Functions of Conserved Cysteines of Soluble Guanylyl Cyclase†

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ABSTRACT: Soluble guanylyl cyclase (sGC), a heme-containing heterodimeric enzyme, is stimulated by NO and catalyzes the formation of the intracellular signaling molecule cGMP. Cysteine residues of sGC have been considered to be important as they were thought to play a significant role in the regulation of the enzyme. The aim of this study was to investigate the possible function of conserved cysteine residues of sGC. Fifteen conserved cysteine residues on sGC were point-mutated to serine, using site-directed mutagenesis. All of the resulting recombinant enzymes were able to synthesize cGMP. Mutation of two cysteines located in the N-terminal, putative heme-binding region of the β_1 subunit yielded proteins that were insensitive to NO. Spectrophotometric analysis of the NO-insensitive mutants purified from Sf9 cells revealed a loss of the prosthetic heme group. Both mutants could be reconstituted with heme and, as a consequence, NO sensitivity of the mutants was restored. Our data show that mutation of two cysteines of the β_1 subunit (Cys-78 and Cys-214) reduces the affinity of sGC for heme. Mutation of the corresponding cysteines on the α_1 subunit did not alter NO responsiveness, indicating that heme-binding is mainly a feature of the N-terminal domain of the β_1 subunit.

By formation of the intracellular signaling molecule cGMP, soluble guanylyl cyclase (sGC)[1] plays a key role in smooth muscle relaxation and inhibition of platelet aggregation. The heterodimeric enzyme consists of two subunits designated α and β with four different isoforms (α_1, α_2, β_1, and β_2) known to date (Garbers et al., 1994; Garbers & Drewett, 1994). sGC is stimulated by nitric oxide (NO), which interacts with the enzyme's prosthetic heme group and leads to a conformational change with subsequent increase in enzyme activity (Ignarro et al., 1984). Recently we were able to show that the homologous C-terminal regions of the subunits, also conserved in the membrane-bound guanylyl cyclases, are sufficient for the formation of cGMP whereas the less conserved N-termini of the subunits are required for NO responsiveness (Wedel et al., 1995).

Cysteine residues are of particular importance regarding structure and function of proteins. Cell surface and secreted proteins frequently exhibit disulfide bonds responsible for structure and stability of particular domains. The reducing conditions in the intracellular environment usually prevent formation of disulfide bridges within cytosolic proteins, although under certain conditions, shortlived disulfide bridging can be induced (Gopalakrishna et al., 1993; Lipton et al., 1993; Duhe et al., 1994) which may exert a regulatory effect on structure and function of an enzyme.

As sulfhydryl groups can undergo oxidation−reduction reactions, it is reasonable to suggest cysteine residues to be primary targets of reducing or oxidizing substances on proteins. Apart from oxidation−reduction reactions, modifications such as alkylation, acylation, ADP-ribosylation, or nitrosylation may also occur on Cys residues, thus possibly influencing an enzyme's regulatory or catalytic properties.

Thiol groups on sGC have received broad attention, especially during the early days of sGC research. Cys residues were thought to play important roles in non-NO-mediated sGC regulation, and during the 1970s and 1980s, substantial work focused on regulation via a "redox-sensitive thiol switch" (Haddox et al., 1978; Craven & DeRubertis, 1978a; Braughler et al., 1979; Braughler, 1980; Böhme et al., 1983). The importance of thiol groups of sGC was shown by various reports: Formation of mixed disulfides occurred as ^{32}S-labeled cysteines were shown to be incorporated into sGC (Brandwein et al., 1981). The use of several thiol blockers led to inhibiton of basal as well as stimulated activity of sGC (Craven & DeRubertis, 1978a,b), suggesting the involvement of thiols in enzyme regulation. As a consequence, regulation of sGC via two different classes of cysteines was postulated (Braughler, 1983), one being responsible for the maintenance of enzyme activity, the other one for stimulation by nitrovasodilators. Yet, a definite function of single cysteine residues on sGC has not been clearly demonstrated so far.

The aim of this study was to investigate the function of conserved cysteine residues of sGC. Fifteen conserved cysteines were mutated into serine. All mutated enzymes were catalytically active and therefore not essential for catalysis and dimerization. Yet two mutants of the β_1 subunit did not exhibit responsiveness to NO. These two NO-insensitive mutants were found to be heme-deficient and to regain NO sensitivity after heme reconstitution. As mutation of the corresponding cysteines of the α_1 subunit did not change NO sensitivity of the enzymes, our results support

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[1] Abbreviations: sGC, soluble guanylyl cyclase; NO, nitric oxide; DEA-NO, diethylamine−NO complex; GSNO, *S*-nitrosoglutathione; WT, wild type; DTT, dithiothreitol; PP-IX, protoporphyrin IX.

