The Ferrous Heme of Soluble Guanylate Cyclase: Formation of Hexacoordinate Complexes with Carbon Monoxide and Nitrosomethane†

James R. Stone‡ and Michael A. Marletta*,‡,§

Department of Biological Chemistry, School of Medicine, and Interdepartmental Program in Medicinal Chemistry, College of Pharmacy, University of Michigan, Ann Arbor, Michigan 48109-1065

Received July 10, 1995; Revised Manuscript Received October 16, 1995⊗

ABSTRACT: The soluble form of guanylate cyclase (sGC) is the only definitive receptor for the signaling agent nitric oxide (·NO). The enzyme is a homologous heterodimer in which each subunit binds 1 equiv of 5-coordinate high-spin heme. ·NO increases the V_{max} of sGC up to 400-fold, probably by binding directly to the heme. Carbon monoxide (CO) forms a 6-coordinate complex with the heme and weakly activates the enzyme. Using stopped-flow spectrophotometry, the on-rate and off-rate for the binding of CO to the heme have been determined to be $(3.58 \pm 0.15) \times 10^4$ M^{-1} s^{-1} and 3.5 ± 0.5 s^{-1}, respectively, at 10 °C. The equilibrium dissociation constant (K_d) has been independently determined to be 97 ± 9 μM. Comparison of this K_d with that calculated from the rate constants indicates that the binding of CO to sGC is a simple one-step process, in which the off-rate of CO from the hexacoordinate complex is much faster than typically found in hemoproteins. The K_d of CO for activating the enzyme was also determined and compared to that for binding to the heme. Nitrosomethane forms irreversible complexes with typical ferrous hemoproteins but was observed to bind reversibly to the heme in sGC, with an off-rate $\geq(7.6 \pm 0.2) \times 10^{-3}$ s^{-1}. In general, the ferrous heme in sGC has a low affinity for ligands that form 6-coordinate complexes due primarily to fast ligand off-rates.

The guanylate cyclases are a family of enzymes that catalyze the cyclization of guanosine 5′-triphosphate (GTP)[1] to guanosine 3′,5′-cyclic monophosphate (cGMP) (Garbers & Lowe, 1994; Waldman & Murad, 1987). There are two general classes of the cyclase, particulate guanylate cyclase (pGC) and soluble guanylate cyclase (sGC). Isoforms of pGC are activated by peptide ligands that bind to an extracellular receptor domain (Garbers & Lowe, 1994), while sGC serves as the predominant if not the only receptor for the novel signaling agent nitric oxide (·NO) [for recent reviews, see Bredt and Snyder (1994), Marletta (1993, 1994), and Nathan (1992)]. In the presence of ·NO, the V_{max} of sGC is increased up to 400-fold. 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. The enzyme contains iron−protoporphyrin IX (heme b) as a cofactor, with a stoichiometry of two per heterodimer (Stone & Marletta, 1995). The two subunits of the heterodimer are homologous, and each subunit is believed to bind 1 equiv of heme. The heme is 5-coordinate high spin in both the ferrous and ferric states with histidine as the sole axial ligand (Stone & Marletta, 1994). The heme readily forms a complex with ·NO, and electronic absorption and electron paramagnetic resonance spectral studies have indicated that this nitrosyl complex is 5-coordinate (Stone & Marletta, 1994; Stone et al., 1995). It was originally speculated that activation of sGC by ·NO was a direct result of the formation of a 5-coordinate nitrosyl−heme complex (Ignarro et al., 1984). The severing of the bond to the *trans*-axial ligand and the resulting changes in porphyrin structure were postulated to mediate a conformational change to the protein, resulting in activation of the enzyme. This mechanism of activation is consistent with all published observations (Stone et al., 1995).

Carbon monoxide (CO) has been shown to bind to the heme of sGC to form a 6-coordinate complex (Gerzer et al., 1981; Stone & Marletta, 1994). At high concentrations, CO also activates purified sGC containing 1 heme per heterodimer by about 4-fold, in comparison to the 130-fold activation achieved with ·NO (Stone & Marletta, 1994). Analogous to the activation of sGC by ·NO, CO has been proposed to serve as an endogenous mediator of intercellular communication (Marks, 1994; Verma et al., 1993). In this report, the on-rate and off-rate for the binding of CO to the heme of sGC have been determined using stopped-flow spectrophotometry. In addition, the equilibrium dissociation constant (K_d) has been independently determined. Comparison of this K_d with that calculated from the rate constants indicates that the binding of CO to sGC is a simple one-step process in which the off-rate of CO from the hexacoordinate complex is much faster than typically found in hemoproteins. The K_d of CO for activating the enzyme was also determined and compared to that of CO for binding to the heme.

Nitrosomethane (CH_3NO) typically binds to high-spin ferrous hemoproteins to form a 6-coordinate complex (Mahy & Mansuy, 1991; Mansuy et al., 1977a,b). The off-rate of

† This work was supported by the Hitchings Award of the Burroughs-Wellcome Fund and by NIH Grant T32 GM07863.

* Author to whom correspondence should be addressed at the College of Pharmacy, 428 Church St., University of Michigan, Ann Arbor, MI 48109-1065.

