A Point-Mutated Guanylyl Cyclase with Features of the YC-1-Stimulated Enzyme: Implications for the YC-1 Binding Site?†

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ABSTRACT: Guanylyl cyclases (GCs) and adenylyl cyclases (ACs) play key roles in various signaling cascades and are structurally closely related. The crystal structure of a soluble AC revealed one binding site each for the substrate ATP and the activator forskolin. Recently, YC-1, a novel activator of the heterodimeric soluble GC (sGC), has been identified which acts like forskolin on AC. Here, we investigated the respective substrate and potential activator domains of sGC using point-mutated subunits. Whereas substitution of the conserved Cys-541 of the β_1 subunit with serine led to an almost complete loss of activity, mutation of the respective homologue (Cys-596) in the α_1 subunit yielded an enzyme with an increased catalytic rate and higher sensitivity toward NO. This phenotype exhibits characteristics similar to those of the YC-1-treated wild-type enzyme. Conceivably, this domain which corresponds to the forskolin site of the ACs may comprise the binding site for YC-1.

Within practically all cells, guanylyl cyclases (GCs)[1] and adenylyl cyclases (ACs) are parts of fundamental signaling cascades. These enzymes catalyze the formation of cyclic nucleotides from the respective nucleotide triphosphates, i.e., cGMP from GTP and cAMP from ATP. In contrast to the homodimeric membrane-bound GCs, sGC and the ACs contain a heterodimeric catalytic domain which is formed by two cytosolic portions (termed C_1 and C_2) of one molecule in the ACs and by two distinct subunits (termed α and β) in the soluble GCs.

sGC is stimulated by the signaling molecule nitric oxide (NO), and the subsequent increase in the level of cGMP induces important functional changes within the cardiovascular and neuronal systems, i.e., vasorelaxation, inhibition of platelet activation and aggregation, and modulation of synaptic transmission (*1*−*3*). The overall structure of the heterodimeric enzyme includes at least two distinct functional domains. The N-terminal region was identified as the heme-binding domain (*4*, *5*) in which the heme group acts as the receptor for NO. Binding of NO to the heme induces conformational changes resulting in a more than 200-fold activation of the enzyme (*6*, *7*). The C-terminal portion of each subunit contains one homologous cyclase catalytic region. The dimer of both catalytic regions constitutes the catalytic center where binding of GTP and conversion to cGMP take place. On the other hand, GTP was shown to induce an increase in the extent of NO dissociation from the heme group and to lead to a change of the Raman spectra of NO-sGC, demonstrating a close association of both domains (*8*, *9*).

The crystallization of the catalytic center of a soluble AC greatly forwarded the knowledge of structural aspects of cAMP and cGMP formation (*10*−*12*). The catalytic center of AC consists of the homologous C_1 and C_2 domains arranged in a "head-to-tail"-like fashion. The interface between the C_2 and C_1 domains forms two pseudosymmetric regions; one of these regions comprises the binding site for the substrate ATP, and the other one which has probably evolved from a former ATP binding site binds the diterpene activator forskolin. As there is accumulating evidence that the catalytic centers of ACs and GCs are closely related (*13*, *14*), one may speculate about a yet unidentified ligand at the pseudosymmetrical counterpart of the GTP binding site of sGC, which in the mammalian ACs houses forskolin.

Recently, we and others have shown that YC-1, a benzylindazole, activates purified sGC and sensitizes the enzyme for its physiological activator NO in vitro (*15*−*17*) and in intact human platelets (*18*) and smooth muscle (*16*). Although YC-1 most likely does not bind to the enzyme's prosthetic heme group but does bind to an allosteric site (*17*, *19*), the exact binding site has not been identified so far. As the YC-1 effects on sGC, i.e., direct stimulation and potentiation of stimulated activity, are similar to those of forskolin on the ACs, YC-1 may well be considered as a ligand candidate for the sGC site corresponding to the forskolin site on the ACs.

This study shows that a single point mutation in the region of the α_1 subunit (α_1C596S) which corresponds to the forskolin binding site in the ACs greatly enhanced the nonstimulated activity and increased the sensitivity toward NO by reducing the NO dissociation rate. We conclude that this very conserved domain in the α_1 subunit has a profound regulatory influence on the catalytic center regarding the catalytic rate as well as the NO dissociation from the heme

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[1] Abbreviations: sGC, soluble guanylyl cyclase; AC, adenylyl cyclase; NO, nitric oxide; DEA−NO, diethylamine−NO complex; WT, wild type.

