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Articles

Spectral and Kinetic Studies on the Activation of Soluble Guanylate Cyclase by Nitric Oxide†

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ABSTRACT: The soluble form of guanylate cyclase (sGC) is the only definitive receptor for the signaling agent nitric oxide (·NO). The enzyme is a heterodimer of homologous subunits in which each subunit binds 1 equiv of 5-coordinate high-spin heme. ·NO increases the V_{max} of sGC up to 400-fold and has previously been shown to bind to the heme to form a 5-coordinate complex. Using stopped-flow spectrophotometry, it is demonstrated that the binding of ·NO to the heme of sGC is a complex process. ·NO first binds to the heme to form a 6-coordinate nitrosyl complex, which then converts to a 5-coordinate nitrosyl complex through one of two ways. For 28 ± 4% of the heme, the 6-coordinate nitrosyl complex rapidly (∼20 s^{-1}) converts to the 5-coordinate complex. For the remaining 72 ± 4% of the heme, the conversion of the 6-coordinate nitrosyl complex to a 5-coordinate nitrosyl complex is slow (0.1−1.0 s^{-1}) and is dependent upon the interaction of ·NO with an unidentified non-heme site on the protein. The heme (200 nM) was completely converted to the 5-coordinate state with as little as 500 nM ·NO, and the equilibrium dissociation constant of ·NO for activating the enzyme was determined to be ≤250 nM. Gel-filtration analysis indicates that the binding of ·NO to the heme has no effect on the native molecular mass of the protein. Correlation of electronic absorption spectra with activity measurements indicates that the 5-coordinate nitrosyl form of the enzyme is activated relative to the resting 5-coordinate ferrous form of the enzyme.

Nitric oxide (·NO)[1] is a free radical that has recently been shown to mediate intercellular communication in such processes as vasodilation and neuronal signal transduction [for recent reviews, see Bredt and Snyder (1994), Marletta (1993, 1994), and Nathan (1992)]. The only receptor for ·NO characterized thus far is the soluble form of guanylate cyclase (sGC) (Garbers & Lowe, 1994; Waldman & Murad, 1987). sGC catalyzes the cyclization of guanosine 5′-triphosphate (GTP) to guanosine 3′,5′-cyclic monophosphate (cGMP). sGC is a heterodimer, possessing an α subunit with a mass ranging from 73 to 88 kDa and a β subunit with a mass of 70 kDa. Each subunit of the heterodimer binds 1 equiv of a *b*-type heme (iron protoporphyrin IX) (Stone & Marletta, 1995a). The heme is 5-coordinate high spin in both the ferrous and ferric states with histidine as the sole axial ligand (Stone & Marletta, 1994, 1995a). The 5-coordinate ferrous heme has the unusual property of not binding oxygen.

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[1] Abbreviations: sGC, soluble guanylate cyclase; ·NO, nitric oxide; TEA, triethanolamine; DTT, dithiothreitol; GTP, guanosine 5′-triphosphate; cGMP, guanosine 3′,5′-cyclic monophosphate; Hb, hemoglobin; Mb, myoglobin; BSA, bovine serum albumin.

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It has been proposed that activation of sGC by ·NO is a direct result of the formation of a 5-coordinate nitrosyl−heme complex (Ignarro et al., 1984). This hypothesis is based on the observation that the ability of ·NO to activate purified sGC is dependent on the presence of heme (Ignarro et al., 1982a). Enzyme containing heme is extensively activated by ·NO. Removal of the heme moiety results in loss of the ability of ·NO to activate the enzyme. Reconstitution of the enzyme with heme partially restores the ability of ·NO to activate the enzyme. Finally, it has been demonstrated that purified heme-deficient enzyme is activated by preformed nitrosyl−heme complexes and also by protoporphyrin IX (lacking iron) (Ignarro et al., 1982b). This last observation led to the speculation that the nitrosyl complex formed on sGC is 5-coordinate and thus structurally resembled protoporphyrin IX. Upon binding of ·NO to the heme iron, the *trans*-axial ligand is believed to dissociate from the heme. The release of this axial ligand and the changes in porphyrin structure were postulated to mediate a global conformational change in the protein, which results in activation.

While the heme in sGC does not bind oxygen, it does bind ·NO. Electronic absorption and electron paramagnetic resonance spectral studies have indicated that this nitrosyl complex is, in fact, 5-coordinate (Stone & Marletta, 1994; Stone et al., 1995). This nitrosyl complex has previously been shown to form under conditions typically used to assay for activation of the enzyme *in vitro* (Stone et al., 1995). In this report, electronic absorption spectra and cGMP determinations were obtained simultaneously on a single sample, before and after the addition of ·NO. This experiment demonstrates that the 5-coordinate nitrosyl form of the enzyme is activated relative to the 5-coordinate resting state of the enzyme. Also, the kinetics of binding of ·NO to the heme of sGC have been investigated using stopped-flow spectrophotometry. The binding of ·NO to the heme is a complex process which involves the interaction of ·NO with a non-heme site on the protein.