‡ Department of Biological Chemistry.

§ Interdepartmental Program in Medicinal Chemistry.

⊗ Abstract published in *Advance ACS Abstracts,* December 1, 1995.

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

0006-2960/95/0434-16397$09.00/0

nitrosomethane from the ferrous heme in these complexes is so slow that the binding is considered to be irreversible. In this report, the interaction of nitrosomethane with the heme of sGC has been investigated. Nitrosomethane binds in a reversible manner to the ferrous heme of sGC and is only the third ligand known to bind to this ferrous heme.

MATERIALS AND METHODS

Materials. CO was obtained from Liquid Carbonic Specialty Gases (Chicago, IL). All other materials unless otherwise stated were obtained from Sigma.

Purification of sGC from Bovine Lung. sGC was purified from bovine lung in a state containing 1.5 equiv of heme per heterodimer as described previously (Stone & Marletta, 1995).

Stopped-Flow Kinetics. sGC (0.75 μM heme) in 25 mM TEA, 5 mM DTT, and 50 mM NaCl, pH 7.4, under air was rapidly mixed with an equal volume of the same buffer containing varying concentrations of CO. The CO solutions were prepared by bubbling buffer for 30 min with a CO/N_2 mixture. The concentration of CO in solution was determined using a solubility of 0.003479 g of CO per 100 g per H_2O at 10 °C when the partial pressure of CO in the headspace is 760 mmHg (Dean, 1992). The gas mixtures were prepared using a 150 mm Gas Proportioner equipped with high-resolution valves (Air Products). Data were acquired on a HI-TECH Scientific SF-61 stopped-flow spectrophotometer controlled by KISS software (Kinetic Instruments, Ann Arbor, MI) at 10 °C. The binding of CO to the heme was monitored as an increase in the absorbance at 423 nm. For each CO concentration, the experiment was performed in triplicate, and the resulting traces were averaged. The final traces were then fit to a single exponential using KISS software.

Measurement of the K_d of CO for Binding to the Heme. Upon completion of each stopped-flow experiment, a spectrum was obtained of the equilibrium mixture. For each concentration of CO, the final spectrum had subtracted from it a spectrum of sGC mixed with buffer lacking CO. These difference spectra were used to determine ΔAbs(440), ΔAbs(423), and subsequently ΔΔAbs(423−440). A plot of ΔΔAbs(423−440) versus the CO concentration was fit with a typical saturation function, $\Delta\Delta Abs = \{\Delta\Delta Abs(max) \times [CO]\}/\{K_d + [CO]\}$, to determine the K_d of CO for binding to the heme.

Determination of the K_d of CO for Activating sGC. Buffer (95 μL) containing 50 mM TEA, 1.5 mM GTP, 4.5 mM $MgCl_2$, and 2 mM DTT, pH 7.4, was placed in a septum-sealed conical tube and bubbled for 20 min with a mixture of CO and N_2 so as to give a particular partial pressure of CO in the headspace and hence a particular concentration in solution based on the solubility stated above. The CO/N_2 mixtures were prepared as described above. The tubes were incubated at 10 °C for 10 min, and then 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. The resulting data were fit with the equation below for nonessential activation as described by Segel (1975).

$$V_{max}(app) = \frac{V_{max}(basal)\{1 + \beta[CO]/\alpha K_d\}}{1 + [CO]/\alpha K_d}$$

$V_{max}(app)$ is the apparent V_{max} at a particular concentration of CO; $V_{max}(basal)$ is the V_{max} of the unactivated enzyme (basal rate); β is the ratio of the V_{max} at saturating CO over the V_{max} of the unactivated enzyme (fold activation); α is the ratio of the K_m for GTP−Mg^{2+} in the presence of saturating CO over the K_m for GTP−Mg^{2+} in the absence of CO; and K_d is the equilibrium dissociation constant of CO for activating the enzyme. The values for K_m for GTP−Mg^{2+} for the unactivated enzyme and for the CO-activated enzyme were determined by incubating sGC (0.75 μg/mL) at 10 °C for 10 min in 50 mM TEA, pH 7.4, 2 mM DTT, and 3.0 mM $MgCl_2$ with variable concentrations of GTP−Mg^{2+} under either N_2 or CO. The incubations were quenched and assayed for cGMP as described above.

Binding of Nitrosomethane to sGC. The binding of nitrosomethane to sGC was monitored on a Cary 3E spectrophotometer at 10 °C. Nitrosomethane was generated *in situ* by reduction of nitromethane with dithionite (Mansuy et al., 1977a). Ferrous sGC (0.9 μM heme) in 25 mM TEA, 50 mM NaCl, and 5 mM DTT, pH 7.4, was placed in a septum-sealed cuvette under argon. The electronic absorption spectrum was recorded before and after the addition of 20 mM nitromethane. Upon subsequent addition of 10 mM dithionite, the electronic spectrum was recorded at 2 min intervals for 30 min. Each spectrum recorded after the addition of dithionite had subtracted from it the spectrum taken 30 min after the addition of dithionite. These difference spectra were used to determine ΔAbs(350), ΔAbs(438), ΔAbs(423), and subsequently ΔΔAbs(423−438). The natural log of ΔΔAbs(423−438) was then plotted versus time to determine a lower limit for the off-rate of nitrosomethane.

