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Direct fusion of subunits of heterodimeric nitric oxide sensitive guanylyl cyclase leads to functional enzymes with preserved biochemical properties: evidence for isoform specific activation by ciguates

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Abstract

Nitric oxide sensitive guanylyl cyclase (NOsGC) is a heterodimeric enzyme consisting of an α and a β subunit. Two heterodimeric enzymes are known to be important for NO-signalling in humans: α_1/β_1 and α_2/β_1 . No difference had so far been detected with respect to their pharmacological properties, but as we show in the present paper the new drugs cinaciguat and ataciguat activate the α_1/β_1 form more effectively. Recent evidence suggests that homodimeric complexes of α and β subunits exist in vivo and that these non-heterodimerizing subunits have a separate function from cGMP signaling. To isolate the effect of the α_1/β_1 or α_2/β_1 heterodimeric enzyme in overexpression experiments from potential effects of non-heterodimerizing α_1 , β_1 or α_2 subunits, we cloned constructs that guarantee a 1:1 stoichiometry between α and β subunits and rule out the presence of homodimers. The carboxy-terminus of the β_1 subunit was directly fused to the amino-terminus of either the α_1 or α_2 subunit. The two different “conjoined” NOsGCs faithfully reproduced the biochemical and pharmacological properties of the α_1/β_1 and α_2/β_1 heterodimeric enzymes including the differential activation by ciguat-activators. Conjoined NOsGCs can be used for isoform specific overexpression in transgenic animals and therapeutic overexpression may be an application in the future. In both cases possible side effects of homodimeric α or β subunits are avoided. Crystallization with the goal of structure determination may also be easier for conjoined NOsGCs because enzyme preparations are more homogenous and are free of “contaminating” homodimers.

1. Introduction

Nitric oxide sensitive guanylyl cyclase (NOsGC) is the major physiological receptor for nitric oxide (NO) throughout the cardiovascular and central nervous system (for reviews see [1] and [2]). Two heterodimeric enzymes are known to be important for NO-signalling in humans: α_1/β_1 and α_2/β_1 . Both catalyze the production of the second messenger molecule cGMP from GTP. Only the α_2/β_1 isoform can interact with the PDZ containing protein PSD-95 via its carboxy-terminal sequence and seems to adhere to specialized membrane compartments in some tissues [3]. No other difference has so far been detected in the biochemical and pharmacological properties of both purified enzyme isoforms [4].

It has been shown that co-expression of the α_1 and β_1 subunit in overexpression systems leads to the formation of α_1 homodimers and β_1 homodimers in addition to heterodimers [5]. Since the homodimers are inactive with respect to cGMP formation Zabel *et al.* suggested the possibility of a physiological equilibrium between homo- and heterodimeric NOsGC complexes and a possible regulation of NOsGC activity by the extent of homodimerization versus heterodimerization. In support of such a concept we have recently shown that NOsGC activity decreases during cerebral postnatal development because of a reduction in heterodimerization and have demonstrated the occurrence of non-heterodimerizing α_1 or β_1 subunits in vivo [6]. Intriguingly there is recent evidence to suggest that both the isolated α_1 subunit and the isolated β_1 subunit have a separate function from cGMP signalling: The α_1 subunit seems to act as an important mediator of the procarcinogenic effect of androgens [7-8]. The β_1 subunit has been suggested to regulate chromatin condensation and cell cycle progression [9].

To isolate the effect of the α_1/β_1 and α_2/β_1 heterodimeric enzyme in overexpression experiments from potential effects of non-heterodimerizing α_1 , β_1 or α_2 subunits, we cloned

constructs that guarantee a 1:1 stoichiometry between α and β subunits and rule out the presence of homodimers. Here we provide the pharmacological and biochemical characterization of these “conjoined” NOsGCs. Conjoined NOsGCs will allow facilitated overexpression in transgenic animals and will exclude overexpression of non-heterodimerizing subunits that have effects independent of cGMP signalling. Crystallization with the goal of structure determination should be easier for conjoined NOsGC because enzyme preparations are more homogenous and are free of “contaminating” β_1 homodimers or α homodimers. In addition to providing a novel tool for research, we provide the first evidence of a pharmacological difference between α_1/β_1 and α_2/β_1 heterodimeric enzymes: The ciguat-activators which are currently being developed clinically for the treatment of heart failure and other cardiovascular disease states are more effective activators of the α_1/β_1 isoform.

2. Materials and Methods

2.1 Materials

3-(5'-Hydroxymethyl-2'-furyl)-1-benzylindazole (YC-1), 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) and 2,2-diethyl-1-nitroso-oxyhydrazine (DEA/NO) and all other chemicals, in the highest grade of purity, were obtained from Sigma Aldrich (Munich, Germany). Cinaciguat (BAY 58-2667) was a generous gift from Johannes-Peter Stasch (Bayer Schering Pharma, Wuppertal, Germany). Ataciguat (HMR 1766) was a generous gift from Sanofi-Aventis (Frankfurt, Germany). Products for Sf9 cell culture were from Invitrogen (Karlsruhe, Germany). Restriction enzymes and polymerases were obtained from NEB (Frankfurt, Germany).

2.2 Cloning of fluorescent tagged NOsGC subunits

The β_1 subunit cDNA was amplified by RT-PCR from rat heart using cDNA Polymerase Mix and the primer pair P141 (5'-CCG ACA CCA TGT ACG GTT TTG TGA-3') and P180 (5'-GGG CCC AGT TTT CAT CCT GGT TTG TTT CCT-3'). The resulting PCR product was subcloned into pcDNA3.1/V5/His-TOPO. From this construct the β_1 subunit cDNA was cloned HindIII/ApaI into pEYFP-N1. β_1 YFP construct was cloned SpeI/XbaI from pEYFP-N1 into pFASTBAC (Invitrogen; Karlsruhe, Germany).

The NOsGC α_1 subunit was amplified from rat cDNA using the primer pair: P178 (5'-ACA CCG GCT AAT AAG GAG GAA ACC AC-3') and P179 (5'-ATC TAC CCC TGA GGC CTT GCC TAA GAA-3'). The resulting PCR product was subcloned into pcDNA3.1/V5/His-TOPO (Invitrogen; Karlsruhe, Germany). From this construct the α_1 cDNA was cloned HindIII/XhoI

into HindIII/SalI pEYFP-N1 (Clontech; Saint-Germain-en-Laye, France). The carboxy-terminally YFP tagged α_1 subunit was cloned NheI/XbaI into SpeI/XbaI pFASTBAC.

The α_2 NOsGC subunit was cloned BamHI/SspI from pFASTBAC [10] into BglII/BamHI (Klenow fill in) in pEGFP-N1 (Clontech; Saint-Germain-en-Laye, France). From this construct the α_2 subunit was cloned Eco47III/NotI into pECFP-N1(Clontech; Saint-Germain-en-Laye, France) to get the α_2 CFP construct. The carboxy-terminally CFP tagged α_2 subunits was cloned Eco47III/NotI into StuI/NotI pFASTBAC vector. ECFP was exchanged with EYFP from pEYFP-C1 (Clontech; Saint-Germain-en-Laye, France) using AgeI/KpnI restriction sites.

2.3 Cloning of conjoined NOsGC

For $\beta_1\alpha_1$ conjoined NOsGC: the α_1 subunit in pcDNA3.1/V5/His-TOPO was amplified by PCR using the primer pair: P270 (5'-GAT CGT GTA CAA GAT GTT CTG CAG GAA GTT CAA AG-3') and P269 (5'-GAT CGT CTA GAT TAA TCT ACC CCT GAG GCC TTG CC-3') to create new restriction sites 5' (BsrGI) and 3'(XbaI) of the α_1 gene. The resulting PCR product was subcloned into pCR2.1TOPO (Invitrogen; Karlsruhe, Germany). From this construct the α_1 NOsGC subunit was cloned BsrGI/XbaI from pCR2.1TOPO 3' of β_1 YFP in pEYFP-N1 to get a β_1 YFP α_1 construct. YFP was deleted by the restriction with BsrGI/ApaI and blunt ends were created by mung bean nuclease (NEB; Frankfurt, Germany) treatment. The $\beta_1\alpha_1$ conjoined NOsGC in pEYFP-N1 was cloned SpeI/XbaI in pFASTBAC.

For $\beta_1\alpha_2$ conjoined NOsGC: NOsGC α_2 rat was cloned BstEII (Klenow fill in)/BamHI from pFASTBAC [10] into pEYFP-C1 EcoRI (Klenow fill in)/BamHI. The YFP α_2 cDNA in pEYFP-C1 was digested with BsrGI/NheI to delete YFP from the vector. In this construct β_1 YFP was cloned NheI/BsrGI from pEYFP-N1 resulting in the conjoined β_1 YFP α_2 (in pEYFP-C1). YFP

was deleted by the restriction with AgeI/BspEI and blunt ends were created by Pfu polymerase treatment. The $\beta_1\alpha_2$ conjoined NOsGC in pEYFP-C1 was cloned SpeI/NotI in pFASTBAC.

2.4 Cloning of STREP tagged (α_1) NOsGC subunits and conjoined NOsGC

The cDNA encoding the carboxy-terminal 228 amino acids of the rat NOsGC α_1 subunit was amplified from the construct above using an antisense primer designed to fuse a carboxy-terminal STREP tag to the α_1 subunit. The following primer pair was used: P289 (5'-AGC AGC TCT GGC AAG GAC AAA T-3') and P290 (5'-TCT AGA TTA TTT TTC GAA CTG CGG GTG GCT CCA ATC TAC CCC TGA GGC CTT GCC TAA G-3'). The resulting PCR product was subcloned into pCR2.1TOPO. The DNA encoding the STREP tagged carboxy-terminal part of the α_1 subunit in pCR2.1TOPO was cloned BstEII/XbaI into the $\beta_1\alpha_1$ conjoined NOsGC in pFASTBAC replacing the respective carboxy-terminus. Rat α_1 NOsGC with a carboxy-terminal STREP tag was obtained by cloning the amino-terminal part of the rat α_1 NOsGC subunit in pCR2.1TOPO SpeI / BstEII in the preceding pFASTBAC construct thereby deleting the β_1 subunit from the conjoined NOsGC construct.