MATERIALS AND METHODS

Materials. ·NO (99.0%) was obtained from Matheson. sGC was purified from bovine lung in a state containing 1.5 equiv of heme per heterodimer as described previously (Stone & Marletta, 1995a). All other materials unless otherwise stated were obtained from Sigma.

Stopped-Flow Kinetics. sGC (0.4 μM heme) in 25 mM TEA, 5 mM DTT, 50 mM NaCl, pH 7.4, under argon was rapidly mixed with an equal volume of a solution of ·NO (0.5−5.0 μM) containing 25 mM TEA, 50 mM NaCl, pH 7.4, under argon. In preparing the ·NO solutions, ·NO was first bubbled through saturated KOH to remove higher oxides of nitrogen. The buffer was made anaerobic by flushing with argon in a tonometer. ·NO was then introduced into the tonometer with a gas-tight syringe to give a partial pressure in the headspace that would result in the desired concentration in solution. The ·NO concentrations were determined using a solubility of 0.007560 g of ·NO per 100 g of H_2O at 10 °C when the partial pressure of ·NO in the headspace is 760 mmHg (Young, 1981). Data were acquired on a Hi-Tech Scientific SF-61 stopped-flow spectrophotometer controlled by KISS software (Kinetic Instruments Inc., Ann Arbor, MI) at 10 °C. The binding of ·NO to the heme was monitored at several different wavelengths between 399 and 434 nm. In all stopped-flow experiments, a spectrum was taken after equilibrium was established. For each set of experimental conditions, the experiment was performed in triplicate, and the resulting traces were averaged. The final traces were then fit to parallel exponentials using KISS software. A model was devised to explain the binding of ·NO to the heme of sGC. Using this model and the software Program A (Professor David Ballou, University of Michigan), simulations of the stopped-flow traces were obtained.

Native Molecular Mass of sGC. Determination of the native molecular mass of the protein was performed on a BioLogic Chromatography System (Bio-Rad) equipped with a 15 cm x 8 mm Toso Haas QC-PAK TSK GFC 300 GL gel-filtration column at 4 °C. The mobile phase was 25 mM TEA, 50 mM NaCl, and 5 mM DTT, pH 7.4, with a flow rate of 1 mL/min at a pressure of 550 psi. The sample injection volume was 50 μL. The standards used were apoferritin (443 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), and egg albumin (45 kDa). All standards were 1 mg/mL and were detected by the absorbance at 280 nm. For sGC (0.2 μM), the resting enzyme was detected by the absorbance at 436 nm, and the enzyme as the ferrous−nitrosyl complex was detected by the absorbance at 405 nm. The nitrosyl complex was formed by placing enzyme under an atmosphere of 1% ·NO in argon and incubating at 4 °C with agitation for 20 min. For both forms of sGC, the experiment was performed in triplicate, and the resulting chromatograms were averaged.

Dependence of Specific Activity on [·NO]. Buffer (95 μL) containing 50 mM TEA, 1.5 mM GTP, 4.5 mM $MgCl_2$, and 2 mM DTT at pH 7.4 was placed in septum-sealed 1.5 mL conical tubes and bubbled for 20 min with argon. The tubes were incubated at 10 °C for 8 min, and then ·NO gas was injected with a gas-tight syringe to give a particular partial pressure in the headspace and hence a particular concentration in solution based on the solubility listed above. The solutions were mixed with a vortex and incubated for an additional 2 min to allow for equilibration of the ·NO. The reaction was initiated with 5 μL of sGC (15 μg/mL). After 10 min, the reaction was quenched by the addition of 400 μL of 125 mM zinc acetate and 500 μL of 125 mM sodium carbonate. The concentration of cGMP in the quench mixture was then determined using a radioimmunoassay (Amersham), following the procedure supplied by the manufacturer.

