NO activation of guanylyl cyclase

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Nitric oxide (NO)-sensitive guanylyl-cyclase (GC) is the most important receptor for the signaling molecule NO. Activation of the enzyme is brought about by binding of NO to the prosthetic heme group. By monitoring NO-binding and catalytic activity simultaneously, we show that NO activates GC only if the reaction products of the enzyme are present. NO-binding in the absence of the products did not activate the enzyme, but yielded a non-activated species with the spectral characteristics of the active form. Conversion of the nonactivated into the activated conformation of the enzyme required the simultaneous presence of NO and the reaction products. Furthermore, the products magnesium/cGMP/pyrophosphate promoted the release of the histidine-iron bond during NO-binding, indicating reciprocal communication of the catalytic and ligand-binding domains. Based on these observations, we present a model that proposes two NO-bound states of the enzyme: an active state formed in the presence of the products and a nonactivated state. The model not only covers the data reported here but also consolidates results from previous studies on NO-binding and dissociation/deactivation of GC.

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Introduction

The nitric oxide (NO)-sensitive guanylyl cyclase (GC) plays a key role in the NO/cGMP signaling cascade involved in physiological processes like smooth muscle relaxation, inhibition of platelet aggregation and synaptic plasticity (Waldman and Murad, 1987; Moncada *et al*, 1991; Garthwaite and Boulton, 1995; Ignarro *et al*, 1999). The NO-sensitive GC is activated by the freely diffusible messenger NO formed by the endothelial and neuronal NO synthases. GC activation leads to enhanced formation of the intracellular messenger cGMP, whose effects are mediated by cGMP-dependent protein kinases, cGMP-regulated ion channels and by cGMP-dependent phosphodiesterases. cGMP-degrading phosphodiesterases do not only degrade the

cGMP but also critically determine the shape of the cGMP signal (Müllershausen *et al*, 2001).

NO-sensitive GC is a heterodimeric protein composed of two subunits, α and β , and a prosthetic heme group. Two isoforms with similar regulatory properties but different subcellular distribution exist (Russwurm *et al*, 2001). Studies on the regulation of the enzyme including this one have been performed with the $\alpha_1\beta_1$ enzyme.

The mechanism of activation of GC by NO is currently under debate; it is generally accepted that NO binds to the prosthetic heme group of the enzyme, which finally leads to activation and enhanced cGMP formation. In the unligated state, the heme group displays an absorbance maximum of 431 nm, indicative of five-coordinated ferrous high-spin heme with a histidine as axial ligand. Histidine 105 of the β_1 subunit has been identified as the axial heme ligand (Wedel *et al*, 1994; Zhao *et al*, 1998). Binding of NO shifts the absorbance maximum to 399 nm (Stone and Marletta, 1994). Together with the observation that the iron-free heme precursor protoporphyrin IX stimulates GC (Ignarro *et al*, 1982), it has been concluded that activation of the enzyme by NO is brought about by the rupture of the histidine to iron bond, resulting in a five-coordinated nitrosyl heme complex. The release of the histidine is thought to induce so far uncharacterized conformational changes leading to activation of the enzyme.

Latest insights into the mechanism of GC activation by NO came from rapid UV/vis spectroscopy at 4°C, demonstrating the formation of an NO-bound intermediate with an absorption maximum at 420 nm (Zhao *et al*, 1999). This absorbance maximum was assigned to a six-coordinated heme iron with histidine bound to the so-called proximal site of the heme and NO bound to the distal site (Figure 1). As the subsequent conversion into the five-coordinated nitrosyl-heme was dependent on the presence of NO, it was concluded that a second NO-binding event occurs in the course of GC activation. The nature of the second NO-binding site remained unknown; theoretically, a non-heme-binding site may be involved but binding of the second NO molecule to the heme appeared more likely. However, before the study of Lawson *et al* (2000) was published, a plausible explanation for a second NO-binding event was missing. The finding of an NO bound to the proximal site of the heme in the crystal structure of cytochrome *c'* from *Alcaligenes xylosoxidans* questioned the dogma of NO-binding exclusively to distal heme sites, and gave rise to the following NO-binding sequence (Lawson *et al*, 2003): NO binds to the distal site of the heme first, thereby weakening the histidine to iron bond and enabling a second NO molecule to bind to the proximal site of the heme. From the resulting unstable dinitrosyl-heme, one NO molecule is released, hypothetically the distally bound NO (see Figure 1). The existence of a six-coordinated NO-bound heme with intact histidine-iron bond in GC has also been reported by others (Makino *et al*, 1999; Sharma and Magde, 1999). However, in contrast to the study by Zhao *et al* (1999), the further conversion into the final five-coordinated