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the notion of an exclusive role of the β_1 subunit in heme binding.

MATERIALS AND METHODS

Materials. 2,2-Diethyl-1-nitrosooxyhydrazine sodium salt (DEA-NO) was purchased from NCI Chemical Carcinogen Repository. Protoporphyrin IX and hemin were obtained from Sigma, and Tween 20 was purchased from Boehringer Mannheim. [α-^{32}P]GTP (800 Ci/mmol) was from NEN−DuPont.

Construction of Mutants. Site-directed mutagenesis of Cys residues was performed by the Kunkel method (Kunkel, 1985; Kunkel et al., 1987) using the Muta-Gene M13 in vitro mutagenesis kit (Bio-Rad, München, Germany). Single-strand mutagenic primers containing the respective point mutations were purchased from TIB Molbiol, Berlin, Germany.

Expression of Recombinant Guanylyl Cyclase. Expression of the recombinant proteins in COS cells, determination of sGC activity in COS cell cytosol, and immunoblot analysis of the cytosolic proteins were carried out as described (Harteneck et al., 1990). The β_1C78S and β_1C214S mutants were subcloned into the baculovirus expression vector pVL 1392 (Invitrogen). The 2.5-kb cDNAs of the mutants ligated in the *Hin*dIII and *Eco*RI sites of pCMV were digested with *Xba*I and *Ssp*I and subsequently cloned in the *Xba*I and *Sma*I site of pVL 1392.

Sf9 Cell Culture. Sf9 cells were propagated as monolayers in TNM-FH medium (Sigma) supplemented with 10% (v/v) fetal calf serum. Spinner-culture cells were grown in TNM-FH medium supplemented with 10% fetal calf serum, streptomycin (100 μg/mL), penicillin (100 units/mL), and 1% lipid concentrate (Gibco). Generation of recombinant viruses was performed by cotransfection of Sf9 cells with the above-described viruses and with BaculoGold baculovirus DNA (Dianova) by the lipofectin method (Groebe et al., 1990). Positive clones were isolated by plaque assay and identified by expression of the appropriate proteins as detected by immunoblot analysis. Purification of recombinant enzymes was carried out after coinfection of spinner-culture cells (2−3 L were grown to 1.3−1.5 $\times$ 10^6 cells/mL) with the appropriate virus and the α_1 WT virus at a multiplicity of infection of 3 for each virus. Sixty-three hours after infection, cells were collected by centrifugation, resuspended in two volumes of 50 mM NaCl, 1 mM EDTA, 0.2 mM benzamidine, and 50 mM triethanolamine hydrochloride, pH 7.0, and lysed by sonification. The homogenate was centrifuged at 200000*g* for 50 min at 4 °C, and purification from cytosol was performed by anion-exchange and immunoaffinity chromatography as described (Humbert et al., 1990).

UV−vis Absorption. Absorbance spectra of β_1C78S, β_1C214S, and WT guanylyl cyclase were recorded in a photodiode array detector with an 8−μL sample cell (Waters 990, Millipore). Five micrograms of either WT or mutants purified from Sf9 cells was injected onto a column (0.5 $\times$ 6 cm) of Sephadex G25 fine (Pharmacia) connected to the photodiode array detector and equilibrated with 300 mM NaCl and 50 mM triethanolamine hydrochloride, pH 7.0, at a flow rate of 0.15 mL/min.

Heme Reconstitution. Heme removal and reconstitution of β_1C78S, β_1C214S, and WT enzyme with heme or protoporphyrin IX was performed under anaerobic condition in nitrogen atmosphere as follows: For heme removal, enzyme was diluted in 1 mg/mL bovine serum albumin, 0.5% (v/v) Tween 20, 50 mM triethanolamine hydrochloride, pH 7.4, and 3 mM dithiothreitol. Reconstitution with heme or protoporphyrin IX was achieved by adding dithionite-reduced heme to a final concentration of 5 μM (100 μM dithionite) or protoporphyrin IX to a final concentration of 25 μM to the heme-depleted enzyme preparations. All samples were preincubated for 2 min at 37 °C before the addition of substrate and the determination of enzyme activity.