Correlation of Spectra with Activity. sGC (160 nM) in 450 μL of 25 mM TEA, 5 mM DTT, 50 mM NaCl, pH 7.4, was placed in a septum-sealed cuvette under argon at 10 °C. The electronic spectrum was recorded on a Cary 3E spectrophotometer, and then the reaction was initiated by the addition of 100 μL of a cold anaerobic solution of 25 mM TEA, pH 7.4, containing 6.75 mM GTP and 20.25 mM $MgCl_2$. At 2 min intervals, 10 μL aliquots were removed and quenched with 90 μL of water, 400 μL of 125 mM zinc acetate, and 500 μL of 125 mM sodium carbonate. Just after the removal of each aliquot, the electronic absorption spectrum of the remaining sample was recorded. After 8 min, ·NO gas was added to the headspace so as to give 6 μM in solution. The cuvette was agitated briefly for mixing, and then the removing of aliquots and obtaining of spectra were continued for an additional 10 min. The concentrations

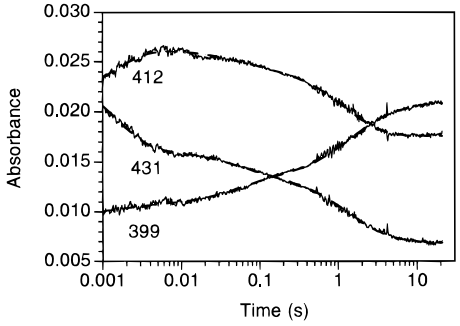

FIGURE 1: Stopped-flow monitoring of the binding of •NO to sGC. The binding of •NO (5.0 μM final) to sGC (0.2 μM final) was followed at three different wavelengths: 431 nm, 412 nm, and 399 nm; original data (—), 4-exponential fit (- - -).

of cGMP in the quench mixtures were then determined using a radioimmunoassay (Amersham), following the procedure supplied by the manufacturer.

RESULTS

Stopped-Flow Kinetics. Spectra taken after the stopped-flow experiments indicated that •NO shifted the Soret band from 431 to 399 nm (data not shown) as previously reported (Stone & Marletta, 1994; Stone et al., 1995). Stopped-flow traces for one concentration of •NO (5 μM) at three different wavelengths are shown in Figure 1. The three traces were each fit to four parallel exponentials. For each of the four phases, the three resulting values for the rate constants were averaged, yielding the following values: $k_1 = 700 \pm 100$ s^{-1}, $k_2 = 20 \pm 6$ s^{-1}, $k_3 = 0.79 \pm 0.11$ s^{-1}, and $k_4 = 0.20 \pm 0.09$ s^{-1}. The three traces in Figure 1 have been refit using these averaged values for the rate constants. The fourth (slow) phase was found not to be reproducible from preparation to preparation. Furthermore, the magnitude of the absorbance change of this phase (A_4) was $\leq$5% of the total absorbance change at 431 nm. Thus, the fourth phase is considered to be artifactual, probably arising from a small and variable population of denatured protein, and consequently has not been considered further. Of particular note in Figure 1 is the direction of the first three absorbance changes (A_1, A_2, A_3) at the three different wavelengths. At 431 nm, the absorbance decreases for all three phases, and at 399 nm, the absorbance increases for all three phases. However, at 412 nm, the absorbance increases for the first phase and decreases for the second two phases. The parameters for the fits in Figure 1 indicated that the ratio of the absorbance change for the third phase over that of the second phase (A_3/A_2) is constant at different wavelengths. Using five different wavelengths between 399 and 434 nm, this ratio was determined to be 2.6 ± 0.5 (data not shown).

To better understand the nature of this binding process, the concentration of •NO was varied from 0.5 to 5.0 μM, and the binding was monitored at 431 nm. The three phases were observed at all concentrations of •NO. Furthermore, over this concentration range of •NO, the magnitudes of all three absorbance changes and the total absorbance change were constant. The ratios of the absorbance change for each phase over the total absorbance change were 54 ± 4% for A_1, 13 ± 2% for A_2, and 33 ± 3% for A_3. The rate constants for the first phase, k_1, and for the third phase, k_3, were observed to be dependent on the concentration of •NO (Figure 2). The rate constant for the second phase, k_2, was found not to vary with the concentration of •NO (data not shown) and was determined to be 20 ± 5 s^{-1}.

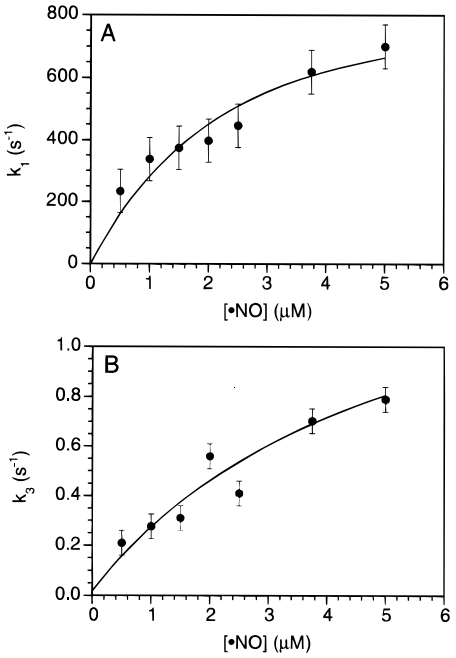

FIGURE 2: Dependence of rate constants on [•NO]. The values for the rate constants were obtained from fitting the original stopped-flow traces (●) and from fitting the computer-generated simulations (—). Panel A: The rate constant for the first kinetic phase (k_1). Panel B: The rate constant for the third kinetic phase (k_3).