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Figure 1 Model for sequential binding of NO to GC. NO-binding first results in a 420 nm six-coordinated state that converts in an NO-dependent manner to the final 399 nm species via a proposed dinitrosyl-heme. The site of the heme to which NO is bound in the final 399 nm state is not identified. For further explanation, see text.

species was not NO dependent. Hence, a second NO-binding event was not required for interpretation of these data.

So far, binding of NO to the prosthetic heme group and activation of the NO-sensitive GC were studied in different approaches. In studies on the enzymatic activity, binding of NO to the prosthetic heme group was not recorded, whereas spectrophotometrical analyses on NO-binding were performed without the substrate. In order to close this gap, simultaneous recordings of NO-binding and activation were performed. Unexpectedly, we found that NO-binding does not necessarily lead to activation of the enzyme. Hence, we demonstrate the existence of two states of the NO-bound enzyme characterized by low and high catalytic activity, but indistinguishable by spectroscopy. Activation of NO-sensitive GC required the simultaneous presence of NO and the reaction products of the enzyme, magnesium, cGMP and pyrophosphate. The observation that NO-binding by itself was remarkably altered by the reaction products of NO-sensitive GC emphasizes the reciprocal communication between the catalytic and the heme-binding domains.

Results

During the previous years, the mechanism of NO-binding to the heme group of GC had been thoroughly investigated. However, NO-binding and activation of the enzyme have not been studied in parallel.

First, we prepared NO-saturated GC. For this purpose, purified GC was incubated with the NO donor PROLI-NO (PROLI-NO) and passed over a desalting column to remove excess NO. Subsequently, enzymatic activity and heme spectra were measured simultaneously in a disposable cuvette. NO saturation of the heme group was estimated by fitting with calculated two-component spectra of various fractions of NO-free and NO-bound GC species using the least-squares method. As can be seen in Figure 2A, the calculated spectrum of 80% NO-bound and 20% NO-free GC fitted the experimentally determined spectrum. From 80% saturation of the heme group, one would expect 80% activation of the enzyme. Much to our surprise, enzymatic activity was only 10% of the maximum rate (Figure 2B). To ensure integrity of the enzyme, we added excess PROLI-NO, which resulted in full activation of the enzyme and complete NO saturation (Figure 2C and D). This experiment showed for the first time that binding of NO to the heme group is not necessarily paralleled by activation of the enzyme. To further elucidate conditions that lead to an NO-saturated yet non-activated enzyme, we used another approach to prepare the nonactivated state.

Figure 2 NO saturation and enzymatic activity of GC after removal of free NO. GC (90 μg, 6 μM) was saturated with NO by incubation with PROLI-NO (12.5 μM). Subsequently, free NO was removed on a desalting column. Spectra and activity of the eluted enzyme were measured simultaneously without or after addition of an excess of PROLI-NO (20 μM). (A) Representative spectrum of the eluted GC (solid line). For comparison, the NO-saturated, NO-free spectra (dashed lines) and a calculated spectrum of 80% NO-saturated GC (dotted line, hidden by the measured spectrum) are also shown. (B) Statistics of cGMP-forming activity of GC (open bar) and NO saturation (black) after removal of free NO. (C) Representative spectrum recorded after addition of PROLI-NO (20 μM) to the eluted GC. (D) Statistics of cGMP-forming activity of GC (open bar) and NO saturation (black) after addition of PROLI-NO (20 μM) to the eluted enzyme. Data in (B) and (D) are means ± s.d. of three independent experiments.

