Inhibition of Deactivation of NO-sensitive Guanylyl Cyclase Accounts for the Sensitizing Effect of YC-1*

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Many of the physiological effects of the signaling molecule nitric oxide are mediated by the stimulation of the NO-sensitive guanylyl cyclase. Activation of the enzyme is achieved by binding of NO to the prosthetic heme group of the enzyme and the initiation of conformational changes. So far, the rate of NO dissociation of the purified enzyme has only been determined spectrophotometrically, whereas the respective deactivation, i.e. the decline in enzymatic activity, has only been determined in cytosolic fractions and intact cells. Here, we report on the deactivation of purified NO-sensitive guanylyl cyclase determined after addition of the NO scavenger oxyhemoglobin or dilution. The deactivation rate corresponded to a half-life of the NO/guanylyl cyclase complex of ~4 s, which is in good agreement with the spectrophotometrically measured NO dissociation rate of the enzyme. The deactivation rate of the enzyme determined in platelets yielded a much shorter half-life indicating either partial damage of the enzyme during the purification procedure or the existence of endogenous deactivation accelerating factors. YC-1, a component causing sensitization of guanylyl cyclase toward NO, inhibited deactivation of guanylyl cyclase, resulting in an extremely prolonged half-life of the NO/guanylyl cyclase complex of more than 10 min. The deactivation of an ATP-utilizing guanylyl cyclase mutant was almost unaffected by YC-1, indicating the existence of a special structure within the catalytic domain required for YC-1 binding or for the transduction of the YC-1 effect. In contrast to the wild type enzyme, YC-1 did not increase NO sensitivity of this mutant, clearly establishing inhibition of deactivation as the underlying mechanism of the NO sensitizer YC-1.

The signaling molecule nitric oxide (NO) has been shown to play an important role in the cardiovascular and nervous systems (1–4). Most of the effects of NO are mediated by the stimulation of the NO-sensitive guanylyl cyclase (GC), the enzyme that catalyzes the formation of cGMP. The subsequent increase in cGMP leads to the activation of cGMP effector proteins like cGMP-activated protein kinases, cGMP-gated Ca**2+ channels and cGMP-regulated phosphodiesterases.

NO-sensitive GC is a heterodimeric enzyme consisting of an α and β subunit (5). Although four enzyme subunits (α1, α2, β1, β2) have been identified so far, only the α1β1 and α2β1 heterodimers have been shown to exist as catalytically active NO sensitive isoforms in vivo (6). Each of the subunits contains the homologous cyclase catalytic domain. With two different cyclase catalytic domains the GTP-converting catalytic region of GC appears to be related to the ATP-converting catalytic region of the adenylyl cyclase (AC). The crystal structure of the catalytic center of AC has been resolved (7, 8). In AC, the homologous cyclase catalytic domains C1 and C2 corresponding to the α and β subunits of NO-sensitive GC, respectively, are arranged in a “head-to-tail”-like orientation with two pseudosymmetric regions within the interface between the C2 and C1 domains. One of these regions comprises the binding site for the substrate ATP. The other region, which has probably evolved from a former ATP binding site, binds forskolin, the activator of ACs.

The NO-sensitive GC contains one prosthetic heme group that acts as the acceptor site for NO (9–12). The heme group reveals an absorption maximum at 430 nm, the Soret band, indicative of a five-coordinated ferrous heme with a histidine as the axial ligand at the fifth coordinating position. NO binds to the sixth coordination position of the heme iron and leads to the rupture of the histidine-to-iron bond yielding a five-coordinated nitrosyl-heme complex with an absorbance maximum at 398 nm. The opening of the histidine-to-iron bond probably initiates a conformational change resulting in up to 200-fold activation of the enzyme. This mechanism of activation is supported by the finding that protoporphyrin IX, the iron-free precursor of heme, stimulates GC independently of NO (10). Conceivably, protoporphyrin IX, a heme lacking iron, mimics the structure of the NO-heme complex in which the iron is moved out of the plane of the porphyrin ring. On the other hand, breakage of the histidine-to-iron bond is required but does not appear to be sufficient for activation, as a mutant without the proximal histidine and, accordingly, without the histidine-to-iron bond did not show an increased catalytic rate (13). Apart from NO, the substance YC-1 has been shown to activate GC ~10-fold and to sensitize the enzyme toward NO and CO (14). Inhibition of NO dissociation has been proposed as the underlying mechanism of sensitization (15).