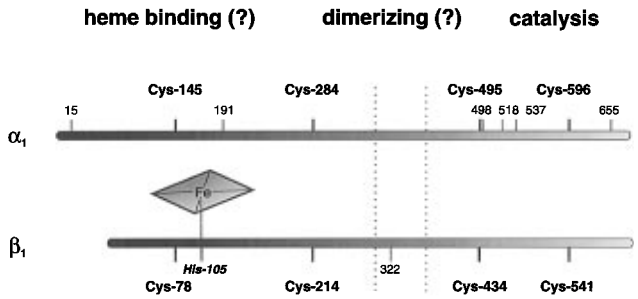

FIGURE 1: Positions of mutated Cys residues on a schematic representation of the $\alpha_1\beta_1$ heterodimer of sGC. Cys residues conserved in all known α and β subunits are printed in boldface type, whereas cysteines conserved between the α or β subunits are indicated by their amino acid number. Additionally, the position of His-105 of the β_1 subunit, the proximal heme ligand, is indicated.

Determination of Guanylyl Cyclase Activities and Protein Concentrations. Soluble guanylyl cyclase was purified from bovine lung as described previously (Humbert et al., 1990). Cyclase activity was measured by the conversion of [α-^{32}P]-GTP to [^{32}P]-cGMP at 37 °C for 10 or 15 min. Reaction mixtures contained 3 mM Mg^{2+} or Mn^{2+} as divalent metal ion, 3 mM dithiothreitol, 0.5 mg/mL bovine serum albumin, 1 mM cGMP, either 100 or 300 μM GTP, and 50 mM triethanolamine hydrochloride, pH 7.4, in a total volume of 0.1 mL. All measurements were performed in duplicate or triplicate and repeated at least three times. Protein concentrations were determined by the method of Bradford (1976) with bovine serum albumin as standard.

RESULTS

Mutagenesis. To investigate the function of Cys residues on sGC, we point-mutated 15 conserved cysteines to serine using site-directed mutagenesis. Cys residues mutated were either conserved between all known α and β subunits, conserved between α_1 and α_2 subunits, or conserved between β_1 and β_2 subunits [for alignment of amino acids see Harteneck et al. (1991)]. Figure 1 shows the amino acid number and indicates the approximate positions of the mutated cysteines in a schematic representation of the $\alpha_1\beta_1$ heterodimer. The subunits comprise the N-terminal putative heme-binding domain, the putative dimerizing domain, and the C-terminal catalytic domain for the α_1 subunit (residues 1−372, 373−438, and 439−691, respectively) and for the β_1 subunit (residues 1−311, 312−377, and 378−619, respectively) (Garbers & Lowe, 1994; Wedel et al., 1995; Wilson & Chinkers, 1995).

Cysteine mutants were coexpressed with the corresponding WT subunits in COS cells. Expression of the mutants was confirmed by Western blot analysis (data not shown).

Table 1 shows the basal and NO-stimulated activites of all 15 mutants in the cytosol of transfected COS cells in the

Table 1: Enzyme Activities of Mutant sGC in COS Cells[a]

	Mg^{2+}			Mn^{2+}		
	cGMP[b] (nmol min^{-1} mg^{-1})		stimulation factor (V_{GSNO}/V_{Co})	cGMP[b] (nmol min^{-1} mg^{-1})		stimulation factor (V_{GSNO}/V_{Co})
	Co	GSNO		Co	GSNO	
WT	0.46	14	30.4	0.56	5.17	9.2
α_1C15S	0.06	3.02	50.3	0.10	1.72	17.2
α_1C145S	0.68	10.04	14.8	0.41	3.98	9.7
α_1C194S	0.22	7.15	32.5	0.26	2.48	9.5
α_1C284S	0.23	6.08	26.4	0.29	2.29	7.9
α_1C495S	0.65	3.88	6.0	0.35	0.87	2.5
α_1C498S	0.39	13.42	34.4	0.39	4.30	11.0
α_1C518S	0.14	5.97	42.6	0.21	2.28	10.9
α_1C537S	0.21	9.54	45.4	0.19	2.51	13.2
α_1C596S	1.74	3.80	2.2	1.31	1.94	1.5
α_1C655S	0.14	7.90	56.4	0.26	2.49	9.6
β_1C78S	*c*	*c*	*d*	0.12	0.14	1.2
β_1C214S	*c*	*c*	*d*	0.11	0.15	1.3
β_1C322S	0.19	6.95	36.6	0.32	2.51	7.8
β_1C434S	0.44	5.14	11.7	0.59	2.01	3.4
β_1C541S	*c*	0.02	*d*	0.04	0.33	8.8

