hydroxymethyl-2’-furyl)-1-benzylindazole (YC-1) was from Alexis Biochemicals (Lausen, Switzerland). 2,2-Diethyl-1-nitroso-oxyhydrzone (DEANO) and all other chemicals, in the highest grade of purity, were obtained from Sigma. Products for SF9 cell culture were from Invitrogen.

Cloning of $\alpha_1$, Deletion Mutants and Generation of Recombinant Baculovirus—Cloning of the $\alpha_1$ subunit (a kind gift of Dr. Georges Guellaën, Créteil; Ref. 13) and the $\beta_1$ subunit has been described.

* This study was supported by Deutsche Forschungsgemeinschaft Grant BE 1865/2-1 and the Forschungsförderungsfond Hamburg. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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The abbreviations used are: sGC, soluble guanylyl cyclase; DEA/NO, 2,2-diethyl-1-nitroso-oxyhydrzone; TEA, triethanolamine.
Nitric Oxide-sensitive Guanylyl Cyclase

Purification of sGC—All purification steps were performed at 4 °C. The cell pellet from 1800 ml of cell solution infected with the respective subunits was homogenized with a cell disruption bomb (Parr, Moline, IL) at 60 bars for 1 h in 180 ml of 50 mM TEA/HCl, pH 8.0 containing 10 mM dithiothreitol, 1 mM benzamidine, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 900 μl of protease inhibitor mixture (Sigma). The homogenate was centrifuged at 40,000 × g for 30 min, and 180 ml of supernatant were collected. All chromatographic steps were performed on a FPLC system (Amersham Biosciences). The protease inhibitor benzamidine (1 mM), dithiothreitol (10 mM), and phenylmethylsulfonyl fluoride (10 μg/ml) were used in all chromatographic steps. The supernatant was immediately applied to a Q-Sepharose column (20 ml volume) at 2 ml/min. Ion exchange buffer A contained 50 mM TEA/HCl, pH 8.0. Ion exchange buffer B contained 5 mM potassium phosphate (pH 7.2). Ion exchange buffer C was prepared by adding 1 M NaCl to buffer B. The column was washed at 3 ml/min with buffer A, buffer B, and 8% buffer C until A280 was stable. A linear gradient from 8% C to 30% C for 828 ml was used to elute sGC. The sGC-containing fractions were pooled by determining sGC activity at basal or NO-stimulated conditions after construction of the ΔN259 mutant, a single nucleotide exchange was done with the QuikChange™ kit (Stratagene, La Jolla, CA) using the following primer pair: P 216, 5'-GGG ACT TTA GTC ACT CCG-3' and P 217, 5'-CCG ACT ACA ACT AGT AGG GCT GTT CAT CAA ACT GCG-3'. The modified nucleotide is underlined. A SpeI/HindIII fragment of the mutated α1 full-length clone was then ligated using SpeI/HindIII into the pFASTBAC vector. Recombinant baculoviruses of respective subunits were generated according to the BAC-TO-BAC™ System (Invitrogen).

RESULTS

To determine the function of the amino-terminal part of the α1 subunit a series of recombinants containing progressive deletions of the amino-terminal sequences of α1 were constructed and expressed in Sf9 cells together with the dimerizing subunit β1. On Western immunoblots using an antibody directed against a carboxyl-terminal sequence of the α1 subunit, full-length α1 and α1 deletion mutants (α1 ΔN259 and α1 ΔN364) exhibited molecular masses corresponding to those predicted from their deduced amino acid sequences (Fig. 1). Expression levels of the full-length α1, α1 deletion mutants, and β1 were very similar (see Fig. 1). Guanylyl cyclase activity was measured in the respective cytosols from Sf9 cells under basal conditions, activation with NO, and activation with the combination of NO and YC-1 (Fig. 2). Guanylyl cyclase activity was similar under all experimental conditions for α1 and the α1 deletion mutant α1 ΔN259. The deletion mutant α1 ΔN364 showed a complete loss of sensitivity toward NO under YC-1 and a slight decrease of guanylyl cyclase activity under basal conditions (see Fig. 2).