Protein Determination. Protein concentrations were determined with the Bradford microassay (Bio-Rad) using BSA as the standard. The assay was calibrated for sGC using quantitative amino acid analysis as described previously (Stone & Marletta, 1994).

RESULTS

Stopped-Flow Kinetics. Two representative traces for the binding of CO to sGC are shown in Figure 1. The total absorbance change is not the same for the two concentrations of CO because the binding site is not being saturated. Also for each trace in Figure 1, a single-exponential fit has been superimposed on the data. From the single-exponential fits of the data, pseudo-first-order (observed) rate constants (k_{obs}) are obtained. A plot of these constants versus the concentration of CO is depicted in Figure 2. The plot is linear, consistent with a simple one-step binding mechanism. The slope of the line, $(3.58 \pm 0.15) \times 10^4\ M^{-1}\ s^{-1}$, is the k_{on} for CO, and the y-intercept, $3.5 \pm 0.5\ s^{-1}$, is the k_{off}. The k_{off} divided by the k_{on} (98 ± 15 μM) is a calculated value for the K_d for the binding process.

Measurement of the K_d of CO for Binding to the Heme. A representative example of a difference spectrum for the binding of CO to sGC is shown in Figure 3A. A plot of ΔΔAbs(423−440) versus CO concentration is shown in Figure 3B. Fitting the data with a typical saturation function,

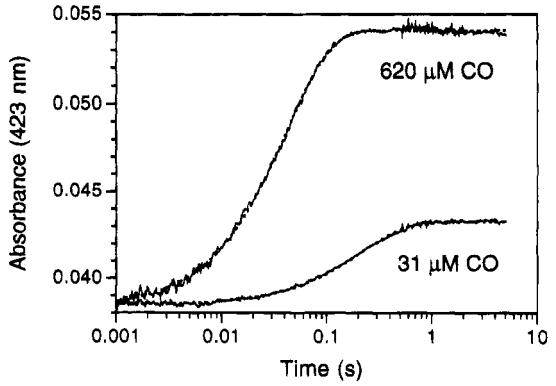

FIGURE 1: Stopped-flow monitoring of the binding of CO to sGC. Original data (—); single-exponential fit (- - -). For both concentrations of CO, the data and the fit are essentially superimposable. Experimental conditions were as described under Materials and Methods. The concentrations of CO shown are the final concentrations after mixing.

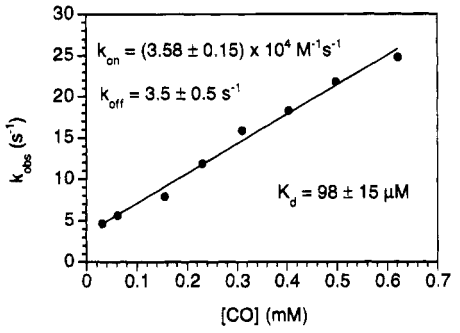

FIGURE 2: Plot of k_{obs} versus [CO]. The pseudo-first-order rate constants (k_{obs}) obtained from the single-exponential fits of the stopped-flow data were plotted versus the final concentrations of CO. The k_{on} is obtained from the slope, the k_{off} from the y-intercept, and the K_d from k_{off}/k_{on}.

$\Delta\Delta$Abs = $\{\Delta\Delta$Abs(max) × [CO]$\}/\{K_d$ + [CO]$\}$, yields a K_d of 97 ± 9 μM for the binding of CO to the heme of sGC.

Determination of the K_d of CO for Activating sGC. The specific activity of the enzyme was determined with varying concentrations of CO (Figure 4). Fitting the data with the equation for nonessential activation yields values for β and αK_d of 3.3 ± 0.3 and 36 ± 8 μM, respectively. In order to calculate the K_d for activation, α was also determined by measuring the K_m for GTP-Mg^{2+} in the presence and absence of 1.2 mM CO (Figure 5). The K_m was determined to be 83 ± 6 μM in the presence of CO and 55 ± 12 μM in the absence of CO, yielding a value for α of 1.51 ± 0.35. Thus, the K_d for activation of sGC by CO is 24 ± 8 μM.