Whereas a relatively precise concept about the NO-mediated activation of GC exists, little is known about the deactivation of the enzyme. Apparently, the rate of NO dissociation from the heme moiety is one of the fundamental parameters for deactivation of the enzyme in biological systems. From other hemo-proteins, it is known that NO dissociation from heme moieties is in general rather slow in the range of minutes or even hours, which in the case of GC would raise the problem of a slow deactivation of the enzyme within a signal transduction cascade. The NO dissociation rate of GC has been determined...
spectrophotometrically after the addition of the NO scavenger oxyhemoglobin (oxyHb). Our analysis of the NO dissociation rate of the purified enzyme yielded a half-life of the NO-GC complex of ~2 min (16); within the same experimental setting, the substrate MgGTP accelerated NO dissociation to a half-life of ~5 s (17). A different spectrophotometric study showed a dissociation rate corresponding to a half-life of ~3 min but no increase in the NO dissociation rate upon the addition of MgGTP (18). In addition to the spectrophotometrically determined NO dissociation, the decline of NO-stimulated cGMP-forming activity has been monitored after the addition of an NO scavenger, which is referred to as the deactivation of NO-sensitive GC throughout the manuscript. Margulis and Sitaramayya (19) reported a deactivation half-life of GC in cytosolic preparations after NO removal of ~4 s at 37 °C. On the other hand, an in vitro study performed in intact cerebellar cells showed a very fast deactivation with a half-life of 0.2 s upon addition of the NO scavenger hemoglobin (20).

In this study, we determined the deactivation rate of the NO-sensitive GC by monitoring enzymatic activity after removal of NO. The rate of deactivation of the purified enzyme corresponded to a half-life of the NO-GC complex of ~4 s, which is in good agreement with the spectrophotometrically determined NO dissociation rate. YC-1, a substance known to sensitize GC toward NO, slowed down deactivation of GC dramatically (120-fold) resulting in a half-life of ~10 min. In contrast, deactivation of an ATP-utilizing GC mutant was unaffected by YC-1. The NO sensitivity of the mutant was not altered by YC-1 emphasizing the concept of an inhibition of NO dissociation as the underlying mechanism of YC-1-induced sensitization of GC.

MATERIALS AND METHODS

Construction of the ATP-utilizing Mutant
Site-directed mutagenesis of the respective amino acids in the α1 and β subunits (α1,R9394Q, β1,E473R, β1,C541D) was performed using the QuikChange site-directed mutagenesis kit (Stratagene) using the instructions of the manufacturer. The sequences of both subunits of the cAMP-forming mutant were verified by sequencing.

Purification of NO-sensitive GC
GC was purified from the bovine lung as described (11). Expression and purification of the ATP-utilizing mutant from Sf9 cells was performed as described previously (6). Protein concentrations were determined by the Bradford method (21).

Preparation of oxyHb
Oxyhemoglobin was prepared from bovine methemoglobin dissolved in 50 mM triethanolamine/HCl, pH 7.4, by reduction with sodium dithionite. Subsequently, the reduced hemoglobin was desalted on a PD-10 column (Amersham Biosciences), and the concentration of oxyhemoglobin (oxyHb) was determined by the Bradford method (21).

Deactivation of Guanylyl Cyclase

As cGMP accumulation rather than the actual GC activity was measured during deactivation, actual activity was calculated by fitting the integral shown in Equation 1,

\[
\text{actual}\_\text{activity}(t) = (\text{maximal}\_\text{activity} - \text{basal}\_\text{activity}) \times e^{-\frac{t}{t_{1/2}}} + \text{basal}\_\text{activity}
\]

To the measured cGMP accumulation using Microsoft Excel Solver with \(t_{1/2}\), maximal activity, and basal activity as variables. The decline in actual activity is shown in the figures beneath the respective cGMP accumulation curves.