Determination of Protein Concentration and Guanylyl Cyclase Activity Assay—Protein concentrations were determined by the method of Bradford using bovine plasma gamma globulin (Protein Assay Standard I, Bio-Rad) as standard. sGC activity of Sf9 cytosol (approximately 40 μg of protein per assay tube) was determined by incubation for 10 min at 37 °C in the presence of 1 mM GMP, 0.5 mM 5′-PGT (about 0.2 μCi), 3 mM MgCl₂, 50 mM TEA/HCl, pH 7.4, 0.25 μl/liter creatine kinase, 5 mM creatine phosphate, and 1 mM 3-isobutyl-1-methylxanthine in a total volume of 0.1 ml as described by Schultz and Böhme (15). Reactions were started by the addition of protein and incubation at 37 °C. All experiments were stopped by ZnCO₃ precipitation, and purification of the enzyme-formed cGMP was performed as described previously (15). Basal enzyme activity measurements were performed in the absence of NO or YC-1. NO-stimulated measurements were performed in the presence of the NO donor DEA/NO, and NO/YC-1-stimulated enzyme activity measurements were performed in the presence of both DEA/NO and YC-1 in variable concentrations. YC-1 was dissolved in 25% (v/v) Me2SO so that the final Me2SO concentration in the enzyme assay did not exceed 2.5% (v/v). At this concentration no effects of Me2SO on enzyme activity were observed. DEA/NO was dissolved in 10 mM NaOH, which also did not affect the enzyme activity.

Generation of the α1-1200 and the β1-89 Antibodies, SDS-PAGE, and Immunoblotting—The α1-1200 antibody was raised against two peptides (EP012493, H₃N-FTPBRSSLFLFNPFP-COOH; and EP012494, H₃N-CFQKDKVEDGNANFLGKASGID-COOH) of the carboxyl-terminal domain of the human α1 subunit, and the β1-89 antibody was raised against the carboxyl-terminal peptide (EP9990255, H₃N-CSKNT-GTEEETQDDDD-COOH). Antibodies were coupled by an additional cysteine to keyhole limpet hemocyanin. Rabbits were immunized on days 0, 14, 28, and 56 and were finally bled on day 50. Successful antigen response was estimated by enzyme-linked immunosorbent assay. For monitoring the purity of enzyme preparations and for the determination of apparent molecular masses of the purified enzyme, SDS-polyacrylamide gel electrophoresis was performed in 10% slab gels, and proteins were stained with Coomassie Blue G-250. For immunoblotting, protein fractions were subjected to 10% SDS-PAGE and then transferred electrophoretically to a nitrocellulose membrane. The membrane was reversibly stained with Ponceau S, and unspecific binding sites were saturated by immersing the membrane for 1 h in TBST buffer (10 mM Tris/HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20) containing 5% nonfat dry milk. The membranes were incubated for 1.5 h in TBST buffer containing 1% dry milk. Negative control reactions were run in the presence of synthetic peptides used for immunization in different combinations (5 μg/ml). The membranes were washed three times for 10 min with TBST and subsequently incubated for 1 h with horseradish peroxidase-labeled anti-rabbit IgG antibodies (diluted 1:4000, Sigma). After three washes with TBST the membranes were processed with the ECL Western blotting detection system according to the recommendations of the manufacturer (Amersham Biosciences).

Statistical Analysis—All results were controlled for their statistical significance by one-way analysis of variance followed by a Newman-Keuls post test. A value of p < 0.05 was considered to be statistically significant.