The cDNA encoding the carboxy-terminal 133 amino acids of the rat NOsGC α_2 subunit was amplified from pFASTBAC [10] using a primer designed to fuse a carboxy-terminal STREP tag to the α_2 subunit. The following primer pair was used: P280 (5'-AAG ACC CAT TCA GAT GCG GAT AGG-3') and P281 (5'-TTA TTT TTC GAA CTG CGG GTG GCT CAA GAG GCT AGT TTC TCG GAG GAA CAT CG-3'). The resulting PCR product was subcloned into pCR2.1TOPO. The STREP tagged α_1 subunit fragment from pCR2.1TOPO was cloned NheI/NotI into the $\beta_1\alpha_2$ conjoined NOsGC in pFASTBAC (see above).

Rat α_2 NOsGC with a carboxy-terminal STREP tag was obtained by cloning the amino-

terminal part of the rat α_2 NOsGC subunit in pFASTBAC [10] NheI/BssHII in the preceding pFASTBAC construct thereby deleting the β_1 subunit from the conjoined NOsGC construct.

The β_1 subunit was also fused with a carboxy-terminal STREP tag. The fluorescent YFP from β_1 YFP in pFASTBAC was replaced SmaI/PvuI with the STREP tag cloned StuI/PvuI from the STREP tagged $\beta_1\alpha_1$ conjoined NOsGC in pFASTBAC.

2.5 Generation of Recombinant Baculovirus, Sf9 Cell Culture and Expression of Recombinant Guanylyl Cyclase Subunits

Recombinant baculovirus was generated according to the BAC-TO-BACTM System (Invitrogen; Karlsruhe, Germany). Sf9 cells were cultured in Sf-900 II serum-free medium supplemented with 1% penicillin/streptomycin and 10% fetal calf serum. Spinner cultures were grown at 27 °C at 140 rpm shaking and diluted to $2 \cdot 10^6$ cells/ml for infection.

20 ml or 500 ml of cell solution were infected with the respective recombinant baculovirus stock with multiplicities of infection of 1.

2.6 Cytosol Preparation, Determination of Protein Concentration and Immunoprecipitation-

74 h after infection, cells were harvested and collected by centrifugation (1500 g for 10 min at 4 °C). All following steps were performed at 4 °C or on ice. The cell pellet from 20 ml was resuspended in 2 ml of homogenization buffer containing 50 mM TEA/HCl, pH 7.4, 10 mM DTT, 1 mM EDTA and completeTM protease inhibitor cocktail (Roche; Penzberg, Germany). For immunoprecipitation experiments PBS containing 1 mM DTT and completeTM protease inhibitor cocktail was used. The cells were lysed by sonication. Cytosolic fractions were obtained by

centrifugation for 30 min at 21000 g at 4 °C. Protein concentrations were determined by the method of Bradford using bovine serum albumin as standard. For immunoprecipitation 1.5 mg protein of the cytosol and 5 µg anti-GFP antibody (Roche; Penzberg, Germany) were used. The method is described in detail in Haase *et al.* [6].

2.7 SDS-PAGE, Coomassie Blue staining and immunoblot analysis

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 with 1 µg purified enzyme in 10% slab gels, and proteins were stained with Coomassie Blue according to Kang *et al.* [11]. For immunoblotting, 1 µg purified enzyme and 50 µg cytosolic proteins were subjected to 10% SDS-PAGE and then transferred to nitrocellulose membranes. The following antibodies against the three NOsGC subunits were used for detection: α_2 -84 (1:750) [12], β_1 (1:4000) and α_1 (1:5000) (both Sigma Aldrich; Munich, Germany). The method is described in detail in Haase *et al.* [6].

2.8 Purification of NOsGC

All purification steps were performed at 4 °C. The cell pellet from 500 ml of cell solution infected with the respective NOsGC subunits or conjoined NOsGC was homogenized by sonification in 30 ml homogenization buffer containing 50 mM TEA/HCl, pH 7.4, 10 mM DTT, 1 mM EDTA and completeTM protease inhibitor cocktail. Avidin (250 nM, IBA; Goettingen, Germany) was added to the homogenate and incubated for 30 min at for 4 °C. The homogenate was centrifuged at 15000 g for 2 h and 30 ml of the supernatant was collected. All

chromatographic steps were performed on an Äkta purifier system (GE Healthcare; Munich, Germany). The supernatant was immediately applied to a Strep-Tactin[®] Superflow[®] high capacity (4 ml volume, IBA; Goettingen, Germany) column (C 10/10 and adapter AC 10, GE Healthcare; Munich, Germany) at 1 ml/min. The Strep purification was done using the Strep-tag[®] Protein Purification Buffer Set and according to manufactures protocol (IBA; Goettingen, Germany).

Fractions showing absorption at 430 nm (Soret-band) were pooled and applied immediately with a flow rate of 15 ml/min to a HiPrep 26/10 Desalting column (GE Healthcare; Munich, Germany) that was equilibrated 5 CV with IEX-1 (50 mM TEA, 10 mM DTT, 1 mM Benzamidin, pH 8.0) buffer before use. Elution was carried out with 2 CV at 15 ml/min with IEX-1 buffer and fractionated in 1.0 ml.

Fractions showing absorption at 430 nm (Soret-band) were pooled and applied immediately with a flow rate of 2 ml/min to a Mono Q 5/50 GL column (GE Healthcare; Munich, Germany) that was equilibrated 10 CV with IEX-1 buffer before use. The column was washed (10 CV) at 2 ml/min with IEX-1. Elution was carried out with 10 CV at 2 ml/min with a linear gradient running from zero to 100 % IEX-2 buffer (50 mM TEA, 10 mM DTT, 1 mM Benzamidin, 1 M NaCl, pH 8.0) and fractionated in 0.5 ml.

Fractions showing absorption at 430 nm (Soret-band) were pooled and concentrated in centrifugal devices with a 30-kDa cut-off (Millipore; Schwalbach, Germany) to 0.5 ml. The concentrated purified enzyme was used for spectroscopic analysis (250 to 500 nm), SDS-PAGE, analytical gel filtration and diluted with 50 mM TEA/HCl, pH 7.4 containing 10 mM DTT, 1 mM EDTA, 0.05 % BSA (w/v) and stored with 10% (v/v) glycerol at -80 °C.

2.9 Analytical gel filtration

200 µg of purified enzyme was applied with a flow rate of 0.3 ml/min to a HiLoad™16/60 Superdex™200 prep grade column (GE Healthcare; Munich, Germany) that was equilibrated 3 CV with gel filtration buffer (50 mM TEA, 250 mM NaCl; pH 8.0) before use. Elution was carried out with 1.5 CV at 0.3 ml/min with gel filtration buffer and fractionated in 1 ml aliquots. Fractions showing absorption at 430 nm (Soret-band) were pooled, concentrated in centrifugal devices with a 30-kDa cut-off and analyzed by Western blot.

2.10 Guanylyl Cyclase Activity Assay

The determination of NOsGC enzyme activity of Sf9 cytosol or purified protein (10 ng/assay tube) was described in detail in Haase *et al.* [6]. Basal enzyme activity measurements were performed in the absence of NO or NOsGC activators. NO stimulated measurements were performed in the presence of 100 µM of the NO donor DEA/NO and NO/YC-1 stimulated measurements were performed in the presence of both 100 µM DEA/NO and 100 µM YC-1. Heme independent activators (cinaciguat or ataciguat) were tested in a concentration of 10 µM in the presence and absence of 10 µM ODQ.

2.11 Statistical analysis

The results are expressed as means ± SEM. of at least three experiments using different animals. All results were controlled for their statistical significance by Student's t-test. A value of $p < 0.05$ was considered to be statistically significant.

3. Results

Recombinant expression of a functional NOsGC enzyme in a baculovirus/Sf9 system requires the co-expression of α and β subunits. NOsGC subunits also form homodimeric complexes when they are co-expressed in Sf9 cells [5]. Immunoprecipitation experiments (Fig. 1) confirmed the data from Zabel et al. [5] that β_1 and α_1 have the ability to form homodimers. In addition our data show that the α_2 subunit also forms homodimers. Thus co-expression of α and β subunits will always lead to a mixture of active NOsGC heterodimers and catalytically inactive NOsGC homodimers with other potential functions [7-9]. To isolate pure heterodimeric enzymes, we cloned constructs that guarantee a 1:1 stoichiometry between α and β subunits and rule out the presence of homodimers. Studies in our lab on fluorescent labeled NOsGC subunits [13] have shown that fluorescent proteins tagged to the carboxy-terminus of the β_1 subunit and amino-terminus of the α subunit are close enough to each other to allow energy transfer (distance 10-100 Å). Based on these data, we directly fused the carboxy-terminus of the β_1 subunit with the amino-terminus of the α subunit without a fluorescent protein as a linker as in Haase et al. [13] and established a direct $\beta_1\alpha_1$ fusion construct which we designated “conjoined NOsGC”. Conjoined NOsGC expression yielded an active and NO sensitive GC enzyme in crude Sf9 cell cytosolic fractions (Fig. 2). Guanylyl cyclase activity was measured under basal conditions, activation with NO and activation with the combination of NO and YC-1 as in previous similar studies [14-15]. There was a trend towards increased guanylyl cyclase activity for the conjoined versus the heterodimeric NOsGC under all experimental conditions that did not reach statistical significance ($p < 0.05$) (Fig. 2). For further comparison of conjoined NOsGC and heterodimeric NOsGC we used a STREP (S) tag II [16] affinity purification. Coomassie Blue-stained SDS-PAGE analysis of purified co-expressed NOsGC isoforms and conjoined NOsGC are shown in