In these experiments, the enzyme was preincubated with substoichiometric amounts of the very fast releasing NO donor PROLI-NO; subsequently, enzymatic activity and NO saturation were assessed simultaneously. Figure 3A shows representative spectra obtained with the PROLI-NO concentrations indicated. Again, NO saturation was estimated by comparison with the calculated spectra of NO-bound and NO-free GC; the respective NO saturation values are given in Figure 3B. Under the conditions applied, 1 μM PROLI-NO led to an NO saturation of 70%, whereas the enzymatic activity amounted up to only 20% of the maximal catalytic rate. An excess of NO (10 μM PROLI-NO) caused full activation of the enzyme. The complete set of experiments is depicted in

Figure 3 cGMP-forming activity and NO saturation of GC evoked by substoichiometric PROLI-NO. GC (6 μg, 0.4 μM) was preincubated with increasing PROLI-NO concentrations as indicated; spectra and enzymatic activity were recorded simultaneously. **(A)** Representative UV/vis spectra (solid lines) of GC preincubated with the indicated PROLI-NO concentrations were fitted with calculated spectra (dotted lines) to determine NO saturation of the enzyme. The position of the absorption maxima of the NO-free and the NO-bound enzyme are indicated by the arrows at 431 and 399 nm, respectively. **(B)** NO-saturation values of GC of the representative experiment depicted in **(A)** were obtained as described in Materials and methods section. **(C)** cGMP-forming activities of the samples depicted in **(A)** were determined in 1 min assays. **(D)** Relative activities were plotted versus relative NO-saturation values of $n=28$ simultaneous measurements of GC preincubated with increasing PROLI-NO concentrations; the NO saturation and activity of each sample is depicted as a black circle. The expected linear correlation is indicated by the line.

Figure 3D, clearly showing that NO saturation and enzymatic activity do not correlate linearly as expected (indicated by the line). Hence, with substoichiometric amounts of NO, we were able to produce the NO-bound but nonactivated conformation of the enzyme.

As we speculated about a reciprocal communication between the catalytic and NO-binding domains in the course of activation, we performed the same experiment in the presence of Mg^{2+} and inorganic pyrophosphate (PP_i) normally produced during the catalytic reaction of the enzyme. As can be seen in Figure 4A and B, the spectra evoked by PROLI-NO in the presence of Mg^{2+}/PP_i were almost indistinguishable from the spectra obtained in their absence (see Figure 3A). However, the presence of Mg^{2+}/PP_i during NO-binding led to activation of the enzyme (Figure 4C), resulting in a strong correlation of NO saturation and catalytic activity (Figure 4D). Hence, NO, together with Mg^{2+} and PP_i , is sufficient to generate the active conformation of the enzyme.

To identify the components of the enzyme's reaction products required for activation upon NO-binding, we tested the combinations of the three reaction products Mg^{2+} , PP_i and cGMP (Figure 5). For easier comparison, the control experiment without Mg^{2+} , PP_i or cGMP (Figure 3D) and the Mg^{2+}/PP_i experiment (Figure 4D) are shown as Figure 5A and H, respectively. Clearly, enzymatic activity correlated with NO-binding when either Mg^{2+}/PP_i (Figure 5H) or

$Mg^{2+}/cGMP$ (Figure 5G) were present during the preincubation, whereas neither Mg^{2+} , PP_i or cGMP alone did promote activation upon NO-binding (Figure 5B–D), nor did the combination cGMP/ PP_i (Figure 4E), Mg^{2+} /inorganic phosphate (P_i) or cGMP/ P_i (data not shown). These results demonstrate that formation of the active conformation of GC not only requires binding of NO but also the presence of Mg^{2+}/PP_i or $Mg^{2+}/cGMP$ during NO-binding. Experiments performed in the presence of the substrate $Mg^{2+}GTP$ displayed correlation of enzyme activity with NO saturation (Figure 5F), which is not surprising because, in the presence of the substrate $Mg^{2+}GTP$, the enzyme continuously catalyzes the formation of the reaction products cGMP, PP_i and Mg^{2+} .

To further study the effect of the reaction products or substrate on the NO-binding process in detail, we monitored the formation of the 420 nm state of GC's heme group that had been observed at 4°C under NO-limiting conditions and attributed to a six-coordinated NO-heme with the intact histidine-iron bond (see Figure 1; Zhao *et al*, 1999). In accordance with the findings by Zhao *et al* (1999), addition of diethylamine NONOate (DEA-NO) to the purified enzyme at 4°C resulted in the formation of the 420 nm intermediate, which was further converted to the 399 nm species (Figure 6A). However, the substrate $Mg^{2+}GTP$ abolished the formation of the 420 nm six-coordinated species under