Previously (6, 14), Cloning of diverse α1 deletion mutants was carried out by digestion with different restriction endonucleases. For construction of the α1, ΔN259 mutant, a Hsin/HindIII fragment of full-length α1 was cloned using Stul/HindIII into the pFASTBAC vector. Before cloning into pFASTBAC the BamFI 5'-ends were filled in with Taq polymerase (Invitrogen). For construction of the ΔN259 mutant, a single nucleotide exchange was done with the QuikChange™ kit (Stratagene, La Jolla, CA) using the following primer pair: P 216, 5'-GGG ACT TTA GTC ACT CCG-3' and P 217, 5'-CCG ACT ACA ACT AGT AGG GCT GTT CAT CAA ACT GCG-3'. The modified nucleotide is underlined. A SpeI/HindIII fragment of the mutated α1 full-length clone was then ligated using SpeI/HindIII into the pFASTBAC vector. Recombinant baculoviruses of respective subunits were generated according to the BAC-TO-BAC™ System (Invitrogen).
full-length $\alpha_1$ and 585 ± 246 nm for $\alpha_1 \Delta N_{259}$. $\alpha_1 \Delta N_{364}$ was NO-insensitive. Concentration-response curves for YC-1 were performed both in the absence (Fig. 4A) and presence (Fig. 4B) of DEA/NO (100 μM). The EC₅₀ values for YC-1 showed no significant differences for full-length $\alpha_1$ and $\alpha_1 \Delta N_{364}$ and were 25 ± 7 and 19 ± 6 μM in the absence and 0.89 ± 0.05 and 1.94 ± 0.64 μM in the presence of DEA/NO, respectively. $\alpha_1 \Delta N_{364}$ was YC-1-insensitive (see Fig. 4A).

All mutant and wild type enzymes were purified to apparent homogeneity. Coomassie Blue-stained SDS-PAGE analyses are shown in Fig. 5. Spectroscopic analysis of the purified wild type enzyme revealed absorption maxima at 432 nm in the absence and 400 nm in the presence of the NO donor DEA/NO (100 μM) (Fig. 6A). Analysis of the purified $\alpha_1 \Delta N_{259}/\beta_1$ enzyme revealed an almost identical spectrum with very similar absorption maxima (431 nm and 399 nm, respectively; see Fig. 6B). Purified $\alpha_1 \Delta N_{364}/\beta_1$ enzyme showed absorption maxima at 432 nm in the absence and 393 nm in the presence of the NO donor DEA/NO (Fig. 6C). Although these maxima were almost identical to the wild type enzyme, the ratio of the absorption at 432 nm to 280 nm was lower, indicating lower heme content (see Fig. 6C). During the purification of the wild type and the $\alpha_1 \Delta N_{259}/\beta_1$ enzyme, fractions from each column were pooled by determining sGC activity at basal and NO-stimulated conditions. For the NO-insensitive $\alpha_1 \Delta N_{364}/\beta_1$ enzyme fractions could only be tested for basal enzyme activity after each column. Thus we selected for heme-containing, NO-sensitive enzyme in the case of wild type and $\alpha_1 \Delta N_{259}/\beta_1$ enzyme. To control whether this effect accounts for the lower amount of heme in $\alpha_1 \Delta N_{364}/\beta_1$ enzyme, we purified $\alpha_1 \Delta N_{259}/\beta_1$ enzyme and assayed the fractions after each column only for basal enzyme activity. This resulted also in a significantly lower ratio of the absorption at 432 nm to 280 nm but absorption maxima very similar to those obtained before (432 nm in the absence and 399 nm in the presence of DEA/NO).

To compare the kinetic properties of the purified enzyme complexes, cGMP formation was determined in the presence of increasing GTP concentrations. A Lineweaver-Burk plot of the data revealed apparent $K_m$ values that showed no significant differences between full-length $\alpha_1$ (134 ± 19 μM), $\alpha_1 \Delta N_{259}$ (119 ± 13 μM), and $\alpha_1 \Delta N_{364}$ (163 ± 12 μM) (Fig. 7). $V_{\text{max}}$ values showed no significant differences and were 145 ± 16 nmol of cGMP/min × mg for full-length $\alpha_1$, 104 ± 15 nmol of cGMP/min × mg for $\alpha_1 \Delta N_{259}$, and 98 ± 15 nmol of cGMP/min × mg for $\alpha_1 \Delta N_{364}$.