[a] Data are representative of at least three experiments. [b] Shown are nonstimulated (Co) and NO-stimulated (100 mM *S*-nitrosoglutathione, GSNO) enzyme activities. [c] Below the detection limit. [d] Stimulation factor cannot be given.

presence of either Mg^{2+} or Mn^{2+} as divalent cofactor. All Cys mutants were catalytically active in the presence of Mn^{2+}. Twelve out of 15 mutants corresponded well with a marked increase in cGMP formation to the addition of *S*-nitrosoglutathione (GSNO; 100 μM).

Three mutants exhibited significantly reduced NO stimulation: The low stimulation factors of α_1C596S (1.5- and 2.2-fold in the presence of Mg^{2+} or Mn^{2+}, respectively) are partly explained by elevation of basal cGMP production (2.5−4-fold). Two mutants of the β_1 subunit, β_1C78S and β_1C214S, exhibited stimulation factors of 1.2- and 1.3-fold in the presence of Mn^{2+}, respectively; in the presence of Mg^{2+}, cGMP production was below the detection limit.

These two mutants were chosen for further investigation as they apparently had lost NO sensitivity and since both mutants are located in the putative heme-binding domain of the β_1 subunit (see Figure 1). The loss of NO responsiveness may be caused either by heme deficiency of the mutants or by impairment of intramolecular signaling.

Purification of Mutant Enzymes. To investigate the molecular basis of the NO insensitivity of the two mutants, β_1C78S and β_1C214S were expressed in Sf9 cells to allow purification of the recombinant proteins. Purification of these mutants from Sf9 spinner culture was performed as described (Humbert et al., 1990) and yielded between 75 and 100 μg of mutant enzymes with near homogeneity as judged by SDS−PAGE (Figure 2). The identity of the proteins was additionally verified by immunoblotting (not shown).

Basal activity of the purified mutants was in the range of the WT enzyme (Table 2). Whereas the β_1C78S mutant was insensitive to NO after purification, the mutant β_1C214S, in contrast to our results in COS cell cytosol, was stimulated 17-fold by NO (10 μM DEA-NO). Stimulation factors of purified WT, β_1C78S, and β_1C214S enzymes correlated well with stimulation factors obtained in Sf9 cytosols (data not shown). At present, we do not have an explanation for the difference between COS and Sf9 cytosols regarding the NO sensitivity of the β_1C214S mutant.

Spectral Studies. In order to determine the heme content of mutant and WT enzymes, UV−vis absorbance spectra were obtained (Figure 3). Wild-type enzyme exhibited an

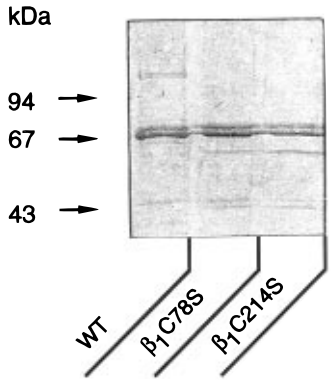

FIGURE 2: SDS−PAGE analysis of the wild-type and mutant sGCs purified from Sf9 cells. Purified WT or mutant enzyme (2 μg) was separated on a 7.5% (w/v) polyacrylamide gel which was subsequently stained with Coomassie Blue.

Table 2: Enzyme Activities of Mutant sGC Purified from Sf9 Cells

	cGMP[a] (nmol min^{-1} mg^{-1})		stimulation factor ($V_{DEA\text{-}NO}/V_{Co}$)
	Co	DEA-NO[b]	
WT	25 ± 5	1693 ± 527	68
β_1C78S	58 ± 2	54 ± 1	1
β_1C214S	58 ± 7	899 ± 85	17

[a] Values are means ± SD from three independent experiments. [b] DEA-NO was used at a concentration of 10 μM.

absorbance maximum typical for hemoproteins at 430 nm (Soret band). A comparably smaller Soret peak was detected in the β_1C214S mutant, which, in addition, was shifted to 423 nm. The β_1C78S mutant did not exhibit a detectable Soret peak. These results are in good accordance with the enzyme activities shown in Table 2. β_1C78S, which did not contain detectable heme, was insensitive to NO. β_1C214S, which exhibited a small Soret peak indicating the presence of heme, exhibited up to 17-fold stimulation by NO.