Figure 4 cGMP-forming activity and NO saturation of GC after preincubation with PROLI-NO in the presence of Mg^{2+} and PP_i . GC ($6 \mu\text{g}$, $0.4 \mu\text{M}$) was preincubated in the presence of Mg^{2+} and PP_i (15 and 0.6 mM , respectively) with increasing PROLI-NO concentrations as indicated. Spectra and activity of the enzyme were determined simultaneously. (A) Representative spectra (solid lines) obtained after NO preincubation were compared to calculated spectra (dotted lines) to measure NO saturation of the enzyme. The position of the absorption maxima of the NO-free and the NO-bound enzyme are indicated by the arrows at 431 and 399 nm, respectively. (B) NO saturation of the samples shown in (A) was determined as described in Materials and methods section. (C) cGMP-forming activities of the samples depicted in (A) determined in a 1 min assay. (D) Relative activities were plotted versus relative NO-saturation values of $n = 21$ samples; the NO saturation and activity of each sample is depicted as a black circle.

Figure 5 Correlation of cGMP-forming activity and NO saturation of GC in the presence of the substrate and reaction products of the enzyme. cGMP-forming activities and degree of NO saturations of GC ($6 \mu\text{g}$, $0.4 \mu\text{M}$) were recorded simultaneously after preincubation with different amounts of PROLI-NO (0, 0.125, 0.25, 0.5, 1, 2, $10 \mu\text{M}$) in the absence (A) and the presence of 15 mM MgCl_2 (B), 1 mM cGMP (C), 0.6 mM PP_i (D), or combinations thereof (E, G–I) as indicated, and in the presence of MgCl_2/GTP (15/5 mM, respectively) (H). Each single pair of NO saturation and activity values is depicted as a black circle (A: $n = 28$, B–I: $n = 21$).

these conditions (Figure 6B). As demonstrated by a single isosbestic point, the NO-free five-coordinated species (431 nm) converted into the NO-bound five-coordinated species (399 nm) without a detectable intermediate. The lack of

the 420 nm peak indicates a much faster formation of the final 399 nm species. We repeated the same experiment in the presence of the reaction products $\text{Mg}^{2+}/\text{cGMP}/\text{PP}_i$, and also under these conditions we were not able to detect the

Figure 6 Monitoring sequential NO-binding in the presence of substrate and products. NO-binding to GC (1.5 μ M) was followed at 4°C by recording UV/vis spectra after addition of DEA-NO (67 μ M; the relatively high DEA-NO concentrations had to be used because of the slow decomposition of DEA-NO at 4°C). Spectra obtained in the absence (A) and the presence of 3 mM MgCl₂ and 1 mM GTP (B), and 3 mM MgCl₂ 1 mM cGMP, 1 mM PP_i (C) at 1, 14, 26, 38 and 62 s are shown.

Figure 7 Model for NO-binding and activation of NO-sensitive GC. NO-binding to the heme initially results in a six-coordinated state (420 nm) that immediately converts into the active state if the reaction products of the enzyme are present. In the absence of the enzyme's products, a nonactivated state is produced in an NO-dependent manner. Implications of the model are given in Discussion.

intermediate (Figure 6C). This demonstrates that neither binding of the substrate Mg²⁺+GTP nor catalytic conversion, but the reaction products of the enzyme mediate the effect of Mg²⁺+GTP on NO-binding.

Discussion

Many aspects of the activation of the NO receptor, NO-sensitive GC, have been unraveled during the last 15 years. From the 'black box' model of an enzyme simply binding NO and then somehow generating cGMP from GTP, more sophisticated models have evolved. The resolution of the structure of the adenylyl cyclase's catalytic core told us details about the catalytic mechanism (Tesmer *et al*, 1997, 1999; Sunahara *et al*, 1998), spectrophotometric analyses revealed precise information about NO-binding and dissociation (Brandish *et al*, 1998; Makino *et al*, 1999; Zhao *et al*, 1999; Ballou *et al*, 2002).