Binding of Nitrosomethane to sGC. Nitrosomethane is unstable in aqueous solution, decomposing by tautomerization to the oxime (CH_2=NOH) and by dimerization (CH_3-(O)N=N(O)CH_3) (Mansuy et al., 1977a). Nitrosomethane can be formed in solution by the reduction of nitromethane (CH_3NO_2) with dithionite. Addition of 20 mM nitromethane did not alter the electronic absorption spectrum of ferrous sGC. However, subsequent addition of 10 mM dithionite caused the Soret to shift from 431 nm to 425 nm, and the single broad peak at 562 nm was replaced by a peak at 568 nm with a shoulder at 542 nm (Figure 6A). This final spectrum is consistent with the formation of a 6-coordinate nitrosomethane complex (Mansuy et al., 1977a). If the order of addition is reversed, and the dithionite is added first, the spectral change is not observed until the addition of nitromethane (data not shown). Figure 6B depicts the difference spectrum for the binding process, with a peak at 423 nm and a trough at 438 nm. The large absorbance change at 350 nm is due to dithionite (λ_{max} = 314 nm). Upon repetitive scanning of the sample at 2 min intervals, it was observed that the spectrum completely reverted to the original spectrum of the 5-coordinate ferrous enzyme within 30 min. Plots of ln $\Delta\Delta$Abs(423−438) versus time and ΔAbs(350) versus time are depicted in Figure 7. The former is a direct measure of the nitrosomethane−sGC complex, and the latter is a direct measure of the dithionite in solution. Under the conditions employed here, the nitrosomethane complex is stable for approximately 10 min, at which time the dithionite in solution is depleted by the reduction of excess nitromethane. After 10 min, the nitrosomethane concentration in solution would begin to fall as no additional nitrosomethane can be generated and that already formed

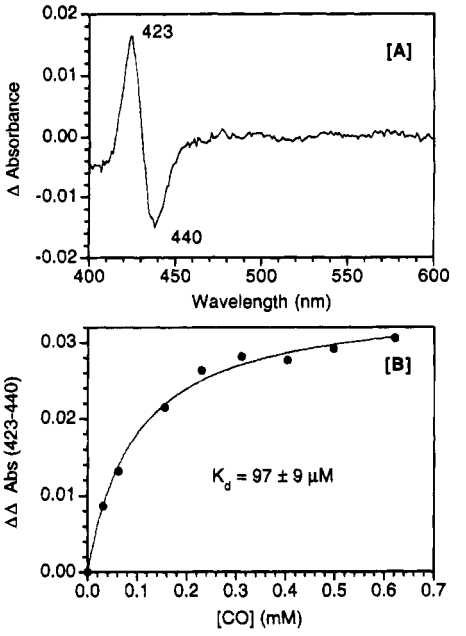

FIGURE 3: Spectral determination of the K_d for CO. Panel A: difference spectrum for the binding of CO to sGC. The electronic spectrum of ferrous sGC was subtracted from that of sGC in the presence of 620 μM CO. The peak at 423 nm and the trough at 440 nm are due to the shifting of the Soret band as CO binds to the heme. Panel B: plot of $\Delta\Delta$Abs(423−440) versus [CO]. The $\Delta\Delta$Abs(423−440) obtained from the difference spectra were plotted against the final concentration of CO. The data were fit with a standard saturation function, $\Delta\Delta$Abs = $\{\Delta\Delta$Abs(max) × [CO]$\}/\{K_d$ + [CO]$\}$, to determine the K_d.

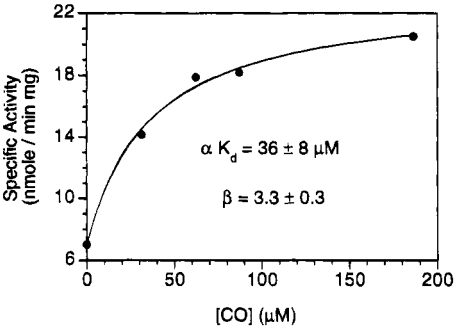

FIGURE 4: Activity of sGC with varying concentrations of CO. sGC was assayed as described under Materials and Methods. The data were fit with nonlinear regression to the equation for nonessential activation. The values are shown for the two parameters determined by the fit.

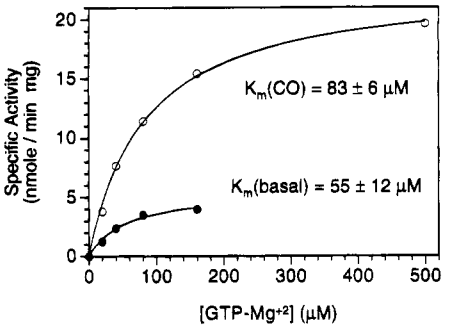

FIGURE 5: Determination of K_m values with and without CO. sGC was assayed as described under Materials and Methods under either nitrogen (●) or CO (○). The data were fit with nonlinear regression to a standard Michaelis–Menten equation: $V = (V_{max} \times [GTP-Mg^{2+}])/(K_m + [GTP-Mg^{2+}])$. The values displayed are the values for the K_m for GTP–Mg^{2+} with the two conditions used.