Heme Reconstitution. In order to find out whether the mutants are still able to bind heme and whether heme reconstitution results in gain of NO responsiveness, we performed heme reconstitution experiments. As delineated in the Materials and Methods section, low concentrations of the detergent Tween 20 led to removal of the prosthetic heme

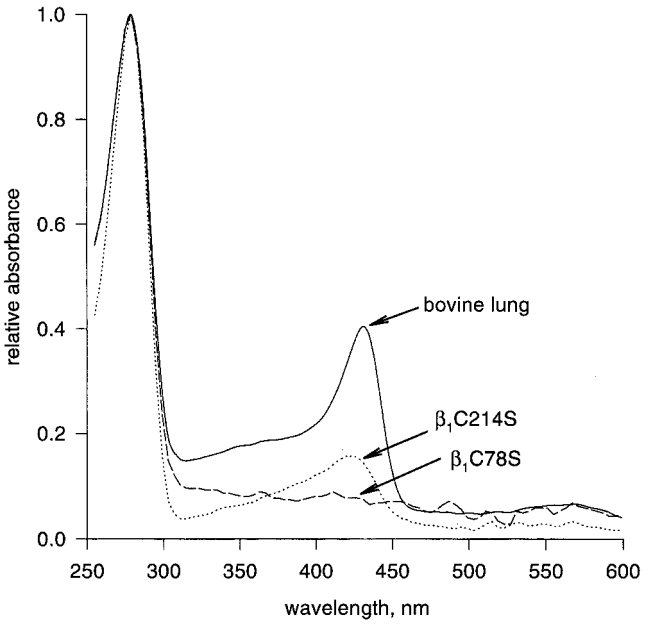

FIGURE 3: UV−vis absorbance spectra of purified mutant and WT sGCs. Deviations in the exact amounts of protein applied have been corrected by rescaling the absorbances to give identical peak heights at 280 nm.

group, whereas further addition of heme resulted in the incorporation of heme into the enzyme (Foerster et al., 1996).

Table 3 shows that presence of Tween 20 alone almost completely abolished NO-induced stimulation of WT enzyme (66-fold in the absence versus 4-fold in the presence of Tween 20). Stimulation by NO (86-fold) was restored by the addition of heme under anaerobic conditions. Wild-type enzyme was also reconstituted with protoporphyrin IX, the iron-free precursor of heme. By mimicking the conformation of the NO−heme complex (Ignarro et al., 1982), protoporphyrin IX stimulated sGC 47-fold independent of NO.

As expected, the presence of Tween 20 did not change the enzyme activity of β_1C78S. Addition of heme led to a 5-fold stimulation by NO. Reconstitution with protoporphyrin IX resulted in a 13-fold stimulation. Higher concentrations of heme or protoporphyrin IX were inhibitory.

Similar to WT enzyme, NO stimulation of the β_1C214S mutant was reduced from 15- to 2-fold in the presence of Tween 20. Upon addition of heme, the mutant exhibited 65-fold stimulation by NO, which is similar as in the WT enzyme. Reconstitution with protoporphyrin IX led to pronounced stimulation of cGMP production (61-fold).

DISCUSSION

In this report, we investigated the participation of cysteine residues in the postulated redox regulation of sGC. We showed that conserved Cys residues on either of the two subunits of sGC were not essential for catalytic activity of the enzyme. All Cys mutants were able to synthesize cGMP, and as dimerization is a prerequisite for the formation of an active enzyme, we conclude that none of the cysteines is involved in dimerization of the two subunits.

Mutation of two homologue cysteines in the most conserved stretch of amino acids of all sGC in the catalytic domain, Cys-596 of the α_1 and Cys-541 of the β_1 subunit (see Figure 1), resulted in enzymes with divergent characteristics: α_1C596S showed elevated basal activity (2.5−4-fold) compared to WT, whereas NO-stimulated activity was reduced. In contrast, β_1C541S showed a strongly reduced overall catalytic rate but, nonetheless, exhibited a WT-like stimulation factor. The position of these two cysteines within the most conserved sequence of amino acids (MPRY*C*LFG) of all guanylyl cyclases argues for an important structural or even catalytic function of these residues. Obviously, the hydroxyl group of serine is capable of partially substituting for the function of the thiol group of cysteine, although this does not explain the rather contrary effects of the mutations on either subunit.