Based on the above observations, a model was constructed to describe the binding of •NO to the heme of sGC (Figure 3). The model is based on the presence of two populations of heme. Both populations bind •NO through a two-step process to form a 6-coordinate complex. The transfer of •NO from the initial site of interaction to the heme is depicted as irreversible, although for the reverse process, rates as high as 1 s^{-1} had no effect on the simulations described below (data not shown). For the smaller population (28%), the 6-coordinate nitrosyl complex converts relatively rapidly to a 5-coordinate nitrosyl complex. For the larger population (72%), the conversion of the 6-coordinate nitrosyl complex to the 5-coordinate nitrosyl complex is much slower and is dependent upon the binding of •NO to a non-heme site on the protein. The stopped-flow traces at 431 nm were then simulated using extinction coefficients of 110, 64, and 25 mM^{-1} cm^{-1} for the 5-coordinate ferrous species, 6-coordinate ferrous nitrosyl species, and 5-coordinate ferrous nitrosyl species, respectively. Simulations based on the model at different •NO concentrations are shown in Figure 4. As designed, the model results in three phases with only the first and third phases being dependent on the concentration of •NO. In Figure 4B, the stopped-flow traces for two different concentrations of •NO at 431 nm have been superimposed with the respective simulations. The measured values for k_1 and k_3 have been plotted versus the concentration of •NO along with a continuum of values (curve) predicted from the model (Figure 2).

Native Molecular Mass of sGC. In order to determine if the binding of •NO to the heme of sGC alters the native mass of the enzyme, gel-filtration analysis was performed on both the ferrous and ferrous—nitrosyl forms of sGC. The gel-filtration chromatograms for the ferrous and ferrous—nitrosyl forms of sGC are shown in Figure 5. The retention

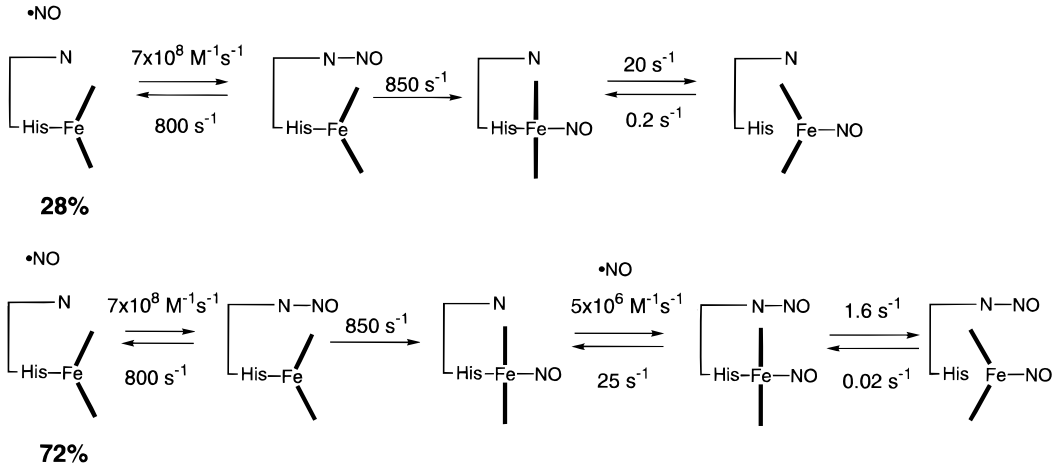

FIGURE 3: Proposed model for the binding of ·NO to sGC. The model is based upon the premise that there are two populations of enzyme, each with two distinct ·NO binding sites, a histidine-coordinated heme and a non-heme site (N). For both populations, ·NO first binds to the heme to form a 6-coordinate nitrosyl complex via a two-step binding process. The 6-coordinate nitrosyl complex then converts to a 5-coordinate nitrosyl complex through one of two ways. For 28% of the heme, the 6-coordinate nitrosyl complex converts relatively rapidly to the 5-coordinate complex. For the remaining 72% of the heme, the conversion of the 6-coordinate nitrosyl complex to a 5-coordinate nitrosyl complex is slow and is dependent upon the binding of ·NO to an unidentified non-heme site on the protein, which is not necessarily the same site used in the initial two-step binding to the heme.