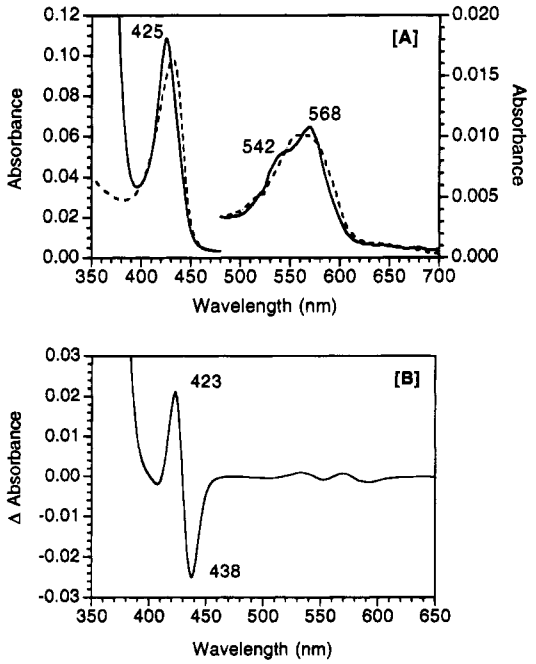

FIGURE 6: Spectral characterization of the nitrosomethane–sGC complex. Panel A: electronic absorption spectrum of the nitrosomethane–sGC complex. sGC (0.9 μM heme) in 25 mM TEA, 50 mM NaCl, 5 mM DTT, and 20 mM nitromethane, pH 7.4, was placed in a septum-sealed cuvette under argon. Spectra were recorded in the presence (—) and absence (- - -) of 10 mM dithionite. Panel B: electronic absorption difference spectrum for the binding of nitrosomethane to sGC. The electronic spectrum of ferrous sGC was subtracted from that of the nitrosomethane complex of sGC. The peak at 423 nm and the trough at 438 nm are due to the shifting of the Soret band as nitrosomethane binds to the heme.

decomposes as described above. Also, beginning at 10 min, the nitrosomethane complex with sGC begins to decompose at a rate that approaches $(7.6 \pm 0.2) \times 10^{-3}$ s^{-1}. This rate cannot be assumed to be the off-rate of nitrosomethane from the heme of sGC. This rate could, for example, represent the decomposition rate of nitrosomethane in solution, and the off-rate could actually be much faster than this. However, this rate can be used as a lower limit for the off-rate of nitrosomethane from the 6-coordinate complex with the heme of sGC.

DISCUSSION

The K_d of CO for binding to the heme of sGC was measured by two independent methods. The value obtained

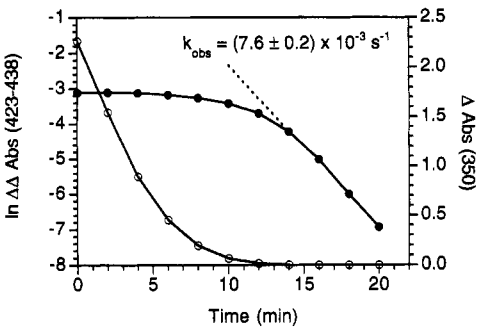

FIGURE 7: Decomposition of the nitrosomethane complex of sGC. The ln ΔΔAbs(423–438) (●) and the ΔAbs(350) (○) values determined from the difference spectra were plotted versus time. The last four points in the plot of ln ΔΔAbs(423–438) were fit to a line by linear regression. The slope of the line is the observed rate constant (k_{obs}) for the decomposition of the nitrosomethane–sGC complex.

Table 1: Comparison of Equilibrium and Kinetic Constants[a]

ligand	protein	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})	K_d
O_2	sGC			>1 mM
	Hb(R)/Mb			0.8–1.3 μM
	cytochrome *c*′			>1 mM
	FixL			30–140 μM
CO	sGC	3.6×10^4	3.5	98 μM
	Hb(R)/Mb	$(2–5) \times 10^5$	0.013–0.019	30–50 nM
	cytochrome *c*′	8–2600	0.0002–0.05	0.6–770 μM
	FixL	$(0.5–1.7) \times 10^4$	0.05–0.09	5–10 μM
CH_3NO	sGC		$\geq 8 \times 10^{-3}$	
	Hb(R)/Mb		$<10^{-7}$	

[a] Values for sGC are for 10 °C. Values for R-state Hb, Mb, cytochrome *c*′, and FixL are for 20–25 °C (Antonini & Brunori, 1971; Gilles-Gonzalez et al., 1994; Kassner, 1991).

by the ratio of k_{off}/k_{on} (98 ± 15 μM) was in excellent agreement with the value obtained from the spectra at equilibrium (97 ± 9 μM). The agreement of these two values is consistent with simple one-step binding of CO to the 5-coordinate heme to generate a 6-coordinate complex. However, the K_d is 3 orders of magnitude higher than the K_d of CO for typical 5-coordinate ferrous imidazole-ligated hemoproteins such as Hb and Mb (Table 1). Comparison of the kinetic constants for CO binding to sGC with those for the binding of CO to Hb and Mb indicates that most of the decrease in affinity is due to an extremely fast off-rate from the sGC 6-coordinate complex (Table 1).

To help gain an understanding of ligand binding processes in sGC, other ligands were sought which could bind to the ferrous heme. A rather obscure but logical choice was nitrosomethane (CH_3NO). In the presence of ferrous 5-coordinate hemoproteins such as Hb, Mb, cytochrome P_{450}, and cyclooxygenase, nitrosomethane binds to the heme to form a 6-coordinate complex (Mahy & Mansuy, 1991; Mansuy et al., 1977a,b). Interestingly, the off-rates of nitrosomethane from these complexes are so slow that the binding is considered to be irreversible. These protein–nitrosomethane complexes are stable for days under air and can be subjected to dialysis or gel filtration to remove any excess dithionite and the nitrosomethane decomposition products. Decomposition of the complex has only been observed upon the addition of ferricyanide, which oxidizes the heme to the ferric state promoting dissociation. Thus, due to the remarkable affinity of nitrosomethane for 5-coordinate ferrous hemoproteins, this ligand had potential for binding to the heme of sGC.