All but two Cys mutants were stimulated by the physiological activator of the enzyme, NO. Mutations of Cys-78 and Cys-214 of the β_1 subunit, which are found in the putative heme-binding domain of sGC, yielded enzymes exhibiting only 1.2- and 1.3-fold stimulation, respectively, in COS cells in the presence of Mn^{2+}. The mutants showed only little or no enzyme-bound heme after purification. Nevertheless, these two mutants gained NO sensitivity after heme reconstitution. Restoration of NO sensitivity shows that neither of the cysteines is absolutely required for the intramolecular signal transduction that conveys the NO−heme-binding signal to the catalytic center of the enzyme. Thus, we conclude that the point mutations result in a reduced affinity for heme.

Previous findings in our laboratory established the importance of His-105 of the β_1 subunit as the proximal ligand of the prosthetic heme group (Wedel et al., 1994). Taking

Table 3: Heme Reconstitution of Mutant sGC

	Tween 20	porphyrin	cGMP[a] (nmol min^{-1} mg^{-1}) Co	cGMP[a] (nmol min^{-1} mg^{-1}) DEA-NO	stimulation factor (V_{DEA-NO}/V_{Co})
WT	−	−	28 ± 2	1850 ± 410	66
	+	−	31 ± 3	112 ± 3	4
	+	heme	26 ± 6	2260 ± 440	86
	+	PP-IX	1440 ± 280		47[b]
β_1C78S	−	−	60 ± 4	57 ± 4	1
	+	−	80 ± 5	90 ± 5	1
	+	heme	66 ± 9	350 ± 60	5
	+	PP-IX	1020 ± 120		13[b]
β_1C214S	−	−	57 ± 6	870 ± 80	15
	+	−	69 ± 9	167 ± 4	2
	+	heme	63 ± 5	4120 ± 1300	65
	+	PP-IX	4240 ± 550		61[b]

[a] Enzyme activites were determined with 3 mM Mg^{2+}. DEA-NO was used at a concentration of 10 μM. Values are means ± SD from three independent experiments. [b] Stimulation factors with PP-IX refer to activity of the heme-depleted enzyme.

into account the cysteine's relative position to the proximal His-105, it is conceivable that both Cys influence heme binding by contributing to the spatial structure of the heme pocket. It is tempting to speculate that these two Cys may be located in close proximity and may even be identical to the vicinal dithiols described by Craven and DeRubertis (1978a).

The data presented here reinforce the findings from our laboratory that catalytic and heme binding domains are separate entities and that heme binding is located in the N-terminal part of the β_1 subunit (Wedel et al., 1995).

There has been an extensive discussion on the stoichiometry and the coordination of the heme group on sGC (Gerzer et al., 1981; Wedel et al., 1994; Stone & Marletta, 1994, 1996 Yu et al., 1994; Burstyn et al., 1995). So far, sGC has been shown to contain 1 mol of heme/heterodimer (Gerzer et al., 1981), although recent reports raise the possibility that there may be 2 mol of heme/mol of enzyme (Stone & Marletta, 1994, 1996). Since there is no spectral evidence for heterogeneously coordinated heme species in the enzyme, these authors postulate a coordination to His residues highly conserved between the α and β subunits, implying a symmetrical distribution of the heme groups between the two subunits.

Mutations of Cys-145 or Cys-284 of the α_1 subunit, which correspond to Cys-78 and Cys-214 of the β_1 subunit (see Figure 1), did not change the catalytic and stimulatory properties of the mutant enzymes compared to wild type. Thus, our results argue against a symmetrical positioning of the heme groups on the two subunits, and indirectly against a stoichiometry of 2 hemes/heterodimer. Moreover, the NO insensitivity of the two Cys mutants of the β_1 subunit strengthens the importance of His-105 of the β_1 subunit in heme binding. Taking into account the mutagenesis data from our previous report, all our data indicate that heme binding is a predominant feature of the β_1 subunit.

Although intracellular redox conditions may well affect the enzyme, the results presented here indicate that cysteine residues are unlikely to represent a direct regulatory feature of sGC. Right now, we cannot rule out a redox-based mechanism involved in the yet poorly understood shutdown of cGMP synthesis, and it will be of high interest to find out about the possible involvement of one or more cysteines in the inactivation of sGC.

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