Guanylyl cyclase activity of the purified enzymes was measured under basal conditions and activation with NO to investigate the status of the enzymes (Fig. 8). Guanylyl cyclase activity was not significantly different under all experimental conditions for $\alpha_1$ and the $\alpha_1$ deletion mutant $\alpha_1 \Delta N_{259}$. In the presence of the NO donor DEA/NO (100 μM) enzyme activity was increased by 242-fold for $\alpha_1$ and 252-fold for $\alpha_1 \Delta N_{259}$. Analysis of the purified deletion mutant $\alpha_1 \Delta N_{364}$ confirmed the complete loss of sensitivity toward NO and demonstrated a slight decrease of guanylyl cyclase activity under basal conditions (see Fig. 8).

Concentration-response curves for YC-1 were performed both in the absence (Fig. 9A) and presence (Fig. 9B) of a submaximally active DEA/NO concentration (100 nM). The EC₅₀ values for YC-1 showed significant differences between full-length $\alpha_1$ and $\alpha_1 \Delta N_{259}$ and were 51 ± 7 and 30 ± 5 μM (p < 0.05) in the absence and 2.62 ± 0.26 and 4.53 ± 0.71 μM (p < 0.05) in the presence of DEA/NO (100 nM), respectively. $\alpha_1 \Delta N_{364}$ was YC-1-insensitive (see Fig. 9A). $V_{\text{max}}$ values showed significant differences only in the absence of DEA/NO and were 9717 ± 216 nmol of cGMP/min × mg for full-length $\alpha_1$ and 5745 ± 420 nmol of cGMP/min × mg for $\alpha_1 \Delta N_{259}$ (p < 0.001) at basal conditions and 24,259 ± 3237 nmol of cGMP/min × mg for full-length $\alpha_1$ and 17,369 ± 3308 nmol of cGMP/min × mg for $\alpha_1 \Delta N_{259}$ in the presence of 100 nM DEA/NO.

**DISCUSSION**

Previous studies have used amino-terminal deletion mutants of bovine NO-sensitive guanylyl cyclase subunits to map functional regions of this enzyme family (11). The deletion of only 131 amino-terminal amino acids of the $\alpha_1$ subunit and co-expression of this deletion mutant with the $\beta_1$ subunit in the baculovirus system led to a 10-fold reduction in enzyme activity in co-infected Sf9 cytosol versus the respective $\alpha_1$ full-length construct and an almost complete loss of NO sensitivity (11). By contrast, in our study there was no significant decline in basal enzyme activity and stimulation by NO after deletion of 259 amino acids of the $\alpha_1$ subunit. It is conceivable that our approach of using endogenously occurring methionines rather than newly introduced methionines as start codons poses less risk of unwanted conformational changes resulting in lower enzyme activity. It is also possible that sequence differences between the bovine and human $\alpha_1$ subunits might explain the discrepancies. Subsequent to the study by Wedel and co-workers (11), the same group analyzed the purified $\alpha_1 \Delta N_{131}/\beta_1$ enzyme complex and showed a loss of affinity in binding of the prosthetic heme group, which binds NO (12). Based on the findings of this study, it has been suggested that heme binding to sGC requires the presence of both subunits ($\alpha_1$ and $\beta_1$) in full-length and that both homologous amino-terminal regions of the $\alpha_1$ and $\beta_1$ subunits participate in binding of the pros-
thetic heme (12). Expression of only the amino-terminal part of the \( \alpha_1 \) subunit expressed in *Escherichia coli* indicated that the amino terminus of the \( \alpha_1 \) subunit (\( \alpha_1-(1-385) \)) is sufficient for heme binding with preserved binding of NO (16). As pointed out by the authors of the latter study (16), this result does not rule out an *in vivo* heme binding site involving residues contributed by both the \( \beta_1 \) and \( \alpha_1 \) subunits as suggested by Foerster et al. (12). Given the degree of homology in the amino terminus of the subunits, it is possible that the \( \alpha_1-(1-385) \) homodimer is an *in vitro* outcome of expression in the absence of the \( \beta_1 \) subunit and that the second \( \alpha_1 \) subunit provides crucial amino acids for heme binding that would come from the \( \beta_1 \) subunit *in vivo* (16). The results of the current study argue against an *in vivo* heme binding site involving amino-terminal residues contributed by both the \( \alpha_1 \) and \( \beta_1 \) subunits as suggested by Foerster et al. (12) and a model where the heme in sGC is sandwiched between the amino-terminal parts of the two subunits (16). The results of the current study rather indicate that the amino-terminal region of the \( \alpha_1 \) subunit is not involved in heme binding. In fact, we could fully reproduce the findings by Zhao and Marletta (16) that the amino-terminal part of the \( \beta_1 \) subunit can be expressed as a soluble, nitric oxide-, and heme-binding protein in *E. coli*. 2 In contrast, the expression of longer constructs of the \( \beta_1 \) subunit including the catalytic domain or co-expression with the \( \alpha_1 \) subunit in *E. coli* led to insoluble protein in the form of inclusion bodies under different experimental conditions. 2 These findings should encourage approaches to solve the structure of the heme binding domain of sGC by focusing on the expression of the amino-terminal part of the \( \beta_1 \) subunit.