NO Concentration Response Curves
Enzymatic activity of the purified mutant (0.04 μg) and the wild type GC (0.08 μg) in the presence and absence of 200 μM YC-1 was determined by incubation for 10 min at 37 °C with 1 mM cAMP, 0.5 mM [α-32P]ATP (10–100 kBP) or 1 mM cGMP, 0.5 mM [α-32P]GTP (10–100 kBP), respectively, and 3 mM MgCl2, 0.5 mM L-dithiothreitol, and 50 mM triethanolamine/ HCl, pH 7.4, in a total volume of 100 μl. The reaction was started by the addition of the DDE-GC concentrations as indicated and transfer of the tubes to 37 °C.
Reactions were stopped by ZnCO$_3$ precipitation; determination of the enzyme-formed cyclic nucleotides was performed as previously described (22). YC-1 was dissolved in Me$_2$SO; the final concentration of Me$_2$SO in the samples did not exceed 2%, which does not affect GC activity. Results are means ± S.D. of three independent experiments performed in duplicates.

Deactivation of GC in Human Platelets

16 ml of venous blood from healthy volunteers were drawn into 4 ml of ACD (85 mM trisodium citrate, 65 mM citric acid, 100 mM glucose), and platelets were prepared as described previously (23) and resuspended in platelet buffer (5 mM HEPES, 150 mM NaCl, 0.55 mM NaH$_2$PO$_4$, 7 mM NaHCO$_3$, 2.7 mM KCl, 0.5 mM MgCl$_2$, 5.6 mM glucose, pH 7.4). Aliquots (90 µl) of the platelet suspension containing 1.5 × 10$^8$ platelets/ml were equilibrated at 37°C for 10 min in the presence of sildenafil and EHNA (100 µM, respectively). After equilibration, 10 µl of S-nitrosogluthathione (GSNO) were added yielding a final concentration of 100 µM. Two seconds after addition of GSNO, 100 µl of buffer containing 100 µM GSNO (as control) or 100 µM oxyHb (for deactivation experiments), were added. Reactions were terminated by addition of 0.3 mM trichloroacetic acid after 1, 2, and 4 s, followed by centrifugation (20,000 g, 10 min, 4°C). Supernatants were extracted twice with 5 volumes of water-saturated diethyl ether, and the aqueous phase was dried under nitrogen at 70°C.

For determination of cGMP, radioimmunoassay was carried out as described (23). Experiments were performed five times in triplicate under identical conditions.

RESULTS

As yet, NO dissociation of purified NO-sensitive GC has only been determined by spectrophotometric analysis (16–18). The resulting deactivation of the enzyme assessed by the decline in enzymatic activity has only been measured in cytosolic fractions and intact cells (19, 20).

In the present study, we have quantified the deactivation of the NO-stimulated purified enzyme (Fig. 1C) by measuring catalytic activity of the enzyme after the addition of the NO scavenger oxyHb. Briefly, GC was preincubated with DEA-NO for 3 min, the substrate GTP and oxyHb were added, and aliquots were stopped after 3, 6, 9, 15, 30, and 60 s. The results

![Fig. 1. Deactivation of NO-stimulated GC after addition of the NO scavenger oxyHb and reactivation by excess NO. A, accumulation of cGMP formed by the NO-stimulated GC (preincubated with NO for 3 min) after the addition of oxyHb was measured at the indicated time points as described under “Materials and Methods” (closed circles). B, actual cGMP-forming activity was calculated from the fitted curve of cGMP accumulation (A) as described under “Materials and Methods.” C, GC used in the deactivation experiments was purified from bovine lung as described under “Materials and Methods.” Shown is the isolated protein (5 µg) resolved on a 7.5% SDS-polyacrylamide gel and stained with Coomassie Brilliant Blue. D, accumulation of cGMP formed by GC (preincubated with NO) before addition of oxyHb (0–120 s) and after restimulation with excess NO (240–360 s) was measured as described under “Materials and Methods.”](http://www.jbc.org/)