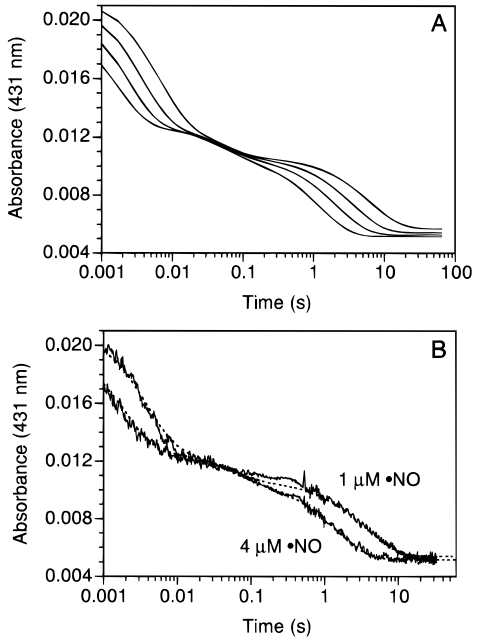

FIGURE 4: Computer simulations for the binding of ·NO to sGC. Panel A: The model in Figure 3 was used to simulate the stopped-flow traces. Depicted above are the computer simulations for four concentrations of ·NO: 0.5, 1, 2, and 5 μM. Panel B: Stopped-flow traces for the binding of ·NO to sGC at 431 nm with the two concentrations of ·NO shown: original data (—), computer simulation (- - -).

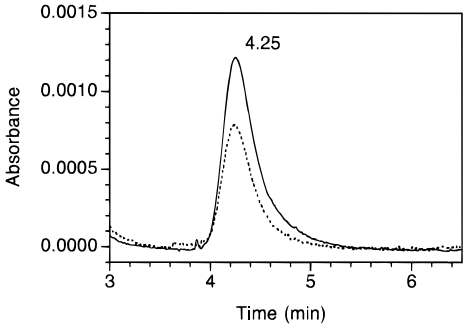

FIGURE 5: Determination of the native molecular mass of sGC. The native mass of sGC was determined by gel-filtration chromatography as described under Materials and Methods. Illustrated are the chromatograms for the ferrous enzyme (—) detected by the absorbance at 436 nm and the nitrosyl complex of sGC (- - -) detected by the absorbance at 405 nm. The retention time was 4.25 min for both forms of the enzyme.

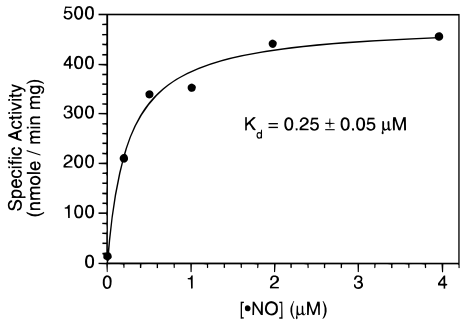

FIGURE 6: Dependence of the ·NO-activated specific activity on the concentration of ·NO. sGC was assayed as described under Materials and Methods. The data were fit to a standard saturation equation: specific activity = V_{max}[·NO]/(K_d + [·NO]). The value listed is the upper limit for the K_d of ·NO for activating sGC.

time is unaffected by the formation of the nitrosyl complex. The standards had retention times of 3.79 min (apoferritin), 4.25 min (β-amylase), 4.56 min (alcohol dehydrogenase), 4.68 min (bovine serum albumin), and 4.99 min (egg albumin). Construction of a standard curve (ln molecular mass vs retention time) allowed for the determination of an apparent native molecular mass for sGC of 200 kDa.

Dependence of Specific Activity on [·NO]. The specific activity of the enzyme was determined in the presence of varying concentrations of ·NO (Figure 6). The data were fit to a standard saturation equation, $V = V_{max}$[·NO]/(K_d + [·NO]), which assumes that the contribution of the basal rate to the measured activity can be ignored. The fit to the data yields an apparent K_d of 250 nM for the activation of sGC by ·NO.

Correlation of Spectra with Activity. Upon addition of ·NO to a sample of sGC, which was in the presence of GTP and Mg^{2+} and, therefore, expressing basal activity, the Soret shifted from 431 to 398 nm, indicating the formation of the

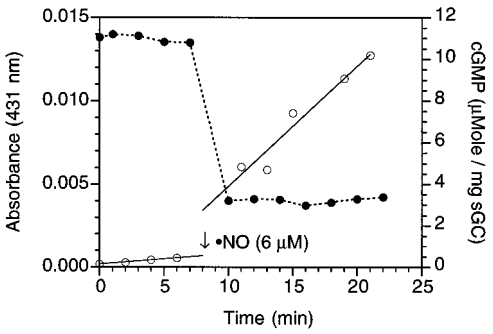

FIGURE 7: Correlation of spectra and activity. The absorbance at 431 nm was determined from the electronic absorption spectra and plotted versus time as was the concentration of cGMP. For the latter plot, the two parts were individually fit to lines by linear regression. The slopes yielded specific activities of 50 ± 3 and 577 ± 82 nmol min^{-1} mg^{-1} with a fold activation of 12 ± 2.