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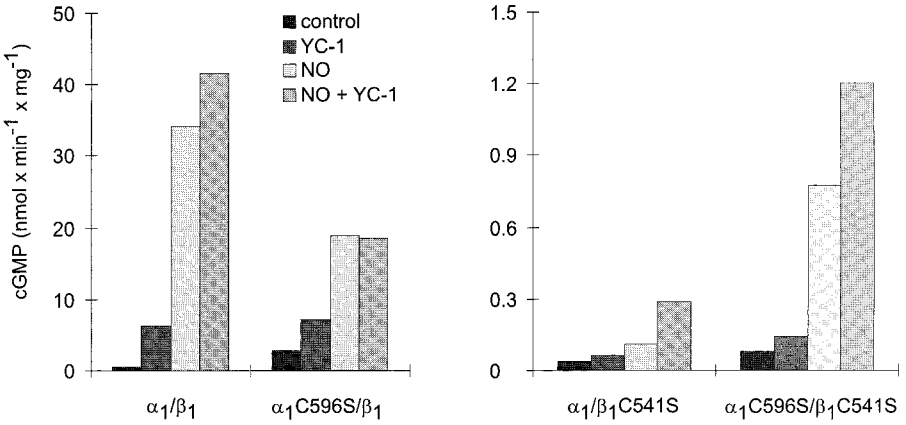

FIGURE 1: Enzyme activities of WT and mutant sGC in Sf9 cell cytosol. Cyclic GMP production was assessed in cytosolic fractions (25 μg of protein) of Sf9 cells coinfected with the indicated mutant and/or WT subunits under nonstimulated and stimulated conditions using either DEA−NO (10 μM), YC-1 (200 μM), or a combination of the two. Data represent one representative experiment out of four (WT and α_1 mutant) and two (β_1 mutant).

group. Our results indicate that this domain may comprise the YC-1 binding site.

EXPERIMENTAL PROCEDURES

Construction of Mutants. Site-directed mutagenesis of Cys residues (α_1C596S and β_1C541S) was performed by the Kunkel method (*20*) as described previously (*21*).

Expression of Recombinant Guanylyl Cyclase. Expression of the recombinant proteins in Sf9 cells, determination of sGC activity in Sf9 cell cytosol, and immunoblot analysis of the cytosolic proteins were carried out as described previously (*21*, *22*). The α_1C596S mutant cloned in pCMV as described previously (*21*) was cut with *Bgl*II and *Ase*I (filled up) and subcloned in the *Bam*HI and *Eco*RI (filled up)-cut pFASTBAC1. Recombination in the virus was achieved with the BAC-TO-BAC Baculovirus Expression System (Gibco BRL).

Sf9 Cell Culture and Infection. Sf9 cells were grown and virus-infected as described previously (*21*). Purification of recombinant enzyme was carried out after coinfection of spinner-culture cells (4 L grown to a density of 1.3−1.5 × 10^6 cells/mL) with the α_1C596S virus and the β_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 2 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.

Purification of Soluble Guanylyl Cyclase and Determination of Guanylyl Cyclase Activity. sGC was purified from bovine lung to apparent homogeneity by an immunoaffinity purification procedure as described previously (*6*). Cyclase activity (0.05 μg of sGC) was assessed via the conversion of [α-^{32}P]GTP to [^{32}P]cGMP at 37 °C for 10 min. Reaction mixtures contained 3 mM Mg^{2+} as the divalent metal ion, 3 mM dithiothreitol, 0.5 mg/mL bovine serum albumin, 1 mM cGMP, 300 μM GTP, and 50 mM triethanolamine hydrochloride (pH 7.4) in a total volume of 0.1 mL. Reactions were stopped by $ZnCO_3$ precipitation, and [^{32}P]cGMP was isolated as described previously (*23*).

For the dissociation experiments, sGC was preincubated with DEA−NO at 37 °C for 3 min. After addition of substrate and 250 μM oxyhemoglobin (prepared as described in ref *15*), reactions were stopped every 15 s to determine the decreasing NO-stimulated enzyme activity. Figure 4 shows the calculated time course of the decreasing enzyme activity.

All measurements were performed in duplicate and repeated at least three times with the exception of the experiments with the β_1 mutant which were carried out twice.