Fig. 3. The two detected signals with molecular mass of 80 and 72 kDa for the co-expressed NOsGC isoforms correspond to the expected molecular mass predicted from their amino acid sequences (79,6 kDa for α_1 S, 78,6 kDa for α_1 , 83,8 kDa for α_2 S, 71,4 kDa for β_1 S and 70,4 kDa for β_1). For the conjoined NOsGC's one signal could be detected with a molecular mass of approximately 150 kDa, which is close to the estimated molecular mass of $\beta_1\alpha_1$ S and $\beta_1\alpha_2$ S conjoined NOsGC (149,1 kDa and 158,6 kDa). STREP tag II purification of the co-expressed NOsGC isoforms with carboxy-terminally tagged β_1 subunits showed an unequal ratio of NOsGC subunits with a shift to the β_1 subunit (Fig.3 A) indicating the purification of substantial amounts of β_1 homodimers besides heterodimeric enzyme. Purification using the carboxy-terminally tagged α subunit co-expressed with a non-tagged β_1 subunit led to an apparent 1 : 1 ratio of α and β subunits (Fig. 3B). Spectroscopic analysis of the purified enzymes (Fig. 4) revealed absorption maxima at 430 nm in the absence and 400 nm in the presence of the NO Donor DEA/NO (100 μ M). The maxima of conjoined NOsGC's (Fig. 4C and 4D) were identical to the respective co-expressed NOsGC isoforms (Fig. 4A and 4B). The ratio of the absorption of 430 nm to 280 nm for all purified NOsGC variants was not significantly different.

To analyze whether the conjoined NOsGC's exist as monomers and correspond in their native molecular mass to the co-expressed NOsGC heterodimer, we did a comparison of analytical gel filtration. The elution profiles were recorded at 430 nm. Conjoined NOsGC's and co-expressed NOsGC isoforms showed a peak at the same elution volume indicating similar molecular masses (Fig. 5 upper panel). Western Blot analysis using antibodies directed against the NOsGC subunits were done to ensure the identity and integrity of the eluted sample (Fig. 5 lower panel).

Guanylyl cyclase activity of the purified enzyme was measured under basal conditions, in the presence of NO (100 μ M DEA/NO) and in the presence of additional YC-1 (100 μ M) to analyze

the properties of the enzyme preparations (Fig. 6). Guanylyl cyclase activity under NO and NO/YC-1 stimulated conditions was not different between conjoined NOsGC and the heterodimeric form. Only basal activity was significantly increased in conjoined NOsGC ($p < 0.05$).

To compare the kinetic properties of the conjoined NOsGC's with the respective heterodimeric NOsGC isoforms, cGMP formation was determined in the presence of increasing GTP concentrations. The Lineweaver-Burk plot of the data under basal conditions (Fig. 7, left) and the presence of 100 μ M DEA/NO (Fig. 7, right) revealed that the kinetic data (Fig. 7, tables) showed no significant differences between the conjoined NOsGC's and the respective heterodimeric NOsGC isoforms.

Concentration response experiments were done using the NO donor DEA/NO (Fig. 8). Although the EC_{50} values for DEA/NO showed no significant differences between the conjoined NOsGC's and the respective heterodimeric NOsGC isoforms, there was a trend towards an increased EC_{50} value (lower affinity) for heterodimeric α_1 S/ β_1 .

Guanylyl cyclase assays were also performed in the presence of the heme independent NOsGC activators cinaciguat (BAY 58-2667) (Fig. 9 left) and ataciguat (HMR 1766) (Fig. 9 right). In the presence of cinaciguat and ataciguat (Fig. 9) the purified heterodimeric α_1 S/ β_1 isoform and $\beta_1\alpha_1$ S conjoined NOsGC showed an expected increase in enzyme activity. Treatment with ODQ led to a further expected activation by cinaciguat and ataciguat for the α_1 S/ β_1 isoform and $\beta_1\alpha_1$ S conjoined NOsGC. In contrast, the purified heterodimeric α_2 S/ β_1 isoform and conjoined $\beta_1\alpha_2$ S NOsGC were just marginally activated by cinaciguat and ataciguat (Fig. 9). The difference in activation by ciguates between the α_2 containing and α_1 containing forms was statistically significant ($p < 0.05$). Treatment with ODQ did not change the isoform specific difference in activation by ciguates between the the α_2 containing and α_1 containing forms.

To compare the kinetic properties of the enzyme isoforms in the presence of cinaciguat and ODQ, cGMP formation was determined in the presence of increasing GTP concentrations and plotted according to Lineweaver-Burk (Fig. 10). Cinaciguat increased the V_{\max} of the heterodimeric α_1S/β_1 isoform significantly ($p < 0.05$), while there was no significant increase for the α_2S/β_1 isoform. The K_m values of both isoforms showed a significant decrease in the presence of cinaciguat ($p < 0.05$). Measurement of EC_{50} values for the activation by cinaciguat confirmed the data for the α_1S/β_1 isoform [17], while no EC_{50} value could be detected for the α_2S/β_1 isoform (data not shown).

4. Discussion

Since 1879 until now the drug of choice for the treatment of acute angina pectoris is and has been glycerol trinitrate [18]. The receptors for this drug in humans are both NO sensitive guanylyl cyclase isoforms α_1/β_1 and α_2/β_1 . No difference has so far been detected in the pharmacological properties of both purified enzyme isoforms [4]. In the present paper we provide the first evidence that ciguat-activators a novel non-NO-releasing drug group activate the α_1/β_1 isoform of NOsGC much more effectively than the α_2/β_1 subtype. This is a surprising finding as the ciguat-activators bind to the heme NO binding domain of the β_1 subunit [17] which is common to both isoforms. It would have been conceivable that a different degree of heme content or oxidation in α_1/β_1 and α_2/β_1 isoforms explains the difference. But our data showing identical heme content between both isoforms (see Fig. 4) and the preserved difference with respect to ciguate activation in the presence of the heme oxidizing substance ODQ argue against this idea. Thus the difference in ciguat-activation between both isoforms seems to lie in the intramolecular signal transduction that converts binding of the ciguat-activators into a conformational changes in the catalytic region leading to an increase in V_{\max} for the α_1/β_1 isoforms. A cinaciguat-analogue has been shown to bind to tyrosine 371 of the α_1 subunit [17]. It is conceivable that this tyrosine in the α_1 subunit and the lack of a homologous tyrosine in the α_2 subunits contributes to the isoform specific V_{\max} increase in the α_1/β_1 isoform. However, the specificity is not absolute since similar to NO the affinity towards the substrate GTP is increased for both isoforms in the presence of cinaciguat as shown by decreased K_m values. We would like to emphasize that an isoform specific V_{\max} increase seen with purified enzyme preparations is not necessarily linked to isoform specificity in vivo. Local substrate concentrations, differing subcellular localization of both isoforms or protein interactions may all lead to differences

between isolated enzymes and the intact cell situation. The selectivity of ciguates for the α_1/β_1 isoform and its pharmacological relevance could and should be further analyzed in α_1 KO mice [19-22].

Given that the potentially isoform specific ciguat-activators are being developed clinically, it seems very important to study the individual roles and specialized functions of the different isoforms. Knockout animals of the different α subunits have provided important insights in the effects of selective abrogation of one isoform [19-22]. It did not seem feasible so far to do the complementary reverse experiment namely selective overexpression of one isoform in a transgenic mouse model. First, co-expression of α and β subunits with similar levels of expression is technically demanding. Second, even if successful it is unclear to what extent overexpressed single α and β subunits interfere with the respective other enzyme isoform. The knockout animals clearly demonstrate interdependence of the stability of the α and β subunits [19]. Third in a transgenic overexpression model homodimers of isolated α or isolated β subunits will form in addition to heterodimers and there is recent evidence to suggest that they have a separate function from cGMP signaling: It has been suggested that the β_1 subunit is a multifunctional protein that regulates chromatin condensation and cell cycle progression in addition to being an obligate component of functional NOsGC-heterodimers [9]. Published data also indicates that the α_1 subunit acts in prostate cancer via a novel pathway that does not depend on the β_1 subunit [7] and is especially important in androgen-independent growth of prostate cancer cells [8].

The genes encoding the α_1 and β_1 subunits are colocalized in all species analyzed so far [23-25] and have a striking tandem arrangement in medaka fish where the last exon of the α_1 subunit and first exon of the β_1 subunit are separated by less than 1000 base pairs [26]. Currently it is unclear what selection pressure preserves this peculiar genomic colocalization throughout

evolution. Although both subunit genes have stayed very close in the phylogenetic tree of animals there is no species known where the genes actually fused to form one polypeptide chain as in other nucleotide cyclases e.g. mammalian adenylyl cyclase. It would have been conceivable that structural requirements preclude such a gene fusion. But as we show in the current paper this is not the case. We hypothesize that the heterodimeric nature of the enzyme has other important functions that are vital for organisms. First, it allows a function of either single α subunits or single β subunits discussed above [7-9]. A second possibility is that regulating expression of different mRNAs and regulating stability of different subunits allows better fine tuning of the NO sensing enzyme: We have recently given further support to the idea initially proposed by Zabel et al. [5] that regulation of NOsGC activity in vivo might involve changes in the extent of heterodimerization [6].