5-coordinate nitrosyl complex. Figure 7 depicts plots of the concentration of cGMP versus time and the absorbance at 431 nm versus time. Upon addition of ·NO, the absorbance at 431 nm decreases as the nitrosyl complex is formed. Coincident with nitrosyl complex formation, the rate of cGMP formation is increased from 50 ± 3 to 577 ± 82 nmol min^{-1} mg^{-1}.

DISCUSSION

During the binding of ·NO to the heme of sGC, three phases were observed (Figure 1). At 431 nm, the absorbance decreases for all three phases, and at 399 nm, the absorbance increases for all three phases, as expected for a binding process that shifts the Soret from 431 to 399 nm. However, at 412 nm the absorbance increases for the first phase and decreases for the next two phases. These observations are consistent with the formation of an intermediate which absorbs more strongly at 412 nm than at 431 or 399 nm. The most probable explanation for these results is that during the first phase, a 6-coordinate nitrosyl complex is being formed. 6-Coordinate nitrosyl complexes typically have Soret bands located in the range of 415−425 nm (Yoshimura & Ozaki, 1984). Thus, it appears that in the binding of ·NO to sGC, initially a 6-coordinate nitrosyl complex forms which then converts to a 5-coordinate nitrosyl complex. The wavelength independence of the ratio of the absorbance change for the third phase over that of the second phase (A_3/A_2) implies that both phases are associated with the same type of spectral change. The most reasonable conclusion is that both the second phase and the third phase are associated with the conversion of the 6-coordinate nitrosyl complex to the 5-coordinate nitrosyl complex. However, judging from the magnitude of A_3/A_2, about $72 \pm 4\%$ of the 6-coordinate complex converts to the 5-coordinate complex through phase 3, and $28 \pm 4\%$ converts through phase 2.

To better understand the nature of this binding process, the concentration of ·NO was varied from 0.5 to 5.0 μM, and the binding was monitored at 431 nm. As expected for an initial binding event, k_1 was found to be dependent on the concentration of ·NO (Figure 2). For the second phase, k_2 was found not to vary with the concentration of ·NO, as would be expected for a slow event occurring after the binding of ·NO. This rate constant was determined to be 20 ± 5 s^{-1}. However, k_3 did show a dependence on the concentration of ·NO (Figure 2). The observation that k_3 is dependent on the concentration of ·NO indicates that, as in the first phase, this third phase also involves the binding of ·NO. However, as described above, the wavelength independence of the ratio A_3/A_2 indicates that both phase 2 and phase 3 are involved in the conversion of 6-coordinate nitrosyl heme to 5-coordinate nitrosyl heme. Since ·NO binds to the heme before phase 3, the ·NO concentration dependence of k_3 must result from the interaction of ·NO with a site other than the heme. By ICP atomic emission, the protein used in these experiments was found to contain neither copper nor iron in excess of the heme (unpublished observation). Thus, the identity of this second ·NO binding site is unknown.

An alternative explanation for the ·NO dependence of k_3 would be that there are two populations of enzyme: one that binds ·NO through k_1 and then converts to the 5-coordinate nitrosyl through k_2, and one that binds ·NO through k_3 followed by a more rapid conversion to the 5-coordinate species. However, if this were occurring, the ratio $A_3/(A_1 + A_2)$ would be independent of wavelength, which is not the case. This ratio is equal to +0.5 at 431 nm, −1.6 at 412 nm, and +1.6 at 399 nm (data not shown). Thus, this model seems unlikely.

A model that can be used to simulate the binding of ·NO to sGC is shown in Figure 3. The model is based on the presence of two populations of heme. Both populations bind ·NO though a two-step process to form a 6-coordinate complex. For the smaller population (28%), the 6-coordinate nitrosyl complex initially formed converts relatively rapidly to a 5-coordinate nitrosyl complex. For the larger population (72%), the conversion of the 6-coordinate nitrosyl complex to the 5-coordinate nitrosyl complex is much slower and is dependent upon the binding of ·NO to a non-heme site on the protein. This model is based on the following observations made during the stopped-flow experiments: (1) k_1 and k_3 are dependent on the concentration of ·NO; k_2 is not; (2) the ratio A_3/A_2 is independent of wavelength; (3) at 412 nm, A_1 is positive while A_2 and A_3 are negative, and (4) A_1, A_2, A_3, and A_{tot} are independent of [·NO] between 0.5 and 5 μM. This last observation necessitated the employment of two-step binding of ·NO to the heme in forming the 6-coordinate nitrosyl complex. If models employing one-step binding were used, A_1 was found to vary with the concentration of ·NO, or else the simulation-derived values for k_1 were too small at the lower concentrations of ·NO. This model can be used to accurately simulate the stopped-flow traces (Figure 4). However, it should be emphasized that this is just one model consistent with the data, and it does not rule out the existence of other models that may also be consistent with the data. For example, the model put forward here implies the binding of ·NO to a non-heme site to form a stable complex, but the dependence of k_3 on [·NO] could also be explained by the oxidation or reduction of a non-heme site by ·NO. Also, the values for the rate constants in Figure 3 comprise one set of values that allows this model to simulate the data. However, other sets of constants may be obtained that serve this task.