YC-1 was dissolved in DMSO. The final DMSO concentration in all samples did not exceed 2% (v/v), a concentration which by itself did not influence sGC activity.

Materials. YC-1 {3-[5′-(hydroxymethyl)-2′-furyl]-1-benzylindazole} was from Cayman Chemicals (Grünberg, Germany). 2,2-Diethyl-1-nitrosooxyhydrazine sodium salt (DEA−NO) was purchased from RBI (Research Biochemicals International, Natick, MA). Oxyhemoglobin was obtained from Sigma (Deisenhofen, Germany). DMSO and sodium dithionite were purchased from Merck (Darmstadt, Germany), and carboxy-PTIO was from Calbiochem (Bad Soden, Germany). [α-^{32}P]GTP (800 Ci/mmol) was from NEN-DuPont (Boston, MA).

RESULTS

In the ACs, the regions of the C_1 and C_2 domains have been identified to be responsible for catalysis and binding of forskolin (*11*, *13*). To study the function of the corresponding, homologous regions of sGC, we used two sGC mutants in which the cysteines out of these remarkably conserved regions in the α and β subunit were changed into serines (*21*).

Figure 1 shows the catalytic activity of the mutants coexpressed with the respective dimerizing subunit in Sf9 cell cytosol. Mutation of the cysteine of the β_1 subunit (Cys-541) yielded an enzyme with a greatly reduced catalytic rate (Figure 1B). Despite the reduction in catalytic activity, the β_1C541S mutant exhibited some sensitivity toward NO, YC-1, and the combination of both activators (3-, 2-, and 8-fold, respectively). These results show that mutation of this conserved cysteine of the β subunit impairs the catalytic rate of sGC, but does not abolish the stimulation by NO or YC-1.

Mutation of the corresponding cysteine of the α_1 subunit (Cys-596), however, led to a marked increase (approximately 7-fold) in the nonstimulated activity of the mutant, whereas

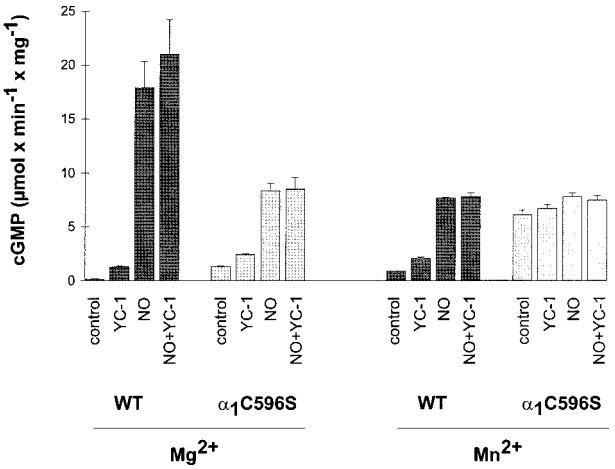

FIGURE 2: Enzyme activities of the purified α_1C596S mutant and WT sGC. sGC was purified from Sf9 cells (α_1C596S) and bovine lung (WT). cGMP production was assessed in the presence of either 3 mM Mg^{2+} or Mn^{2+} under nonstimulated and stimulated conditions with either DEA−NO (10 μM), YC-1 (200 μM), or a combination of the two. Data are means ± the standard deviation from three independent experiments.

the NO-stimulated catalytic rate was only about 50% of that of the wild type (WT; Figure 1A). YC-1 stimulated the activities of the WT and mutant enzymes to almost the same level. Due to the elevated nonstimulated activity of the mutant, the stimulation by YC-1 was only 2.6-fold compared to 14.4-fold for the WT enzyme. In contrast to the WT enzyme, YC-1 did not further increase the activity of the NO-stimulated mutant. In conclusion, the mutant revealed an augmented nonstimulated activity and a reduced level of stimulation by YC-1 or NO.

Coexpression of both mutated subunits (α_1C596S and β_1C541S) yielded an enzyme that exhibited an increased catalytic rate when compared with that of the $\alpha_1-\beta_1$C541S dimer (see Figure 1B). This shows that the point mutation on the α_1 subunit leads to an increased rate of catalysis regardless of the type of β_1 subunit with which it dimerizes.