We show for the first time isoform specific activation of NOsGC by ciguates using purified protein and provide tools to investigate the two different isoforms in two separate entities in overexpression experiments with no interference by single subunits or homodimers. If experiments in transgenic animals are encouraging conjoined NOsGC constructs may also enable experiments directed towards therapeutic overexpression of NOsGC in gene therapy.

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Footnotes

¹ ResearcherID: A-1701-2010

² ResearcherID: A-4297-2010

³ ResearcherID: D-5327-2009

⁴ ResearcherID: A-2551-2010

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Abbreviations

BSA, bovine serum albumin

CFP, cyan fluorescent protein

CV, column volume

DTT, dithiothreitol;

GFP, green fluorescent protein

NO, nitric oxide

NOsGC, NO sensitive guanylyl cyclase

PAGE, polyacrylamide gel electrophoresis

PBS, phosphate buffered saline

SDS, sodium dodecyl sulfate

S, STREP tag II

TEA, triethanolamine

YFP, yellow fluorescent protein

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Legends of figures

Fig. 1. Homodimerization of NOsGC subunits. Cytosolic fractions of Sf9 cells coexpressing YFP-tagged and nontagged NOsGC subunits were used for immunoprecipitation with an antibody against YFP. The precipitates were analyzed by Western blot using antibodies against β_1 (A), α_1 (B) and α_2 (C) subunit of NOsGC.

Fig. 2. Comparison of NOsGC activity in cytosolic fractions of Sf9 cells coexpressing the α and β subunits and expressing the conjoined NOsGC. Guanylyl cyclase activity was measured under basal conditions (black columns), in the presence of 100 μ M DEA/NO (white columns) and in the presence of additional 100 μ M YC-1 (gray columns). Data are expressed as means \pm S.E.M. (n = 4).

Fig. 3. SDS-PAGE analysis of purified NOsGC variants (A - α_1/β_1 S; B - α_1 S/ β_1 and $\beta_1\alpha_1$ S; C - α_2 S/ β_1 and $\beta_1\alpha_2$ S). 1 μ g of purified enzyme (S indicates STREP tag) was electrophoretically separated by SDS-PAGE and stained with Coomassie Blue (a representative gel is shown).

Fig. 4. Spectroscopic analysis of purified guanylyl cyclase enzyme complexes (A α_1 S/ β_1 ; B α_2 S/ β_1 ; C $\beta_1\alpha_1$ S; D $\beta_1\alpha_2$ S). Spectroscopic analysis shows relative absorption values at basal (*solid line*) or NO-stimulated (100 M DEA/NO, *dotted line*) conditions. The ratio of the absorption at 432 nm to 280 nm was 0.81 ± 0.13 for α_1 S/ β_1 , 0.82 ± 0.10 for $\beta_1\alpha_1$ S, 0.92 ± 0.04 for α_2 S/ β_1 , and 0.90 ± 0.05 for $\beta_1\alpha_2$ S (S indicates STREP tag)

Fig. 5. Characterization of the purified NOsGC variants by gel chromatography and Western blot analysis. Elution profiles of NOsGC variants are shown for gel filtration column (upper panel). The respective peak fractions were pooled, concentrated and 1 μ g of protein was used for Western blot analysis using antibodies against α_1 , α_2 and β_1 subunit of NOsGC (lower panel, a representative blot is shown).

Fig. 6. Comparison of NOsGC activity of purified NOsGC heterodimer and conjoined NOsGC. Guanylyl cyclase activity was measured under basal conditions (black columns), in the presence of 100 μ M DEA/NO (white columns) and in the presence of additional 100 μ M YC-1 (gray columns). Data are expressed as means \pm SEM (n = 3).

Fig. 7. Double reciprocal Lineweaver-Burk plot for substrate dependence of respective purified NOsGC variants (see figure key). Substrate dependence was measured in a range from 0.01 to 1 mM under basal conditions (left) and in the presence of 100 μ M DEA/NO (right). In the tables below the diagram the V_{\max} and K_m values are shown. Data represent means of at least three independent experiments performed in duplicate \pm S.E.M.

Fig. 8. Concentration-dependent effect of of DEA/NO on purified guanylyl cyclase activity. Dose-response curve of α_1 S/ β_1 (closed circle), $\beta_1\alpha_1$ S (open circles), α_2 S/ β_1 (closed triangles) and $\beta_1\alpha_2$ S (open triangles) were measured in a range of 0.1 nM to 1 mM DEA/NO. EC_{50} values are given in the table. Data are expressed as means \pm S.E.M. (n = 3).

Fig. 9. Guanylyl cyclase activity of the purified respective NOsGC enzyme complexes in the presence of cinaciguat (left) and ataciguat (right). Guanylyl cyclase activity was measured

under basal conditions (black columns), in the presence of 10 μM cinaciguat or 10 μM ataciguat (grey columns) and in the presence of additional 10 μM ODQ (striped columns). Data are expressed as means \pm SEM ($n = 3$, * $p < 0.05$ $\alpha_1\text{S}/\beta_1$ vs $\alpha_2\text{S}/\beta_1$; $^{\S}p < 0.05$ $\beta_1\alpha_1\text{S}$ vs $\beta_1\alpha_2\text{S}$; $^{\#}p < 0.05$ $\alpha_1\text{S}/\beta_1$ vs $\alpha_2\text{S}/\beta_1$; $^{\S}p < 0.05$ $\beta_1\alpha_1\text{S}$ vs $\beta_1\alpha_2\text{S}$).

Fig. 10. Double reciprocal Lineweaver-Burk plot for substrate dependence of respective purified sGC variants (see figure key) in the presence of 10 μM ODQ and 10 μM cinaciguat. Substrate dependence was measured in a range from 0.01 to 1 mM. Data represent means of at least three independent experiments performed in duplicate \pm S.E.M. ($n = 3$, statistical significance for V_{max} and K_m : * $p < 0.05$ $\alpha_1\text{S}/\beta_1$ vs $\alpha_2\text{S}/\beta_1$).