We find in the current study that deletion of 259 amino acids of the \( \alpha_1 \) subunit leaves the enzyme functionally intact but that deletion of the first 364 amino acids leads to an enzyme complex with preserved heme binding but loss of sensitivity to NO or YC-1. NO still binds to the enzyme variant lacking 364 amino acids since it induces the typical spectral shift of the prosthetic heme group. This indicates that amino acids 259-364 of the \( \alpha_1 \) subunit are either directly important for the transduction of the activation signal by NO or that the deleted region is indirectly involved in the mediation of the NO effect, e.g. by the stabilization of another domain of the enzyme. The cysteine 238 and cysteine 243 region in the \( \alpha_1 \) subunit has been mapped as the likely binding site of the YC-1-related substance BAY 41-2272 using photoaffinity labeling (2). We show in the current study that both cysteines including an additional 14 amino-terminal residues can be deleted without loss of YC-1 sensitivity. Given the data by Stasch and colleagues (2), we think that it is likely that the region adjacent to cysteine 238

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*9* S. Behrends, unpublished data.
and cysteine 243 (from amino acid 259 to 364) of the α₁ subunit represents the binding site of YC-1. The possibility that YC-1 still binds to the α₁ΔN₂⁵⁹ mutant enzyme complex and that the activation signal is not transduced within the enzyme directly or indirectly as discussed for NO is also a plausible explanation of our results.

In the present study, sGC in Sf9 cell cytosol is activated 25-fold, while the purified enzyme is activated 80-fold. The -fold stimulation by YC-1 in the literature varies from 7-fold (17), 10-fold (18), and 14-fold (19) up to close to 100-fold (20). YC-1 activation of sGC is to a very large degree heme-dependent (17, 21), and thus -fold stimulation of YC-1 is a function of the heme content of the enzyme similar to the heme-dependent activator NO. The result that stimulation factors by YC-1 are significantly higher for the purified enzymes than in Sf9 cytosol can be explained by a higher percentage of heme-containing versus heme-free enzyme that may also form by expression in Sf9 cells. Since we have pooled the fractions during our purification protocol according to the determination of sGC activity at basal and NO-stimulated conditions, we have purified selectively heme-containing enzyme.