To further investigate the molecular basis of the augmented catalytic rate, the α_1C596S mutant coexpressed with the WT β_1 subunit in Sf9 cells was purified as described previously (*6*, *21*). The subunits of the purified protein exhibited the expected molecular masses as judged by SDS−PAGE (not shown).

First, we compared the catalytic activities of the purified mutant and WT enzymes in the presence of Mg^{2+} and Mn^{2+}. sGC, as all cyclases, requires divalent metal ions for the conversion of GTP to cGMP, and Mg^{2+} and Mn^{2+} are commonly used to study cyclase activity. Nonstimulated activity of sGC is higher with Mn^{2+} than with Mg^{2+}, whereas under NO-stimulated conditions, sGC exhibits a higher rate of cGMP formation in the presence of Mg^{2+}. Enzyme activities of the purified mutant and WT enzymes in the presence of each metal are shown in Figure 2. In the presence of Mg^{2+}, the purified α_1C596S mutant revealed an almost 9-fold higher enzyme activity than the WT (1.30 and 0.15 μmol of cGMP min^{-1} mg^{-1}, respectively). The mutant was stimulated by YC-1 only 1.9-fold, whereas the WT enzyme was activated 9-fold (2.4 and 1.3 μmol of cGMP min^{-1} mg^{-1}, respectively); the rates of YC-1-stimulated activity of the WT and mutant enzymes were in a comparable range. The presence of Mn^{2+} raised the nonstimulated activity of the WT enzyme about 6-fold (0.8 μmol of cGMP min^{-1} mg^{-1}), and the maximal catalytic rate was reduced by approximately 50% as has been described by others previously. YC-1 only led to a 2-fold increase in WT enzyme activity (2.0 μmol of cGMP min^{-1} mg^{-1}). However, the mutant exhibited an extraordinarily high rate of cGMP formation in the presence of Mn^{2+}, reaching almost the level of the NO-stimulated WT enzyme (6.1 and 7.6 μmol of cGMP min^{-1} mg^{-1}, respectively). This strongly augmented activity was practically not further increased by either YC-1 or NO (6.6 and 7.8 μmol of cGMP min^{-1} mg^{-1}, respectively).

Table 1: K_m Values for WT and α_1C596S Mutant Guanylyl Cyclase[a]

	Mg^{2+}		Mn^{2+}	
enzyme	control	DEA−NO (10 μM)	control	DEA−NO (10 μM)
WT sGC ($\alpha_1-\beta_1$)	111	8.2	6.7	6.7
mutant sGC (α_1C596S$-\beta_1$)	11.6	12.3	10.5	10.4

[a] Data are representative of three experiments and are expressed in units of micromolar.

sGC has been shown to be sensitive to the minute amounts of NO present in the atmosphere (*24*). To rule out the possibility that the mutant's elevated activity resulted from a higher level of prestimulation by atmospheric NO, we assessed cGMP formation in the presence of the NO scavengers oxyhemoglobin or carboxy-PTIO. Enzyme activities of the WT and mutant were similarly reduced by approximately 50% by both NO scavengers (not shown), showing that the higher activity of the mutant under nonstimulated conditions was not due to a higher percentage of NO-stimulated enzyme molecules. Taken together, the point mutation leads to an enzyme that, in the presence of Mg^{2+}, reveals the features of the Mn^{2+}-treated WT enzyme. In the presence of Mn^{2+}, nonstimulated activity of the mutant is immensely elevated to a level similar to that of the NO-stimulated WT enzyme; thus, the mutant appears to adopt the conformation of a fully activated enzyme species.

Next, we studied the affinities of the mutant toward GTP and YC-1. The EC_{50} values for YC-1 in the presence of either metal did not differ from the ones for the WT (approximately 10 μM; data not shown). The K_m values for GTP of the WT and mutant enzymes are shown in Table 1. As has been described previously, the K_m of the WT under nonstimulated conditions (111 μM) is shifted 1 order of magnitude by NO stimulation (8 μM) in the presence of Mg^{2+}. However, the mutant exhibited a low K_m value (12 μM) previously under nonstimulated conditions. This value is in the range of that of the stimulated WT and of the K_m values seen with Mn^{2+} (see Table 1). Again, the mutant reveals properties of the Mn^{2+}-treated WT enzyme.