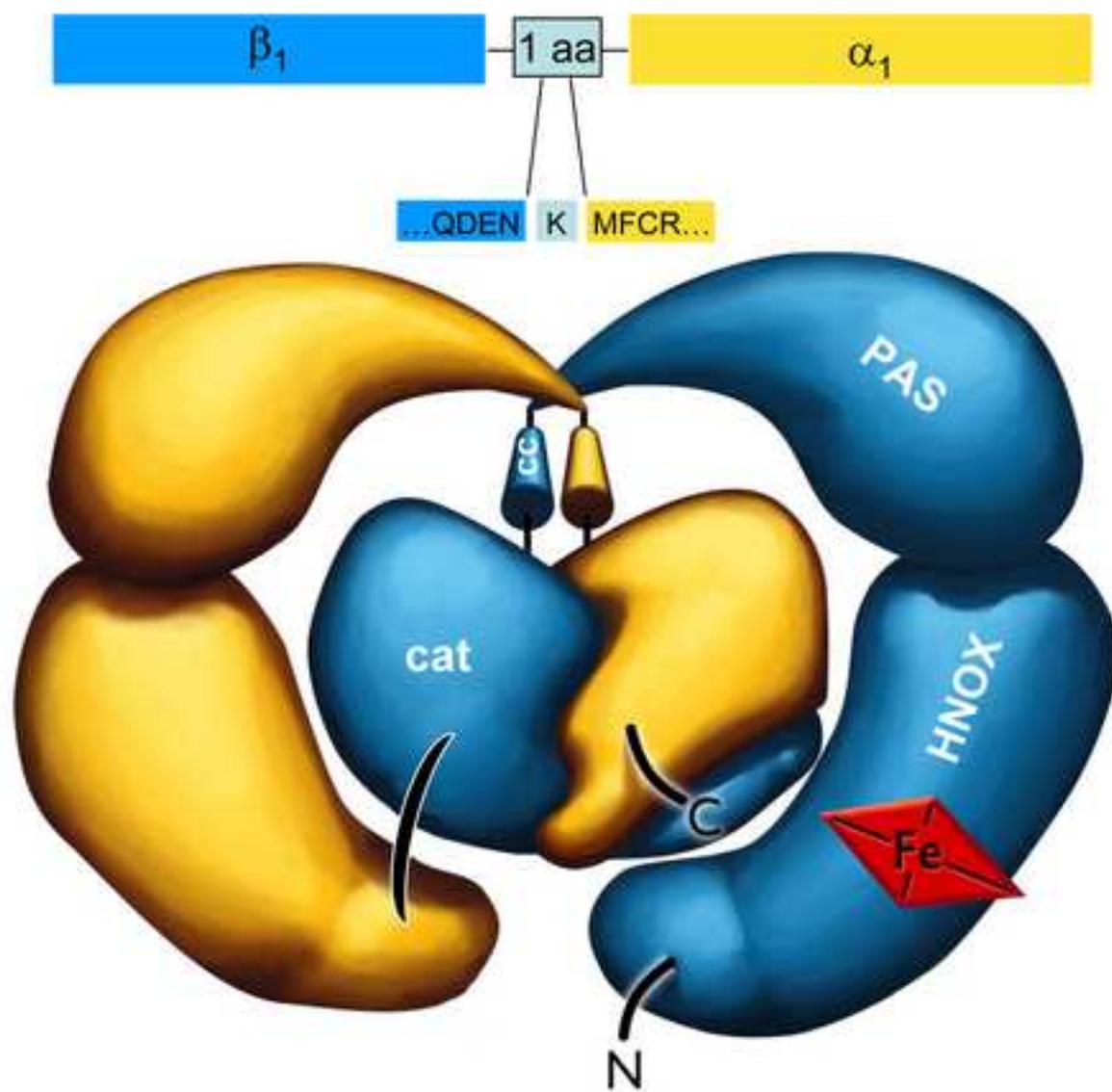


Figure 1

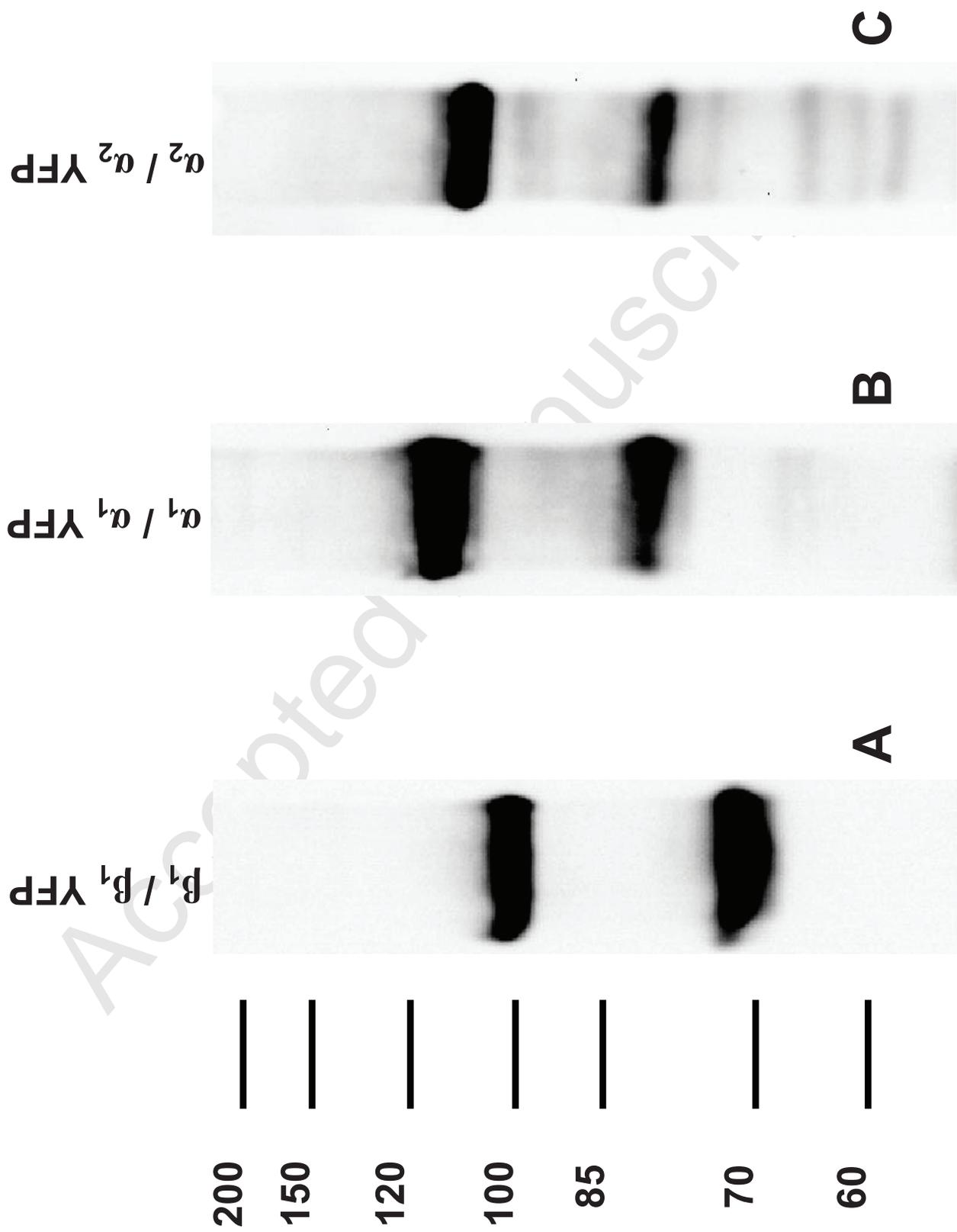


Figure 2

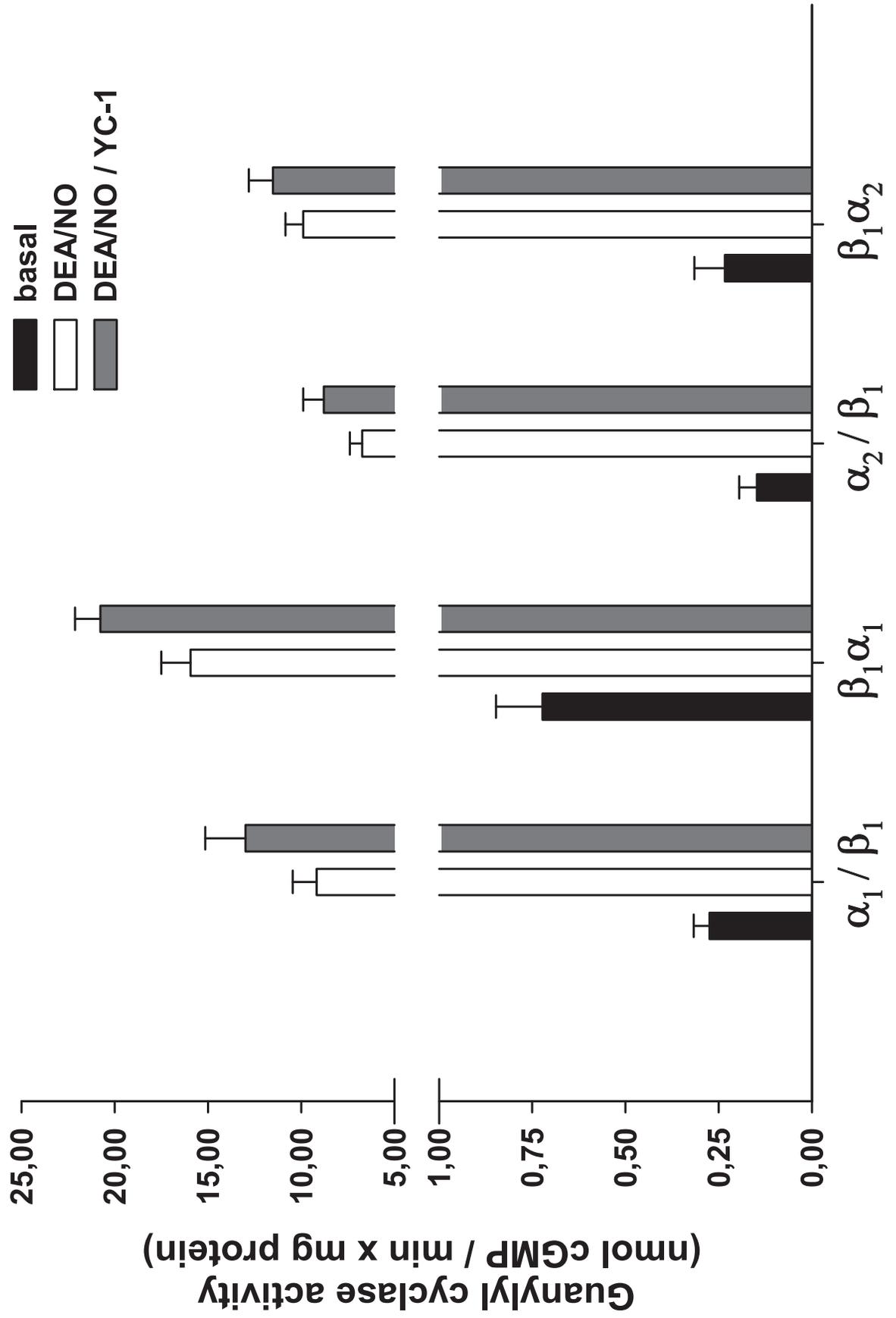


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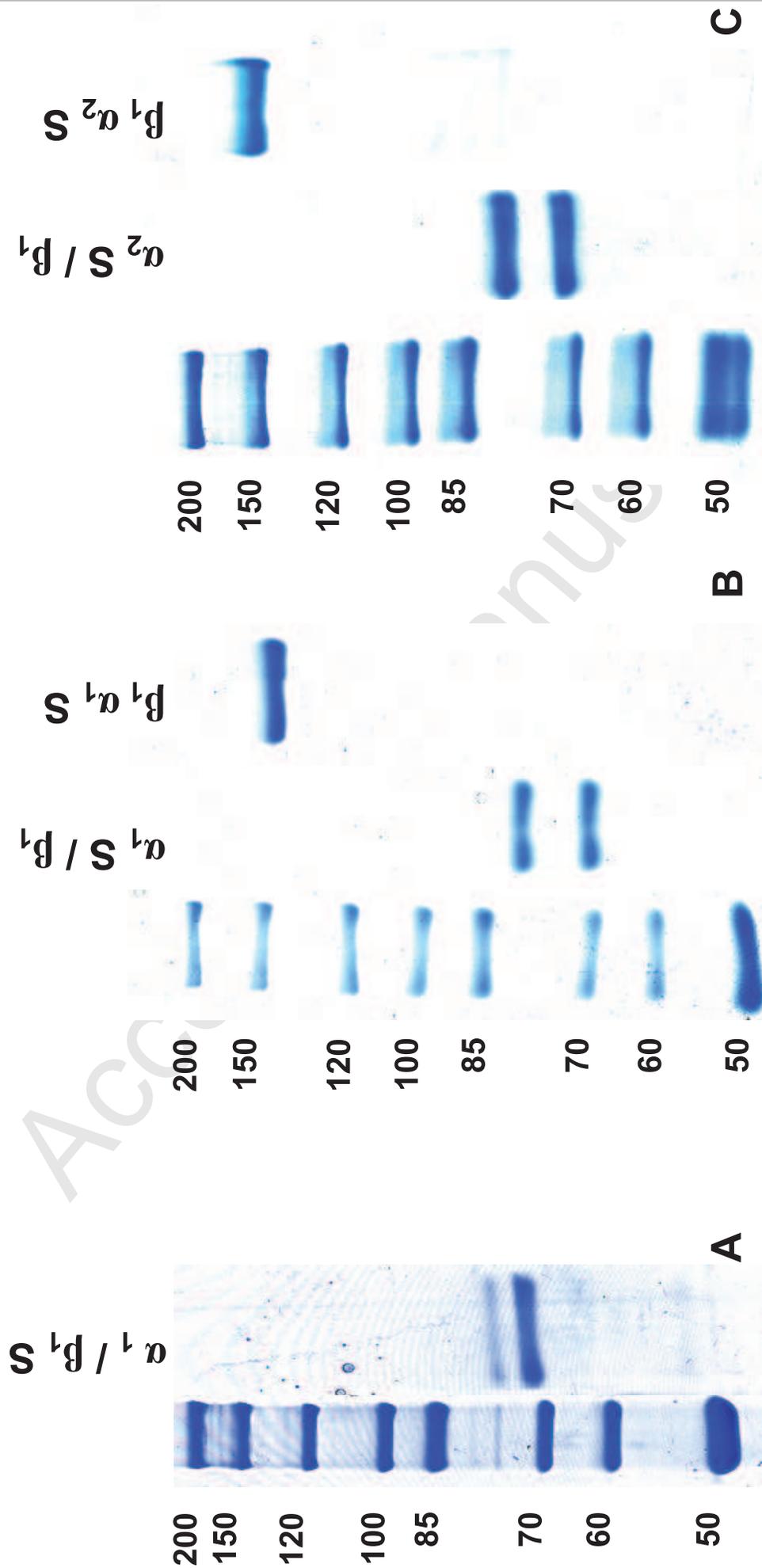