![Fig. 2. Deactivation of NO-stimulated GC after 100-fold dilution. A, accumulation of cGMP formed by the NO-stimulated GC (preincubated with NO for 3 min) after a 100-fold dilution was measured at the indicated time points as described under “Materials and Methods.” B, actual cGMP-forming activity was calculated from the fitted curve of cGMP accumulation (A) as described under “Materials and Methods.” C, accumulation of cGMP formed by the NO-stimulated GC (preincubated with NO for 3 min) after a 100-fold dilution with 10 µM DEA-NO was measured at the indicated time points.](http://www.jbc.org/)
of such an experiment are presented in Fig. 1; the accumulated cGMP at the different time points is shown in Fig. 1 A. The graph in Fig. 1 B calculated to fit cGMP accumulation at these time points as described under “Materials and Methods” shows the decline in cGMP-forming activity over time yielding a half-life of \( \frac{1}{2} \approx 4 \) s. After deactivation, the enzyme could be restimulated by the addition of excess NO as shown in Fig. 1 D.

To rule out any inhibitory effect of oxyHb on the enzyme, we used a different experimental setting in which the preincubated NO-GC complex was diluted 100-fold to lower the DEA-NO concentration from 5 \( \mu \text{M} \) to 50 nM, i.e. from above to below the EC_{50} under the conditions applied. The resulting cGMP accumulation at different time points is shown in Fig. 2 A; Fig. 2 B shows the calculated enzyme activities indicating a half-life of the GC-NO complex of \( \frac{1}{2} \approx 3 \) s. The appropriate control experiment for the whole set of deactivation experiments is shown in Fig. 2 C. Here, the NO-preincubated enzyme was diluted, and additional maximally activating NO was added. Maximal activity was observed already after 3 s and remained constant for the measured 60 s demonstrating that the enzyme was not affected by the dilution performed in the experiments shown in Fig. 2 A and B. More importantly, the results showed that NO-stimulated cGMP formation started without a measurable delay allowing us to monitor deactivation by measuring enzyme activity with short incubations.

In spectrophotometric experiments, we have previously shown that GTP increases the NO dissociation rate by \( \approx 50-100 \) fold. The data suggested that catalysis may have an effect on the deactivation. On the other hand, because of the heterodimeric structure of GC and the antiparallel orientation of the subunits, a second non-catalytic GTP binding site might exist and mediate the GTP effect on NO dissociation. Instead of MgGTP, GC is able to utilize MnGTP and with this substrate exhibits maximal NO-stimulated catalytic rates that are reduced to \( \approx 50\% \) of those determined for MgGTP. The deactivation of GC using MnGTP as substrate is shown in Fig. 3 and yielded a half-life of \( \approx 8 \) s. Next, we studied the deactivation of GC in the presence of the GTP analogs GTP\( \gamma \)S and GMP-PNP. Both analogs were quantitatively converted to cGMP as detected by cGMP radioimmunoassay; the catalytic rates being reduced to about one-third (GTP\( \gamma \)S) and one-tenth (GMP-PNP) of the cGMP-forming rates observed for the substrate GTP. The finding that the GTP analogs could substitute for the substrate allowed us to measure the deactivation of NO-stimulated GC with GTP\( \gamma \)S and GMP-PNP by following the decline of enzyme activity as described above. The resulting half-lives (3–4 s, not shown) determined with either GTP\( \gamma \)S or GMP-PNP were in the range of the half-life observed for MgGTP showing that fast deactivation of GC does occur at drastically reduced cGMP-forming rates.

From the crystal structure of AC, three amino acids have been shown to be responsible for nucleotide specificity, and substitution of the corresponding amino acids of GC with the respective counterparts of AC resulted in a GC conversion of ATP to cAMP (24). To further elucidate a role of the catalytic center within the fast deactivation process, we studied the

![Fig. 3. Deactivation of GC measured with the substrate MnGTP. A, accumulation of cGMP formed by the NO-stimulated GC (preincubated with NO for 3 min) after the addition of oxyHb using MnGTP as a substrate was measured at the indicated time points as described under “Materials and Methods.” B, actual cGMP-forming activity was calculated from the fitted curve of cGMP accumulation (A) as described under “Materials and Methods.”](http://www.jbc.org/)