Nitrosomethane does bind to the ferrous heme of sGC to form a 6-coordinate complex (Figure 6). However, unlike the binding of nitrosomethane to other hemoproteins, the binding to sGC is reversible, with an off-rate $\geq(7.6 \pm 0.2) \times 10^{-3}$ s^{-1}. These results are similar to those observed for the binding of CO to the heme of sGC. CO and nitrosomethane have very different binding geometries. CO prefers to bind in a linear fashion to hemes with an Fe−C−O angle of ~180°. Nitrosomethane, on the other hand, binds through the nitrogen, resulting in an Fe−N−O angle of ~120°. However, despite the different binding geometries, both ligands have remarkably fast off-rates from the heme of sGC. This property of an enhanced off-rate may be a general feature of all ligands that bind to ferrous sGC to form 6-coordinate complexes.

Table 1 lists the kinetic and equilibrium constants for the binding of ligands to sGC at 10 °C as well as those for the binding to R-state Hb and Mb, cytochrome *c′*, and FixL at 20−25 °C. Cytochrome *c′* and FixL are two hemoproteins with spectral and ligand binding properties similar to those of sGC. The ligands listed in Table 1 have in common the property of forming 6-coordinate complexes with hemoproteins. For all of these ligands, sGC, cytochrome *c′*, and FixL have much reduced affinities compared to R-state hemoglobin and myoglobin. For CO, the reduced affinity for sGC is due primarily to an increased off-rate, while the reduced affinity of CO for cytochrome *c′* is due primarily to a reduced on-rate. For FixL, the reduction in affinity of CO is distributed more evenly to both kinetic constants. Also, for ferrous sGC, nitrosomethane has a much increased off-rate compared to Hb and Mb. Thus, sGC, cytochrome *c′*, and FixL are all 5-coordinate high-spin hemoproteins that have reduced affinities for ligands, which form 6-coordinate complexes. However, judging from the kinetic constants, it is unlikely that the low ligand affinities are a result of structural features that are common to all three hemoproteins. Instead, each protein may employ a unique strategy for reducing the affinity of exogenous ligands.

Recently a flash-photolysis study of the binding of CO to the heme of sGC was reported (Kharitonov et al., 1995). A k_{on} of $(1.2 \pm 0.1) \times 10^5$ M^{-1} s^{-1} and a k_{off} of 28 ± 2 s^{-1} were obtained for enzyme at 20 °C in 50% glycerol. No value was reported for the equilibrium dissociation constant. Considering the differences in experimental conditions, these values are in fairly good agreement with those reported here (Figure 2). Most notably, an unusually fast off-rate was observed. However, a very different mechanism for the binding process was postulated. The measured k_{on} was believed to represent the binding of CO to the 5-coordinate heme to form a 6-coordinate complex, as stated here. However, the measured k_{off}, which is faster than that typically found for 6-coordinate complexes, was believed to represent dissociation of CO from a minor population of 5-coordinate CO-coordinated heme. The dissociation of CO from the 6-coordinate complex was assumed to be slow (~10^{-2} s^{-1}), as is the case for typical hemoproteins. Thus, the hypothesis was put forward that the histidine must dissociate first from the heme before CO can dissociate. If all of the steps in this more complicated model are assumed to be reversible, then from derivations using first principles the K_d is approximately equal to the dissociation rate from the 6-coordinate complex divided by the on-rate. However, if the off-rate for CO from the 6-coordinate heme is similar to that for other hemoproteins, then the measured K_d of CO for sGC would be 10−100 nM, and this measured K_d would not be equal to the measured off-rate divided by the on-rate. However, as demonstrated above, the measured K_d is actually 100 μM and agrees quite well with k_{off}/k_{on}. Thus, a simple one-step binding model better describes the binding of CO to sGC than the more complicated model.

The main issue left unsettled in the binding of CO to sGC concerns the nature of the fast off-rate. In fact, it was this fast off-rate that prompted the speculation of a more complicated binding model. For most hemoproteins and heme model compounds, the off-rate of CO from the respective 6-coordinate complexes is 2−3 orders of magnitude slower than it is from sGC (Antonini & Brunori, 1971; Gilles-Gonzalez et al., 1994; Kassner, 1991; Rose et al., 1993). However, there is one model 6-coordinate compound, a "bis-handle" porphyrin, in which the off-rate is 3.5 s^{-1} at 20 °C, close to the rate observed for sGC (Rose et al., 1993). In this complex, the fast off-rate has been ascribed to an unusually weak base *trans* to the CO combined with steric hindrance on the distal side of the heme. Both of these factors are believed to contribute to the unusual ligand binding behavior of cytochrome *c′* (Finzel et al., 1985) and are suspected to play a role in sGC as well. For example, the ability of sGC to form a 5-coordinate nitrosyl−heme complex is consistent with a relatively weak Fe−His bond (Stone et al., 1995). Thus, the binding of CO to the heme of sGC can best be described as simple one-step binding in which the off-rate is faster than commonly observed in hemoproteins due to the unusual heme environment in sGC.