Figure 4

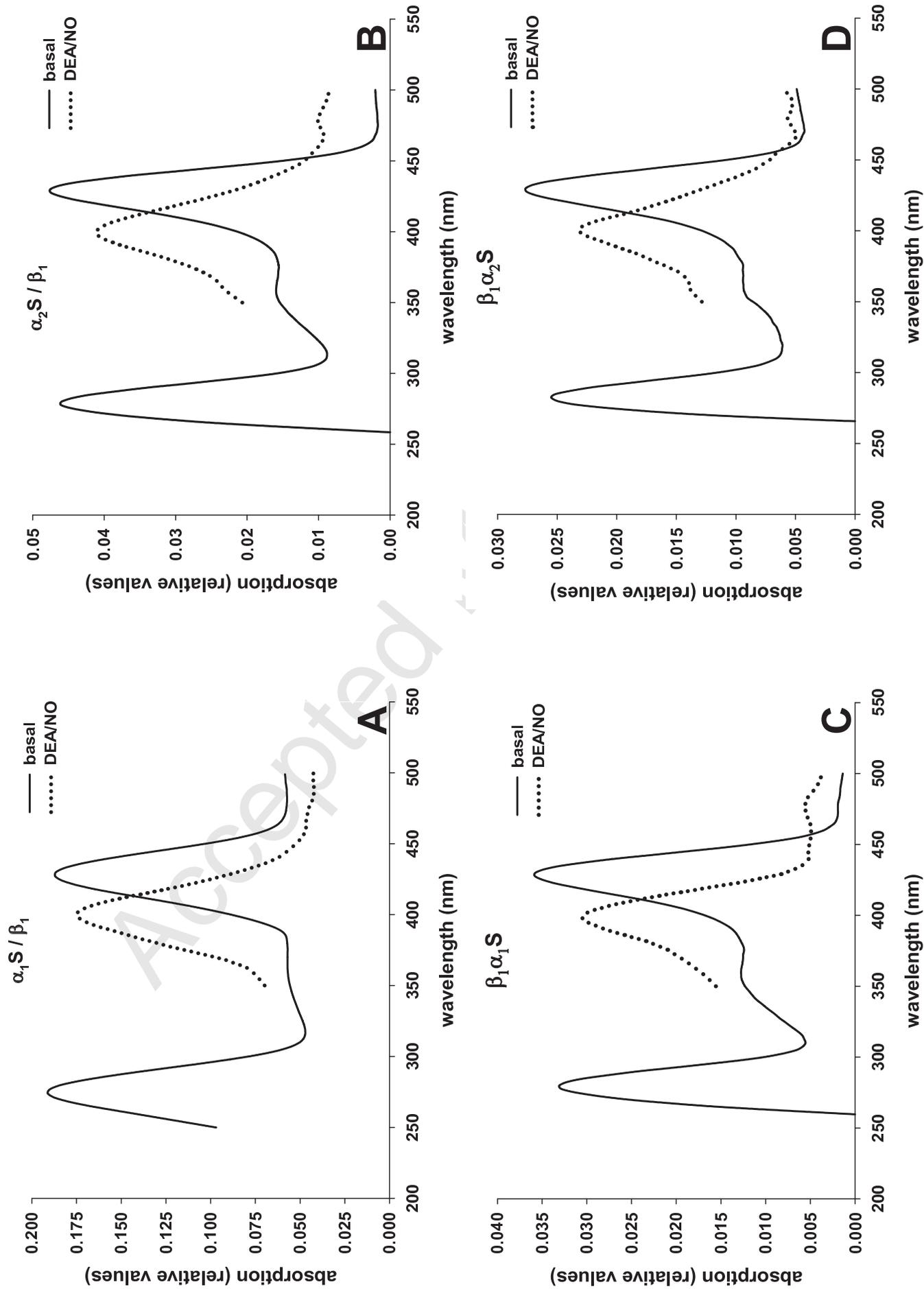


Figure 5

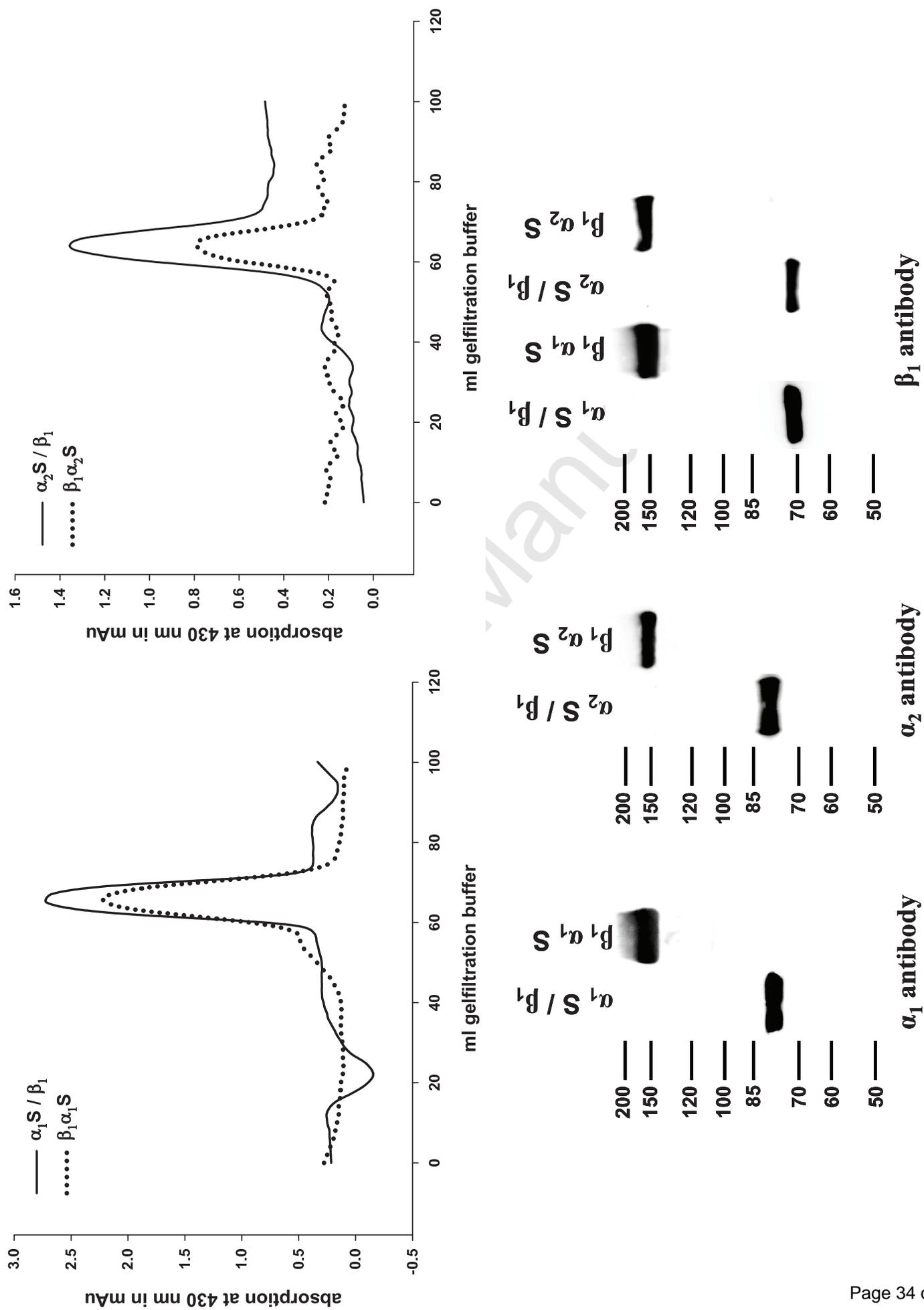


Figure 6

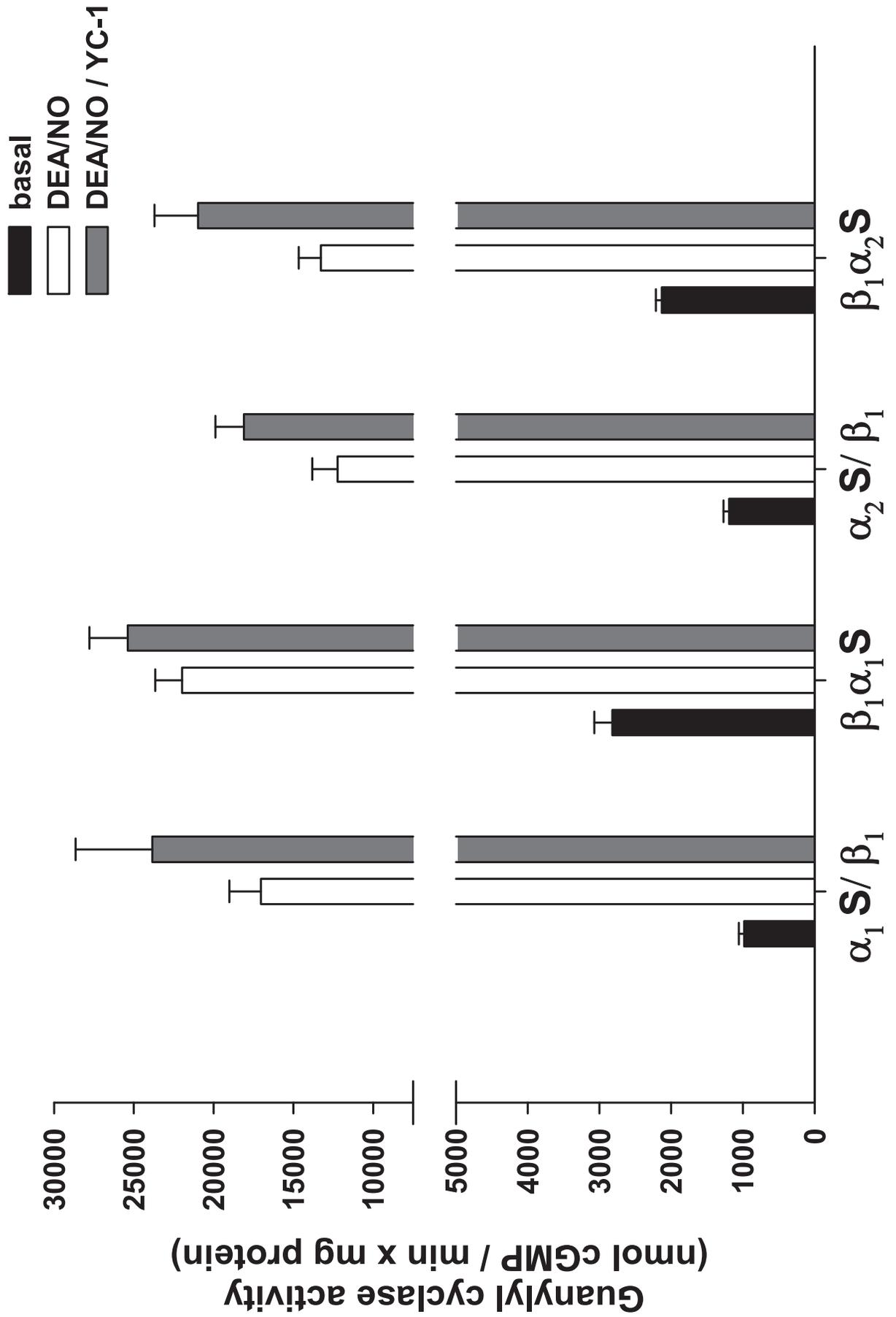
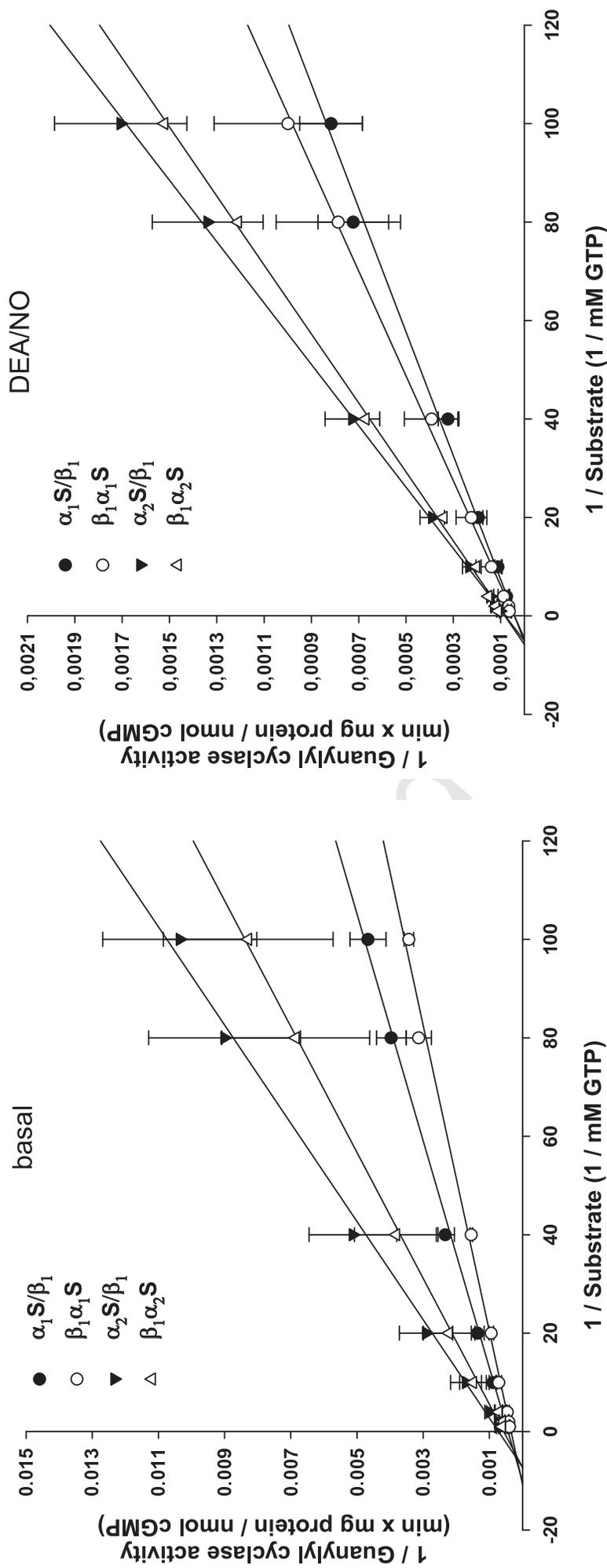


Figure 7



basal	V_{max} in $\mu\text{mol cGMP} / \text{min x mg}$	K_m in $\mu\text{M GTP}$
α_1S/β_1	2.36 ± 0.35	99.64 ± 8.31
$\beta_1\alpha_1S$	2.96 ± 0.35	95.35 ± 11.59
α_2S/β_1	1.49 ± 0.28	136.41 ± 4.89
$\beta_1\alpha_2S$	1.98 ± 0.51	135.37 ± 21.81

DEANO	V_{max} in $\mu\text{mol cGMP} / \text{min x mg}$	K_m in $\mu\text{M GTP}$
α_1S/β_1	24.22 ± 0.23	192.75 ± 36.5
$\beta_1\alpha_1S$	24.73 ± 3.57	213.28 ± 39.72
α_2S/β_1	12.54 ± 0.86	200.80 ± 35.91
$\beta_1\alpha_2S$	14.07 ± 4.24	204.67 ± 69.68