![Fig. 4. Deactivation of the cAMP-forming GC mutant. A, cAMP-forming GC mutant was expressed in Sf9 cells and purified as described under “Materials and Methods.” Shown is the purified protein (5 \( \mu \text{g} \)) resolved on a 7.5% SDS-polyacrylamide gel and stained with Coomassie Brilliant Blue. B, accumulation of cAMP formed by the NO-stimulated GC mutant (preincubated with NO for 3 min) after the addition of oxyHb was measured at the indicated time points as described under “Materials and Methods.” C, actual cAMP-forming activity was calculated from the fitted curve of cAMP accumulation (B) as described under “Materials and Methods.”](http://www.jbc.org/)
Deactivation of the ATP-utilizing GC mutant. The ATP-utilizing GC mutant was expressed in Sf9 cells and purified to apparent homogeneity with an immunoaffinity purification procedure (11). The purified enzyme showed the appropriate molecular masses of the \( \alpha_9251 \) and \( \alpha_9252 \) subunits on SDS gels (Fig. 4A). The ATP-utilizing mutant featured maximal NO-stimulated cAMP-forming rates comparable with those of the cGMP-forming WT enzyme. Determination of the mutant deactivation half-life yielded a value of \( 15 \) s (Fig. 4B and C), which is slower than that of the WT enzyme although markedly faster than the dissociation of the WT enzyme measured spectrophotometrically in the absence of GTP. These experiments suggest that the ATP present is able to substitute for GTP in the deactivation process either by binding to the catalytic or to a putative non-catalytic site.

In the experiments described above we monitored the deactivation of the purified enzyme. A much faster deactivation of the NO-stimulated GC in intact cerebellar cells with a half-life of \( 0.2 \) s has been reported (20). Therefore we measured deactivation of the enzyme in the intact cellular environment and performed the respective deactivation experiments in human platelets. The deactivation measured was clearly faster than that of the purified enzyme; however, the method applied was not sensitive enough to allow an exact quantification of the rate of deactivation (data not shown).

The GC activator YC-1 has been shown to sensitize the enzyme toward NO, and an inhibition of the deactivation has been proposed as the underlying mechanism. Therefore, we performed the deactivation experiment with the purified enzyme in the presence of YC-1. YC-1 slowed down the deactivation tremendously (120-fold) resulting in a half-life of the NO-GC complex of \( 10 \) min (Fig. 5B). Fig. 5C depicts the YC-1-induced shift of the NO concentration response curve by one order of magnitude to the left. This NO-sensitizing effect may very well be explained by the pronounced inhibition of GC deactivation by YC-1.

**Fig. 5.** Effect of YC-1 on the deactivation of GC. A, accumulation of cGMP formed by the NO-stimulated GC (preincubated with NO for 3 min) in the presence of YC-1 (200 \( \mu \)M) after the addition of oxyHb was measured at the indicated time points as described under "Materials and Methods." B, actual cGMP-forming activity was calculated from the fitted curve of cGMP accumulation (A) as described under "Materials and Methods." For comparison the deactivation in the absence of YC-1 is shown (dashed line). C, wild type GC (0.1 \( \mu \)g) was incubated for 10 min with the indicated concentrations of DEA-NO in the presence (closed circles) and absence (open circles) of 200 \( \mu \)M YC-1.

**Fig. 6.** The cAMP-forming GC mutant lacks the YC-1 effect. A, accumulation of cAMP formed by the NO-stimulated GC mutant (preincubated with NO for 3 min) in the presence of YC-1 (200 \( \mu \)M) after the addition of oxyHb was measured at the indicated time points as described under "Materials and Methods." B, actual cAMP-forming activity was calculated from the fitted curve of cAMP accumulation (A) as described under "Materials and Methods." For comparison the deactivation in the absence of YC-1 is shown (dashed line). C, cAMP-forming GC mutant (0.1 \( \mu \)g) was incubated for 10 min with the indicated concentrations of DEA-NO in the presence (closed circles) and absence (open circles) of 200 \( \mu \)M YC-1.
Next, we measured the deactivation of the ATP-utilizing mutant in the presence of YC-1 to find out whether the mutant deactivation rate was slowed down by YC-1 as in the WT enzyme. Much to our surprise, the deactivation was decelerated only modestly by YC-1 (Fig. 6, A and B). Because the YC-1 effect on deactivation of the mutant was almost vanished compared with the one observed in the WT enzyme, the mutant represented an ideal tool to elucidate whether the YC-1 induced sensitization of the WT enzyme toward NO occurs independently of the effect on deactivation. We obtained NO concentration response curves for the ATP-utilizing mutant in the presence and absence of YC-1. As can be seen in Fig. 6C, the EC50 for NO of the mutant was not shifted by YC-1. These results indicate that the YC-1-induced inhibition of the deactivation accounts for the observed sensitizing effect of YC-1 in the WT enzyme.