Previously, it had been assumed that dissociation of ·NO from the heme would be too slow to account for physiological deactivation of the enzyme (Stone & Marletta, 1994). We have observed that ligands such as CO and nitrosomethane, which form 6-coordinate complexes with the heme, have dissociation rate constants from sGC that are

several orders of magnitude greater than those for typical 5-coordinate high-spin hemoproteins (Stone & Marletta, 1995b). Thus, it is reasonable to assume that the dissociation rate of ·NO from the intermediate 6-coordinate complex is also much faster than from typical high-spin ferrous hemoproteins. In the proposed model, the transfer of ·NO from the initial site of interaction to the heme is depicted as irreversible; although for the reverse process, rates as high as 1 s^{-1} had no effect on the simulations (data not shown). If this step is indeed $\sim$1 s^{-1}, then according to the model, the slow step for dissociation would be the reassociation of the histidine with the heme iron (for the major component of the heme population) at 0.02 s^{-1}. If this is the case, then the half-life for deactivation would be 35 s, consistent with the apparent deactivation of sGC observed in studies employing whole organs (Palmer et al., 1987). Thus, dissociation can no longer be assumed to be too slow to account for physiological deactivation of the enzyme. The direct measurement of the dissociation rate of ·NO from the heme of sGC is clearly an important future experiment.

The nature of the difference between the two heme populations in the model is unclear. Since the enzyme used in these studies contained 1.5 equiv of heme per heterodimer, it is possible that the major population represents heme in one of the subunits (fully saturated) and the minor population corresponds to heme in the other subunit (half saturated). Alternatively, the major population could correspond to the heme in enzyme containing 2 equiv of heme per heterodimer, and the minor population to heme in enzyme containing 1 equiv of heme per heterodimer. Further studies are required to address these issues.

Cytochrome *c′* is a hemoprotein with ligand binding properties similar to sGC. The binding of CO to certain isoforms of ferro-cytochrome *c′* is a complex process. To explain the complex kinetics, it was initially suggested that an equivalent of CO was binding to a non-heme site (Cusanovich & Gibson, 1973). Later it was discovered that the complex kinetics could be explained by taking into account a dimer-to-monomer transition that occurred upon the binding of CO to the heme (Kassner, 1991). To elucidate whether ·NO was changing the oligomerization state of sGC, the native molecular mass was determined for the resting 5-coordinate ferrous enzyme and for the 5-coordinate nitrosyl form of the enzyme. Both were found to have an apparent native molecular mass of $\sim$200 kDa, in general agreement with the reported values, which have ranged from 150 to 270 kDa (Waldman & Murad, 1987).

How the activity of the enzyme varies with the ·NO concentration is a central question in cellular signaling with ·NO. According to the stopped-flow experiments, complete nitrosyl complex formation was observed with as little as 500 nM ·NO. Determination of the specific activity with varying concentrations of ·NO yielded an apparent K_d of 250 $\pm$ 50 nM for the activation of sGC by ·NO (Figure 6). The peak physiological concentration of ·NO used for signaling purposes is believed to be 100−500 nM (Malinski & Taha, 1992). Thus, the value of 250 nM is in the physiological range. However, since it was necessary to preincubate the buffer with ·NO for 2 min to allow equilibration before the addition of the enzyme, this value must be viewed as an upper limit for the K_d because it is unclear how much ·NO decomposed before the addition of sGC. Decomposition would occur primarily through the reduction of ·NO by DTT, but could also occur through trace-metal-catalyzed oxidation of ·NO involving trace amounts of contaminating oxygen. Thus, the K_d for ·NO activating sGC is $\leq$250 nM at 10 °C. This agrees with the observation that during the stopped-flow experiments, the amount of 5-coordinate nitrosyl complex formed was constant between 0.5 and 5 μM ·NO.