However, in studies investigating binding of NO to the heme group, the enzyme was treated as simple NO-binding protein without a catalytic domain, whereas in analyses of activation or deactivation the mechanism of NO-binding was ignored. Taken together, the enzyme was perceived as two separate entities, that is, either as an NO receptor or as a cGMP-forming enzyme; the only established communication between the entities is that binding of NO leads to stimulation of catalysis. This assumption of one-way signaling denies the

principle of reciprocity and, accordingly, we considered an influence of the catalytic domain on the NO-binding heme domain. Therefore, the impact of the substrate on the NO-binding process was studied by monitoring the formation of the six-coordinated intermediate of GC formed under NO-limiting conditions at 4°C (Zhao *et al*, 1999). The formation of the stable intermediate was clearly abolished in the presence of the substrate (see Figure 6). Furthermore, as the reaction products had the same effect, we conclude that neither catalytic conversion nor the substrate itself, but the reaction products altered NO-binding. The acceleration of the conversion into the final five-coordinated species by the products strongly supports the assumption of a reciprocal communication between the catalytic and the NO-binding domains, albeit binding of the products to another binding site located pseudosymmetrically to the catalytic center (Tesmer *et al*, 1997) or within the heme-binding regulatory region cannot be ruled out.

Unexpectedly, binding of NO in the absence of the reaction products did not activate the enzyme, but produced an NO-bound, nonactivated GC. Only in the presence of the reaction products Mg²⁺+cGMP/PP_i, NO-binding triggered activation of the enzyme. To explain these apparently confusing results, we propose the following model depicted in Figure 7.

The model predicts two NO-bound states of GC indistinguishable by spectroscopy but characterized by high and low enzymatic activity; one of them is formed in the presence of

the reaction products of the enzyme, the other one in the absence. Arbitrarily, the two states have been assigned to the two possible NO-bound conformations, with NO bound to the distal and proximal sites, respectively. In the presence of the products, NO binds to the distal site of the heme, forming a six-coordinated intermediate (420 nm) that immediately converts into the final five-coordinated and activated species (399 nm, see Figure 7). The NO-binding sequence in the absence of the reaction products is similar to the one in Figure 1 and starts with the formation of the stable six-coordinated intermediate (420 nm; Ballou *et al*, 2002). The next step requires additional NO, as a second NO molecule binds to the proximal site replacing the histidine and repelling the distally bound NO. The formed five-coordinated species is not activated and exhibits an absorption maximum of 399 nm. The NO dependency of this step predicts that the back-reaction, that is, the transition to the dinitrosyl state, requires rebinding of NO, and hence removal of free NO locks the enzyme in the NO-bound state. As a consequence of the model deduced from the data discussed above, the release of the histidine-iron bond does not lead to activation of the enzyme *per se*, but requires the presence of the reaction products of the enzyme or the substrate. Consistency of the model with results obtained here and in previous studies will be discussed below.

In the experiment described first, NO was bound to GC in the absence of substrate or products, and prior to addition of substrate free NO was removed. According to the model, the nonactivated, NO-bound enzyme was prepared and the respective experiment revealed comparatively low catalytic activity in the light of high NO saturation of the heme group. The catalytic activity was not restored by the reaction products formed during the measurement, because the lack of free NO prevented the back-reaction via the dinitrosyl state to the six-coordinated intermediate (420 nm), which is required to finally reach the activated state (see Figure 7). Accordingly, further addition of NO (see Figure 2C) led to full activation of the enzyme as free NO allowed the back-reaction to the six-coordinated state that, now in the presence of the reaction products, immediately converted into the five-coordinated, activated form of the enzyme.

Using a similar procedure, Brandish *et al* (1998) prepared an NO-saturated enzyme for spectrophotometric dissociation studies in the absence of the substrate and removed unbound NO on a desalting column. The binding models described here and in the introduction predict that the NO-bound GC formed in the absence of the substrate requires binding of NO during the back-reaction to form the dinitrosyl state; without free NO the enzyme is locked in the NO-bound state, thereby explaining the slow NO dissociation ($t_{1/2} = 2.9$ min) obtained in the study by Brandish *et al* (1998), which we reproduced under the same experimental conditions ($t_{1/2} = 2$ min). The deactivation of the NO-stimulated enzyme *in vitro* and in intact cells measured by the conversion of GTP to cGMP has been described to be much faster (Bellamy and Garthwaite, 2001; Russwurm *et al*, 2002). However, addition of substrate in the dissociation study (Brandish *et al*, 1998) did not alter NO dissociation rates ($t_{1/2} = 3.2$ min), a result we also obtained ($t_{1/2} = 2$ min) in the described experimental set-up (Brandish *et al*, 1998). The obvious difference between the deactivation and dissociation studies is the time of substrate addition; in the dissociation study, the substrate was added to

the NO-saturated enzyme *after* removal of free NO, whereas in the deactivation study substrate was added *before* removal of free NO. Thus, the dissociation study measured NO dissociation from the five-coordinated nonactivated GC and yielded slow NO dissociation, whereas in the deactivation study the five-coordinated activated form was prepared, which exhibited a much faster deactivation. In accordance with the model, addition of substrate after removal of free NO in the dissociation study did not accelerate dissociation. Taken together, the results from both studies support the concept that the simultaneous presence of NO and substrate or products is required to produce the activated NO-bound form, whereas in the absence of substrate or products the nonactivated state is formed.