To study the mutant's affinity toward NO, we obtained NO concentration−response curves. Compared to that of the WT, the maximal NO-stimulated activity of the mutant was reduced (17.9 and 8.3 μmol of cGMP min^{-1} mg^{-1}, respectively), and YC-1 led only to a marginal potentiation (see Figure 2). For a better comparison of the EC_{50} values, the data depicted in Figure 3 are shown as the percentage of the maximum for one representative experiment. The α_1C596S

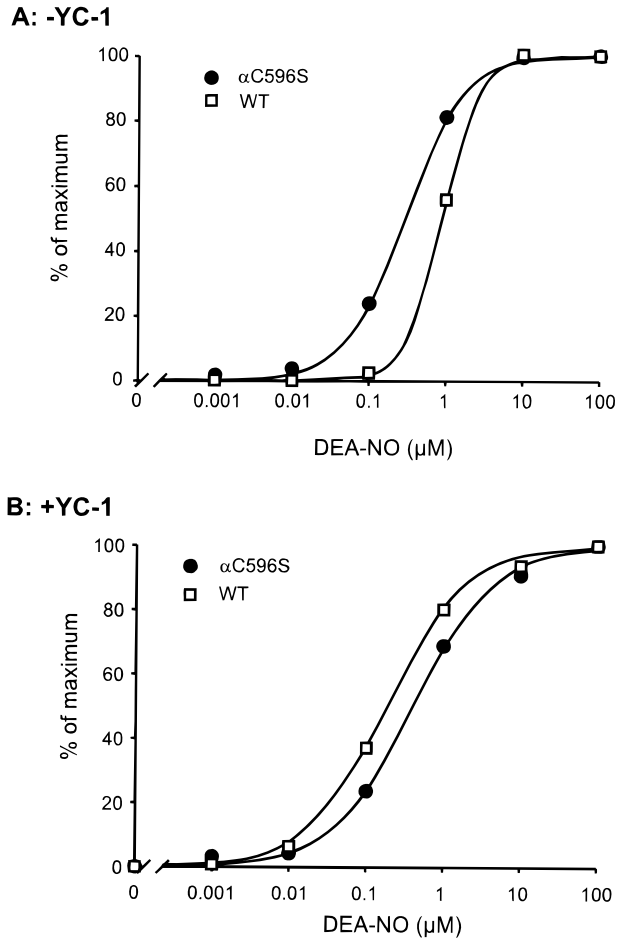

FIGURE 3: NO concentration−response curves of the mutant and WT sGC. cGMP production of the WT enzyme (□) and the α_1C596S mutant (●) was assessed with increasing concentrations of DEA−NO in the absence (A) or presence (B) of 200 μM YC-1. Shown are the data of one representative experiment out of four with similar results.

mutant revealed an increased affinity toward NO which is higher than that of the WT enzyme (EC_{50} values of 0.56 and 1.27 μM, respectively). The difference in the EC_{50} values of both enzymes is 0.71 ± 0.18 μM in four independent experiments. As shown previously (*15*), YC-1 shifted the EC_{50} value of the WT enzyme for NO by about 1 order of magnitude whereas the YC-1-induced increase in NO sensitivity of the mutant was rather small (EC_{50} values of 0.13 and 0.25 μM, respectively). In summary, the mutant reveals an increased affinity for NO in the absence of YC-1.

Next, we wanted to determine whether the increase in NO sensitivity due to the mutation could be explained by a reduction of the NO dissociation rate. Figure 4 illustrates the time course of NO dissociation monitored by cGMP formation of the NO-stimulated mutant after addition of oxyhemoglobin (M. Russwurm et al., manuscript in preparation). Compared to the NO dissociation rate of the WT enzyme which amounts to a half-life of about 3−5 s (*8*), the rate of NO dissociation from the α_1C596S mutant was strongly decreased, yielding a half-life of more than 20 s.

Taken together, the sGC mutant exhibits two remarkable features. First, it mimics the phenotype of the Mn^{2+}-treated WT. Second, the mutant displays the prominent characteristics of the YC-1-treated WT enzyme, i.e., a 10-fold