It is clear from these experiments that ·NO is a very potent activator of the enzyme. From electronic absorption and electron paramagnetic resonance spectral studies, it has been shown that ·NO forms a 5-coordinate complex on the enzyme (Stone & Marletta, 1994; Stone et al., 1995), and that this complex forms under conditions typically used to assay for activation of the enzyme *in vitro*: 25 mM TEA, 5 mM DTT, 5 mM $MgCl_2$, 1.5 mM GTP, at pH 7.4 and 37 °C (Stone et al., 1995). However, in that latter experiment the enzyme was not assayed simultaneously with the collection of spectra. Because of the high enzyme concentration, the substrate would be depleted before a rate could be determined using the discontinuous radioimmunoassay. To circumvent this problem, the total activity was reduced by using the least amount of enzyme for which useful spectra could still be obtained and by performing the experiment at 10 °C. Upon the addition of ·NO, the spectral change observed is the same as previously seen upon the addition of ·NO to sGC (Stone & Marletta, 1994; Stone et al., 1995) and indicates the formation of a 5-coordinate nitrosyl complex. Figure 7 depicts plots of the concentration of cGMP versus time and the absorbance at 431 nm versus time. Upon the addition of ·NO, the absorbance at 431 nm decreases as the nitrosyl complex is formed. Coincident with nitrosyl complex formation, the rate of cGMP formation is enhanced. Thus, the formation of a 5-coordinate nitrosyl complex is correlated with an increase in specific activity.

There are two peculiarities in this experiment that need to be addressed. First, there is an apparent burst of activity after the addition of ·NO. For example, when the concentration of cGMP at $t = 8$ min was calculated from the data in Figure 7, the basal rate gave 0.54 $\pm$ 0.02 (μmol/mg sGC $\pm$ SD) while that after ·NO addition was 2.74 $\pm$ 0.71. This reproducible burst is of variable intensity but only occurs when ·NO gas is added directly to enzyme that is turning over. A burst has never been detected when enzyme is added to buffer containing ·NO. Thus, the burst may be an artifact arising from the interaction of decomposition products of ·NO (such as $\cdot NO_2$) with the enzyme. However, it is possible that there may be a short-lived enzyme species that has a higher specific activity than the one which is being monitored spectrally. The second peculiarity is the low fold activation. In general, lower fold activations were obtained at 10 °C than at 37 °C. This is true even for the experiments performed in the conical tubes, where a 50-fold activation was observed at 10 °C (Figure 6) and a 400-fold activation was observed at 37 °C (Stone & Marletta, 1995a). Thus, the basal rate may be less temperature-dependent than the ·NO-activated rate. Furthermore, the fold activations were lower when incubating in a cuvette at 10 °C than when incubating in 1.5 mL conical tubes at the same temperature. This appears to be due to variable basal rates in these experiments. While the basal rate was consistently 7−15 nmol min^{-1} mg^{-1} in 1.5 mL conical tubes, in the cuvette the basal rate ranged from 30 to 120 nmol min^{-1} mg^{-1}. Whether this is due to the surfaces of the cuvette, the increased protein concentration, or the irradiation with UV/

visible light is at present unclear. However, the specific activity of the 5-coordinate nitrosyl form of sGC determined in Figure 7 is typical of the specific activities obtained when assaying ·NO-activated enzyme at 10 °C in conical tubes where the fold activation is much higher. Thus, it is reasonable to conclude that the 5-coordinate nitrosyl complex, which is the predominant form of the enzyme upon the addition of ·NO, is significantly activated over the resting 5-coordinate ferrous form of the enzyme.

All of the observations made here are consistent with the original proposal for the mechanism of activation of the enzyme by ·NO (Ignarro et al., 1984), namely, that ·NO activates sGC by binding directly to the heme to form a 5-coordinate nitrosyl−heme complex. However, it cannot be ruled out that there are other coordination/redox states of the heme which result in even higher specific activities than that of the 5-coordinate ferrous nitrosyl complex. One candidate would be the 6-coordinate nitrosyl complex which forms transiently during the binding process. Also, it cannot be ruled out that the binding of ·NO to other sites on the protein besides the heme influences the activity of the enzyme as well.

In summary, the binding of ·NO to the heme of sGC is a complex process. ·NO first binds to the heme to form a 6-coordinate nitrosyl complex, which then converts to a 5-coordinate nitrosyl complex through one of two ways. For 28 ± 4% of the heme, the 6-coordinate nitrosyl complex converts relatively rapidly (∼20 s^{-1}) to the 5-coordinate complex. For the remaining 72 ± 4% of the heme, the conversion of the 6-coordinate nitrosyl complex to a 5-coordinate nitrosyl complex is slow (0.1−1.0 s^{-1}) and is dependent upon the interaction of ·NO with an unidentified non-heme site on the protein. Correlation of electronic absorption spectra with activity measurements indicates that the 5-coordinate nitrosyl form of the enzyme is activated relative to the resting 5-coordinate ferrous form of the enzyme. How the formation of this 5-coordinate nitrosyl complex results in activation of the enzyme is currently under study.

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