In the second set of experiments, NO-binding activated the enzyme only in the presence of the reaction products. According to our model, NO-binding in the absence of products leads to the nonactivated NO-bound form of GC. This explains the discrepancy of NO saturation and catalytic activity exemplified by an enzyme 70% saturated with NO, exerting only 20% of the maximal catalytic rates. The finding that higher NO concentrations (10 μ M) led to full activation even in the absence of products during NO-binding apparently contradicts the model. However, under these conditions, free NO was still available after addition of the substrate and allowed the enzyme to convert into the active state via the formation of the dinitrosyl and six-coordinated nitrosyl-histidyl species (420 nm).

Also, the results of the previous experiment analyzing the stability of the 420 nm species are covered by the model. In the absence of products, the conversion into the 399 nm five-coordinated species required additional NO, as shown by the higher stability of the 420 nm intermediate and the slower conversion to the 399 nm species under the NO-limiting conditions applied. In the presence of the products Mg^{2+} /PPi/cGMP, the 420 nm six-coordinated state was not observed, as it immediately converted into the five-coordinated and activated species.

Besides the model discussed above, other models (e.g. those including a non-heme NO-binding site or addition of a second NO molecule to the already bound NO molecule) may be developed to describe the available data on NO-binding and activation of the NO-sensitive GC. However, every model has to account for the following findings: (i) Binding of NO in the absence of products/substrate involves two sequential NO-binding events (Zhao *et al*, 1999). (ii) Two NO-bound five-coordinated states of the enzyme exist, an active and a nonactive state (shown in this study). (iii) The nonactive, NO-bound state converts into the active state only in the presence of products or substrate and additional free NO (shown in this study).

Taken together, the study introduces a new model of ligand binding to the NO receptor, proposing two five-coordinated NO-bound species: one of them is activated, whereas the other one, formed in the artificial absence of substrate or products, is not activated. Further studies on the NO-binding mechanism should take into account that the NO-sensitive GC is not only the NO receptor but also a receptor-coupled enzyme, and analyses performed in the absence of the substrate do not describe ligand binding as it occurs in the intracellular environment, that is, in the presence of substrate or NO-sensitive GC.

Materials and methods

Reagents

DEA-NO and PROLI-NO were from Alexis (Switzerland), Sephadex G25 fine and α -[32 P]GTP were from Amersham Biosciences. All other chemicals were obtained from Sigma-Aldrich.

Purification of NO-sensitive GC

NO-sensitive GC was purified from bovine lung by an immunoaffinity procedure described by Humbert *et al* (1990). Purification from 5 kg fresh lung yielded 5 mg protein at a concentration of approx. 10 mg/ml. Purity was judged by SDS-polyacrylamide gel electrophoresis, and revealed a homogenous $\alpha_1\beta_1$ enzyme. NO-stimulated catalytic activity amounted to approx. 15 μ mol cGMP/min/mg protein. All experiments were performed with different preparations of the enzyme.

Analysis of enzymatic activity after removal of PROLI-NO on a desalting column

Purified GC (90 μ g) was incubated with 12.5 μ M PROLI-NO for 40 s in 100 μ l buffer A (150 mM NaCl, 2 mM DTT, 50 mM triethanolamine/HCl, pH 7.4) and passed over a desalting column (1 ml Sephadex G25 fine, diameter 0.5 cm, flow rate 1 ml/min, buffer A). The protein concentration of the eluate (200 μ l) was determined by the Warburg method. Immediately afterwards, enzymatic activity and UV/vis spectra of the eluate were measured simultaneously in a disposable cuvette in the absence or presence of 20 μ M PROLI-NO. Enzymatic activity was determined by conversion of α -[32 P]GTP to [32 P]cGMP (1 min incubation at 37°C, 60 μ l eluate, 5 mM α -[32 P]GTP (~10 kBq), 10 mM MgCl₂, 2 mM cGMP in a total volume of 75 μ l, Schultz and Böhme, 1984). UV/vis spectra were recorded simultaneously 20 and 40 s after start of the reaction using a Cary 100 spectrophotometer (Varian Inc.). The two spectra did not differ significantly and yielded identical NO saturation values (see below).