The ligand binding properties of sGC reported here should help clarify the interaction of the enzyme with the endogenous activator ·NO. From stopped-flow experiments, it is clear that the initial adduct with ·NO is 6-coordinate (Stone and Marletta, unpublished observations). In consideration of the fast off-rates for CO and nitrosomethane, it is reasonable to assume that the off-rate of ·NO from this initial complex is much faster than typically seen in hemoproteins. However, unlike the other ligands studied, ·NO eventually forms a 5-coordinate complex with the enzyme, and it is the severing of the Fe−His bond that is assumed to be the primary event responsible for the activation of the enzyme. Thus, to understand the interaction of ·NO with the heme, it is critical to know how the initial 6-coordinate complex converts to a 5-coordinate complex. Previously, it had been speculated that dissociation of ·NO from the ferrous heme would be too slow to account for physiological deactivation of the enzyme (Stone & Marletta, 1994). However, in light of the kinetic data reported here for CO and nitrosomethane, it can no longer be assumed that the off-rate of ·NO from the heme in sGC is too slow to account for deactivation.

The binding of nitrosomethane to the heme of sGC also raises more possibilities for the mode of interaction of nitrosothiols with this enzyme. Nitrosothiols are pharmacological vasodilators which have been speculated to serve a physiological role (Stamler, 1994), an idea currently lacking direct evidence. Nitrosothiols are believed to activate sGC *in vitro* via the homolytic cleavage of the N−S bond to generate ·NO. However, in light of the reversible binding of nitrosomethane to the heme of sGC observed here, it is quite possible that nitrosothiols can directly bind to the heme of sGC via the nitrogen to generate a 6-coordinate complex. Formation of the complex could then be followed by

homolytic cleavage of the N−S bond to eventually generate the 5-coordinate ferrous nitrosyl complex, which is believed to be the activated form of the enzyme. This mechanism might explain how these compounds could directly activate sGC.

Since CO activates purified sGC, the K_d for activation of the enzyme by CO was determined and compared to the K_d of CO for binding to the heme moiety. The kinetic model used to determine the K_d for activation of sGC by CO was that of nonessential activation described by Segel (1975). This model assumes a rapid equilibrium kinetic mechanism for the interaction of the substrate GTP−Mg^{2+} with sGC such that the K_m for GTP−Mg^{2+} is equal to the K_d for GTP−Mg^{2+}. The advantage of this model is that one can calculate the K_d for activation of sGC by CO simply by measuring the specific activity of the enzyme at several concentrations of CO and by determining two K_m values. Since the final objective was to compare this K_d to that determined by stopped-flow analysis, all enzyme incubations were performed at 10 °C, the same temperature as the stopped-flow experiments.

The K_d of CO for activating sGC was determined to be 24 ± 8 μM. This value differs from the values of 98 ± 15 μM and 97 ± 9 μM for the K_d of CO for binding to the heme. However, the values do not differ enough to warrant the conclusion that CO is activating the enzyme by binding to a site other than the heme. More likely there is a trivial explanation for the difference. The K_d for activation was determined in the presence of Mg^{2+} and GTP. When determining the K_d for binding to the heme, these agents were absent. It is possible that these two agents affect the affinity of CO for the heme of sGC. Another possibility is that the assumptions used in determining the K_d for activation are invalid. The primary assumption is that the interaction of sGC with GTP−Mg^{2+} can be described by a simple preequilibrium kinetic model in which the K_d for GTP−Mg^{2+} is equal to the K_m for GTP−Mg^{2+}. It may be necessary to employ a more complicated steady-state kinetic model for the interaction of sGC with its substrate. While further experiments are required to determine the nature of the difference between the two measurements for the K_d of CO, the values are close enough to be consistent with CO activating sGC by direct binding to the heme moiety.

It has been proposed that CO generated by heme oxygenase is an endogenous activator of sGC (Marks, 1994; Verma et al., 1993). The observations reported here would seem to indicate that this is unlikely, at least for the $\alpha_1\beta_1$ isoform of sGC. First, the specific activity of the CO-activated enzyme is 29 times lower than that of the ·NO-activated enzyme (Stone & Marletta, 1994). Second and probably more important, the physiological concentration of CO is probably <1 μM. However, the values obtained here for the K_d of CO for sGC range from 24 to 98 μM. Thus, it appears that the low concentrations of CO generated *in vivo* would have very little effect on the $\alpha_1\beta_1$ isoform of sGC. If anything, it appears as if the enzyme has evolved to be quite insensitive to all potential heme ligands except ·NO.