The purified α₁ΔN₃⁶⁴ enzyme complex contained less heme than the wild type enzyme or the α₁ΔN₂⁵⁹ enzyme complex purified under regular conditions. Because of the lack of NO sensitivity, the α₁ΔN₃⁶⁴ enzyme complex had to be purified by

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**Fig. 4.** Concentration-dependent effect of YC-1 on guanylyl cyclase activity in Sf9 cell cytosol after infection with the respective α₁ subunits in combination with β₁. The results show the effect of YC-1 in a range between 0.1 and 300 μM YC-1. A shows dose-response curves for α₁ (closed circles), α₁ΔN₂⁵⁹ (open circles), and α₁ΔN₃⁶⁴ (closed squares) for basal guanylyl cyclase activity, whereas B shows DEA/NO (100 μM)-stimulated curves. Enzymatic activity of α₁ΔN₃⁶⁴ was measured only for the highest YC-1 concentration at basal conditions. All points of YC-1-dependent curves represent means (±S.E.) of at least three independent experiments performed in duplicate.

**Fig. 5.** SDS-PAGE analysis of purified sGC variants. 1 μg of purified enzyme was electrophoresed by 10% SDS-PAGE and stained with Coomassie Blue. On the right side of each panel a low molecular mass standard (LMW) is shown.

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controlling fractions after each column for basal guanylyl cyclase activity only. In the case of the wild type enzyme and the \( \alpha_1 \Delta N_{259} \) enzyme complex we have pooled the fractions during our purification protocol according to the determination of guanylyl cyclase activity at basal and NO-stimulated conditions and have thus purified selectively heme-containing enzyme. Attempts to establish a purification protocol with wild type enzyme that would be applicable to all three enzyme variants.
by pooling fractions after each column according to their absorbance at 430 nm in the spectrophotometer were not successful. Especially at the crucial initial stages of the purification protocol the measurement of the absorbance at 430 nm gave no reliable results with respect to sGC-containing fractions when compared with NO-activated guanylyl cyclase activity measurements. To find out whether the relatively low heme content of the \( \alpha_1 \) enzyme complex was due to the impossibility of

![Fig. 8. Guanylyl cyclase activity of the respective purified guanylyl cyclase enzyme complexes. Guanylyl cyclase activity was measured under basal conditions (only 3 mM Mg\(^{2+}\), black columns) and in the presence of 100 \( \mu \)M DEA/NO (white columns). The columns represent means \( \pm \) S.E. of at least three independent experiments.](image)

![Fig. 9. Concentration-dependent effect of YC-1 on purified guanylyl cyclase activity. The results show the effect of YC-1 in a range between 0.1 and 200 \( \mu \)M YC-1. A shows dose-response curves for \( \alpha_1 \) (closed circles), \( \alpha_1 \Delta N_{259} \) (open circles), and \( \alpha_1 \Delta N_{364} \) (closed squares) for basal guanylyl cyclase activity, whereas B shows DEA/NO (100 nM)-stimulated curves. Enzymatic activity of \( \alpha_1 \Delta N_{364} \) was measured only for the highest YC-1 concentration at basal conditions. All points of YC-1-dependent curves represent means (±S.E.) of five independent experiments performed in duplicate.](image)
pooling fractions according to NO-activated guanylyl cyclase activity, we purified the α1 ΔN259 enzyme under the same conditions. This resulted in an enzyme preparation with a similarly reduced heme content. While this argues in favor of the hypothesis that the different purification procedure is responsible for the relatively low heme content, we cannot rule out the possibility that the α1 ΔN364 mutant enzyme complex shows reduced heme binding affinity.

In summary, we show that 1) the amino-terminal part of the α1 subunit is not involved in heme binding and 2) amino acids 259–364 of the α1 subunit represent an important functional domain for the transduction of the NO activation signal and likely represent the target for NO-sensitizing substances like YC-1.

Acknowledgments—The expert technical assistance of Jutta Starbatty, Jenny Behrens, and Alexandra Zielinski is gratefully acknowledged.

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