**DISCUSSION**

From other heme-containing proteins it is known that NO dissociation is fairly slow in the range of minutes to hours. As a much faster dissociation rate of NO from a five-coordinated ferrous hemoprotein was unimaginable, mechanisms other than simple dissociation being responsible for the deactivation of NO-sensitive GC have been proposed (25). Yet, it appears than simple dissociation being responsible for the deactivation of the enzyme yields a half-life of 5 s versus 3 min, determined spectrophotometrically for the purified enzyme varied considerably (17, 18). Here, we assessed NO dissociation by measuring the resulting deactivation of the enzyme; by monitoring enzymatic activity of purified NO-sensitive GC after the addition of an NO scavenger or after dilution of NO we found a half-life of ~4 s. This is in agreement with the data obtained in our spectral study on the purified enzyme in the presence of GTP (17) and another study performed in cytosolic fractions after the addition of oxyHb (19).

GTP has been shown to increase NO dissociation in spectroscopic experiments (17). Furthermore, GTP induced a change in resonance Raman spectrum of the NO-GC complex (26). In principle, the increase in dissociation caused by GTP may indicate that GTP bound to the catalytic center is required for fast NO dissociation. On the other hand, MgGTP might mediate its effects on NO dissociation by a second non-catalytic MgGTP binding site. The pseudosymmetric counterpart of the catalytic site, which in AC binds forskolin, appeared as a tempting candidate for such a binding site. To find out whether the MgGTP effect on NO dissociation is mediated by the GTP-converting center or by a putative second non-catalytic site, we used a mutant with a changed substrate specificity utilizing ATP instead of GTP. The dissociation rate of this mutant yielded a half-life of ~15 s, which is slower than the half-life of the WT enzyme. However, the difference is moderate; therefore, it cannot be decided whether ATP is able to substitute for GTP on the catalytic or the putative non-catalytic nucleotide binding site thereby accelerating NO dissociation.

In a recent study, a considerably faster deactivation of NO-sensitive GC in intact cells upon the addition of oxyHb was reported with a half-life of 0.2 s (20). In our analogous experiments performed in platelets, deactivation was markedly faster than with the purified enzyme and compatible with the results obtained in granular cerebellar cells. Thus, although a possible impairment during the course of the purification of the enzyme cannot be ruled out, the differences in deactivation of the purified enzyme and of the enzyme in intact cells suggest the existence of deactivation accelerating factors in intact cells. Therefore, we studied the YC-1 effect on the deactivation of NO-sensitive GC. This new GC sensitizer led to a dramatic inhibition of the deactivation yielding a ~120-fold prolonged half-life of the GC-NO complex. Surprisingly, the deactivation of the ATP-utilizing mutant was almost unaffected by YC-1. In a recent report, the N-terminal heme-binding region of the α subunit has been proposed to be responsible for YC-1 binding (27).

Thus, substitution of these amino acids either prevents the transduction of the YC-1 effect or the respective amino acids in the catalytic domain are directly or indirectly involved in YC-1-binding. The fact that NO sensitivity of the ATP-utilizing mutant was unaffected by YC-1 clearly establishes the inhibition of deactivation of GC as the underlying mechanism for the YC-1-induced sensitization.

In summary, the slow deactivation of NO-sensitive GC observed in the presence of YC-1 and the extremely fast deactivation of the enzyme observed in intact cells suggest that NO dissociation and the subsequent deactivation can be altered physiologically and pharmacologically. A change in the rate of deactivation of the enzyme would alter the amplitude and duration of the cGMP response induced by a given NO concentration thereby adapting the state of NO sensitivity of certain tissues to special physiological and pathophysiological conditions. Further experiments have to elucidate whether the opposite effects on the deactivation of NO-sensitive GC are mediated by a common site affecting NO dissociation from the heme group of the enzyme.

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