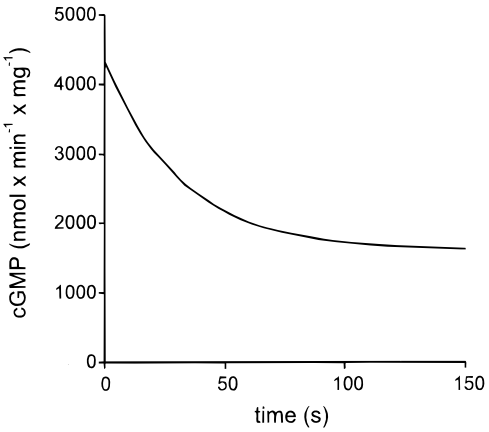

FIGURE 4: NO dissociation of the α_1C596S mutant. The α_1C596S mutant was preincubated with DEA−NO, and cGMP formation was assessed after the addition of oxyhemoglobin. Shown are the calculated enzymatic activities; the half-life of the NO−α_1C596S complex is approximately 24 s.

increased nonstimulated catalytic rate and the higher affinity for NO (*15*, *17*).

DISCUSSION

By mutation of a single amino acid residue in each of the highly conserved regions of the two sGC subunits, we produced recombinant enzymes that revealed quite remarkable changes in their catalytic and regulatory properties. Substitution of Cys-541 of the β_1 subunit with serine led to an enzyme with greatly reduced catalytic activity that was still stimulated by NO and YC-1. Our data underline the findings of Sunahara et al. (*13*) that this amino acid of the β_1 subunit is one of the residues being critically involved in guanine binding. To our surprise, the corresponding mutation on the α_1 subunit (α_1C596S) showed a 10-fold increase in the nonstimulated catalytic rate, demonstrating a profound influence of this α_1 region on the catalytic reaction.

Besides for the substrate MgGTP, sGC, like all adenylyl and guanylyl cyclases, requires a second metal ion as a cofactor (*25*). In the recently determined crystal structures of soluble AC (*10*, *11*), the binding site for this second metal ion has not been identified. Interestingly, the properties of the α_1C596S mutant in the presence of Mg^{2+} were practically identical to those of the Mn^{2+}-treated WT enzyme (see Figure 2). Our data suggest that the Cys−Ser exchange leads to a change in the enzyme conformation which now resembles that of the Mn^{2+}-treated WT enzyme. In the presence of Mn^{2+}, however, the mutant's nonstimulated activity was increased to a level almost as high as that measured by the NO-stimulated WT enzyme. As NO only led to a marginal further increase in the catalytic rate, we assume that the conformation of the mutant in the presence of Mn^{2+} corresponds to that of the NO-induced conformation of the WT enzyme.

The most prominent features of the α_1C596S mutant are the elevated enzyme activity and the increase in affinity for NO which is most likely due to the reduction of the NO dissociation rate. These properties are reminiscent of the characteristics of the YC-1-treated WT sGC. As the region of the α_1 subunit in which the amino acid was substituted corresponds to the forskolin site in the AC, it is very tempting to speculate about YC-1 binding to the respective site on

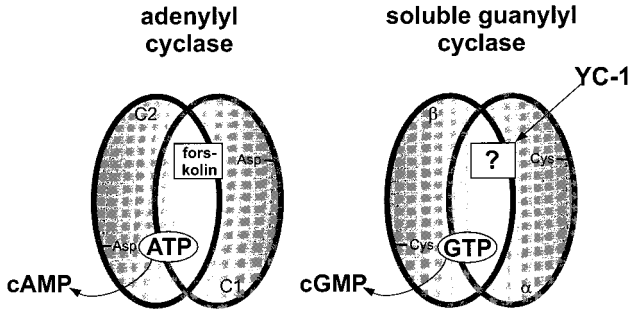

FIGURE 5: Schematic representation of a putative regulatory site on sGC. Shown are schematic representations of the catalytic domains of AC and sGC. The mutated cysteines and their respective counterparts in the ACs are denoted. Forskolin binds to a site pseudosymmetrical to the catalytic site. The cysteine mutation in the respective region of sGC leads to an increase in the nonstimulated catalytic rate and a decrease in the NO dissociation rate, suggesting that YC-1 exerts its effect by binding to this site.

sGC. The forskolin site which is pseudosymmetric to the ATP site possibly originates from a former nucleotide binding site which now has lost its ability to bind ATP (*12*).

In summary, we suggest the model shown in Figure 5. In analogy to the forskolin site in the ACs, the corresponding potential regulatory site on sGC may have evolved from a former catalytic GTP binding site. By binding to this site, YC-1 would affect the enzyme's catalytic properties as well as influence the NO dissociation. Further studies have to show whether this site can be pharmacologically utilized to modulate the properties of sGC in vivo.

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