Figure 8

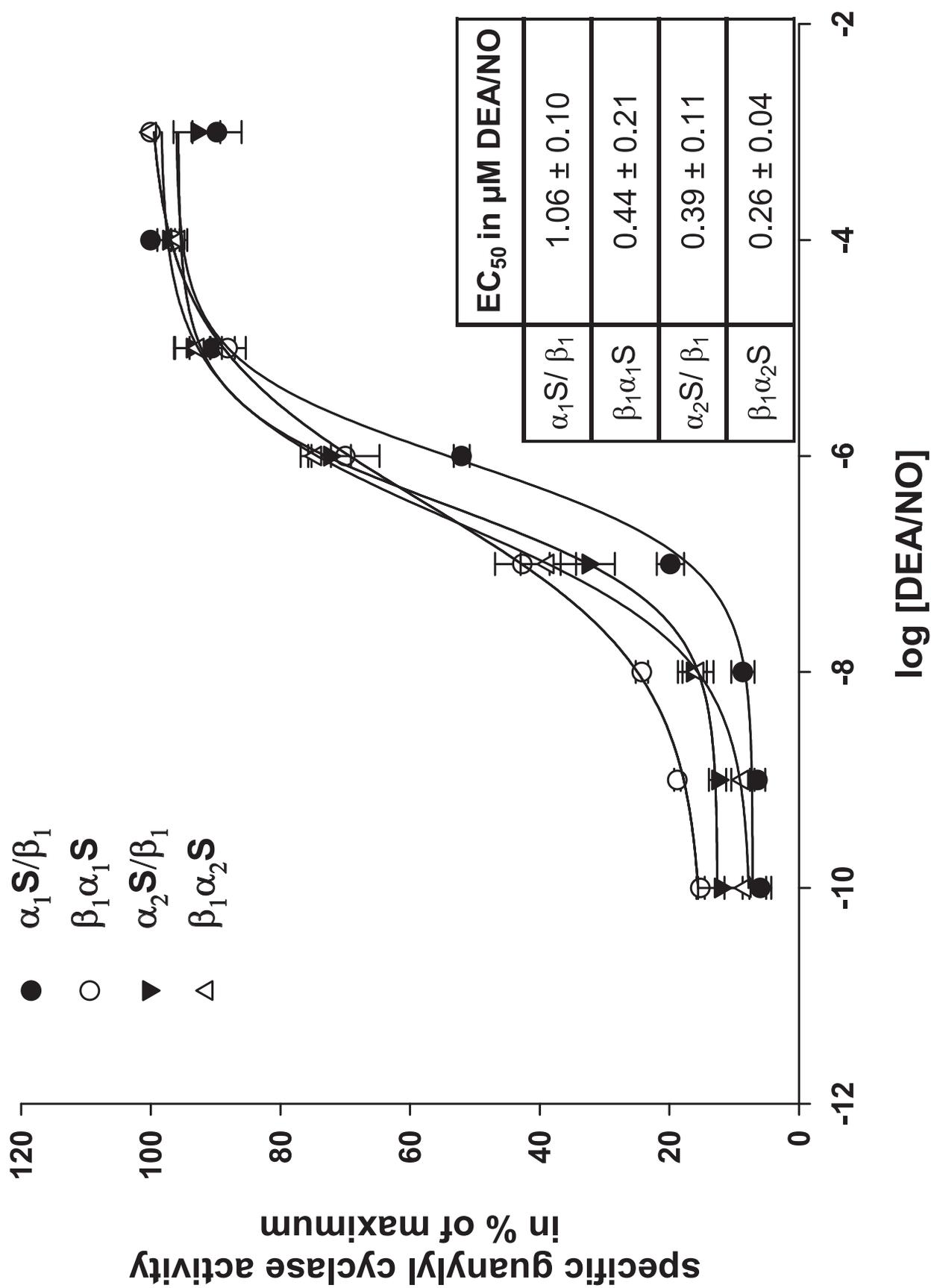


Figure 9

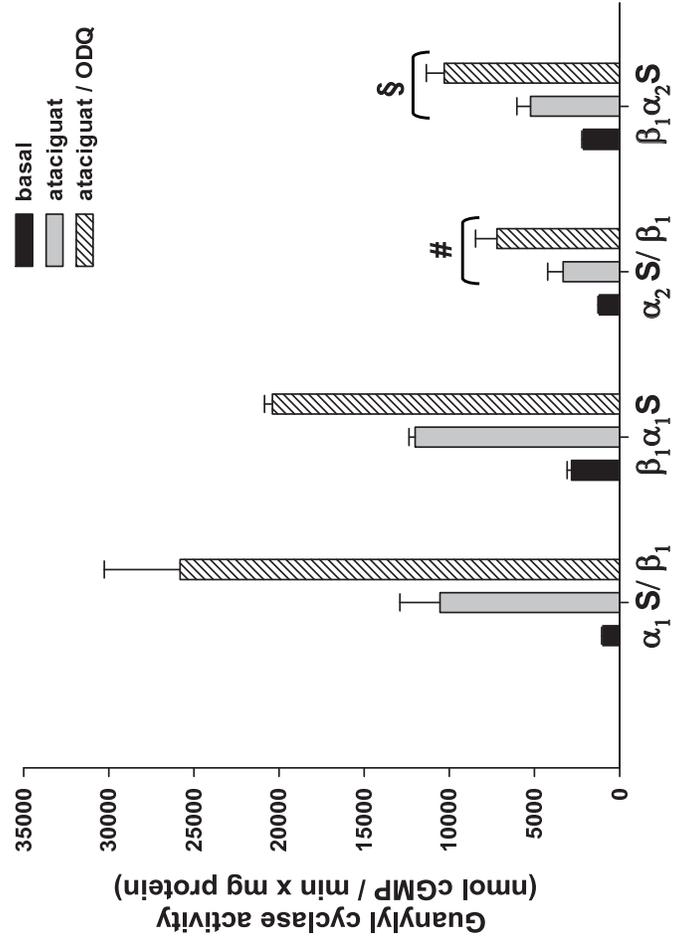
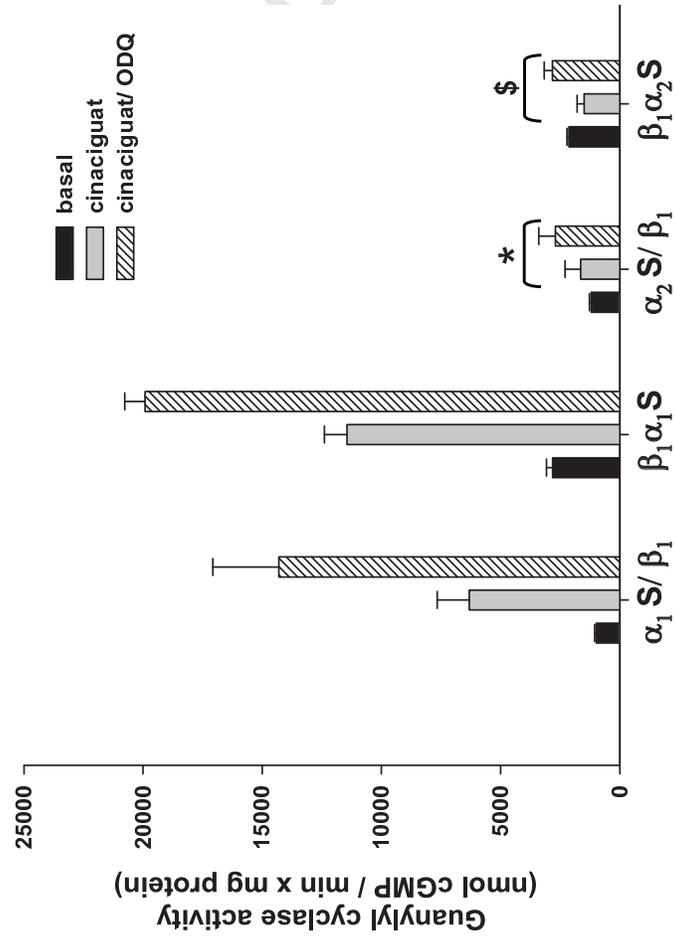
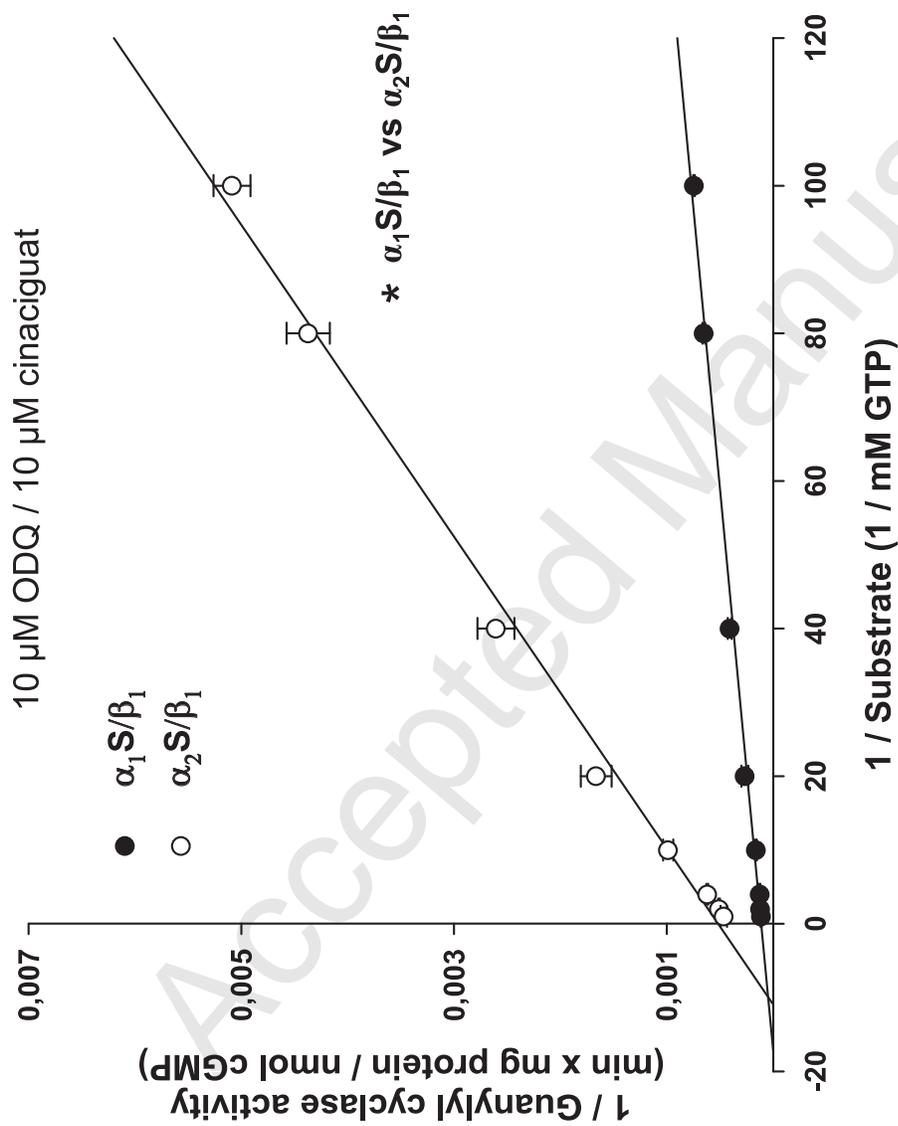


Figure 10



cinaciguat	V_{\max} in μ mol cGMP / min x mg	K_m in μ M GTP
$\alpha_1 S/\beta_1$	9.44 ± 0.16	61.92 ± 1.57
$\alpha_2 S/\beta_1$	1.95 ± 0.13	91.70 ± 2.04