Determination of enzymatic activity evoked by substoichiometric amounts of PROLI-NO

In a disposable cuvette, GC (6 μ g, final concentration 0.4 μ M) in 2 mM DTT, 50 mM triethanolamine/HCl, pH 7.4, was preincubated (40 s, 37°C) with increasing PROLI-NO concentrations as indicated, and in the absence and presence of 15 mM MgCl₂, 1 mM cGMP, 0.6 mM PP_i or 0.6 mM Na₂HPO₄ (P_i) if indicated (final volume 85 μ l). Subsequently, 15 μ l substrate (5 mM α -[32 P]GTP (~10 kBq), MgCl₂ and cGMP if not present during preincubation) were added and cGMP-forming activities were measured (1 min, 37°C, Schultz and Böhme, 1984). UV/vis spectra of the same sample were obtained simultaneously 10, 30 and 50 s after addition of substrate (8453 diode array, Agilent technologies). The spectra obtained at 30 s were used to calculate NO saturation values (see below).

For the experiment in the presence of Mg²⁺ + GTP, GC (12 μ g, final concentration 0.4 μ M) in 2 mM DTT, 50 mM triethanolamine/HCl, pH 7.4, was preincubated (40 s, 37°C) with increasing PROLI-

NO concentrations as indicated in the presence of 5 mM α -[32 P]GTP (~10 kBq), 15 mM MgCl₂ and 1 mM cGMP (final volume 200 μ l). Subsequently, a 100 μ l aliquot was stopped to determine the amount of cGMP produced during the preincubation, and measurements of NO-saturation and cGMP-forming activity of the remaining sample were performed as described above.

The following maximum catalytic rates (in μ mol cGMP/min/mg) obtained in these experiments were used for normalization: control conditions (Figures 3 and 5A) 15 $\pm$ 2; Mg²⁺ (Figure 5B) 15 $\pm$ 2; cGMP (Figure 5C) 15 $\pm$ 2; PP_i (Figure 5D) 14 $\pm$ 2; cGMP + PP_i (Figure 5E) 13 $\pm$ 0.3; Mg + GTP (Figure 5F) 25 $\pm$ 1.4 (performed with a different enzyme preparation, one experiment performed with the same preparation as the other depicted experiments yielded 16 μ mol cGMP/min/mg); Mg + cGMP (Figure 5G) 17 $\pm$ 1; Mg²⁺ + PP_i (Figures 4 and 5H) 16 $\pm$ 1; Mg²⁺ + cGMP + PP_i (Figure 5I) 16 $\pm$ 1.

Determination of NO saturation of GC by calculation of mixed two component spectra

For quantification of NO saturation of the enzyme, spectra of NO-free and completely NO-saturated GC were obtained under the same conditions as the spectrum of the partially saturated GC. A mixed two-component spectrum of NO-saturated and -free GC was calculated using the formula:

$$A_{\text{mixed}}(\lambda) = A_{\text{NO-saturated GC}}(\lambda) \times k_{\text{NO-saturation}} + A_{\text{NO-free GC}}(\lambda) \times (100\% - k_{\text{NO-saturation}})$$

where $A(\lambda)$ is the absorption at wavelength λ , $k_{\text{NO-saturation}}$ the NO-saturation value for each wavelength λ in 1 nm steps between 350 and 480 nm.

Using the least-squares method with $k_{\text{NO-saturation}}$ as independent variable, the calculated spectrum was fitted to the measured spectrum of the partially NO-bound GC to obtain the $k_{\text{NO-saturation}}$ value.

Analysis of sequential NO-binding to GC

UV/vis spectra (8453 diode array, Agilent technologies) of GC (22 μ g, final concentration 1.5 μ M, in 3 mM DTT, 50 mM triethanolamine/HCl, pH 7.4, total volume 100 μ l) were obtained after addition of DEA-NO (final conc. 67 μ M, the relatively high DEA-NO concentrations had to be used because of the slow decomposition of DEA-NO at 4°C) during a 1 min incubation at 4°C in the absence or presence of 3 mM MgCl₂, 1 mM cGMP, 1 mM PP_i and 1 mM GTP as indicated.

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