Ever since it was proposed that ·NO activates sGC by forming a 5-coordinate nitrosyl complex, it has generally been assumed that the structural change induced by ·NO, which is most responsible for activating the enzyme, is the severing of the bond between the iron and the *trans*-axial ligand (Ignarro, 1991), now known to be a histidine (Stone & Marletta, 1994). It has further been speculated that the histidine which ligates the iron becomes the catalytic base once released (Traylor & Sharma, 1992). This last proposal is unlikely simply due to the homology between the catalytic domains of sGC and the catalytic domains of pGC and adenylate cyclase, neither of which have been shown to bind heme (Garbers & Lowe, 1994). The first direct evidence against the catalytic-ligand theory was the observation that the purified protein could be activated by CO, which forms a 6-coordinate complex with the heme (Stone & Marletta, 1994). The proponents of the catalytic-ligand theory then suggested that a small population (~2%) of the CO-coordinated heme was actually 5-coordinate and that this small population was responsible for all of the observed increase in activity (Kharitonov et al., 1995). The unusually fast off-rate of CO from the heme was cited as evidence that this small population with a 5-coordinate CO complex existed. However, as discussed above, comparison of the equilibrium data with the kinetic data for CO binding indicates that the fast off-rate is not due to the formation of a small population of sGC with a 5-coordinate CO complex. Thus, based on the sequence and kinetic data, it is doubtful that the heme ligand is the catalytic base, and it appears that CO mildly activates sGC simply by binding to the heme to form a 6-coordinate complex.

The activation of sGC by CO raises an interesting question. If the primary structural change which results in activation is the severing of the Fe−His bond, then how can CO activate the enzyme even mildly by forming a 6-coordinate complex? Perhaps it is not the severing of the Fe−His bond that is important, but the geometry of the porphyrin plane. In the resting enzyme, the 5-coordinate heme should be puckered slightly toward the histidine, and the relative enzyme activity is 1. For the 6-coordinate complex with CO, the heme should be nearly flat, and the relative activity is 4. For the 5-coordinate nitrosyl complex, the heme should be puckered away from the histidine toward the nitrosyl group, and the relative activity is 130 (Stone & Marletta, 1994). Thus, the geometry of the porphyrin plane is probably the structural feature most responsible for regulating enzyme activity, not the coordination state of the iron.

In summary, the on-rate, off-rate, and K_d for the binding of CO to the heme of sGC are consistent with a simple one-step binding process. The K_d of CO for activating the enzyme is comparable to that of CO for binding to the heme. Nitrosomethane binds reversibly to the heme in sGC. In general, the ferrous heme in sGC has a low affinity for ligands that form 6-coordinate complexes, due primarily to fast ligand off-rates. How this unusual property of the heme contributes to the physiological activation of the enzyme by ·NO is currently under investigation.

ACKNOWLEDGMENT

We acknowledge Professor David Ballou (University of Michigan) for helpful discussions concerning stopped-flow spectrophotometry.

REFERENCES

Antonini, E., & Brunori, M. (1971) in *Hemoglobin and Myoglobin in Their Reactions with Ligands*, North-Holland Publishing Co., Amsterdam.

Bredt, D. S., & Snyder, S. H. (1994) *Annu. Rev. Biochem. 63*, 175−195.

Dean, J. A. (1992) in *Lange's Handbook of Chemistry*, McGraw-Hill, New York.
Finzel, B. C., Weber, P. C., Hardman, K. D., & Salemme, F. R. (1985) *J. Mol. Biol. 186*, 627−643.
Garbers, D. L., & Lowe, D. G. (1994) *J. Biol. Chem. 269*, 30741−30744.
Gerzer, R., Böhme, E., Hofmann, F., & Schultz, G. (1981) *FEBS Lett. 132*, 71−74.
Gilles-Gonzalez, M. A., Gonzalez, G., & Perutz, M. F. (1994) *Biochemistry 33*, 8067−8073.
Ignarro, L. J. (1991) *Biochem. Pharmacol. 41*, 485−490.
Ignarro, L. J., Wood, K. S., & Wolin, M. S. (1984) *Adv. Cyclic Nucleotide Protein Phosphorylation Res. 17*, 267−274.
Kassner, R. J. (1991) *Biochim. Biophys. Acta 1058*, 8−12.
Kharitonov, V. G., Sharma, V. S., Pilz, R. B., Magde, D., & Koesling, D. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 2568−2571.
Mahy, J. P., & Mansuy, D. (1991) *Biochemistry 30*, 4165−4172.
Mansuy, D., Chottard, J. C., & Chottard, G. (1977a) *Eur. J. Biochem. 76*, 617−623.
Mansuy, D., Gans, P., Chottard, J. C., & Bartoli, J. F. (1977b) *Eur. J. Biochem. 76*, 607−615.
Marks, G. S. (1994) *Cell. Mol. Biol. 40*, 863−870.
Marletta, M. A. (1993) *J. Biol. Chem. 268*, 12231−12234.
Marletta, M. A. (1994) *Cell 78*, 927−930.
Nathan, C. (1992) *FASEB J. 6*, 3051−3064.
Rose, E., Boitrel, B., Quelquejeu, M., & Kossanyi, A. (1993) *Tetrahedron Lett. 34*, 7267−7270.
Segel, I. H. (1975) in *Enzyme Kinetics, Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems*, pp 227−231, John Wiley & Sons, Inc., New York.
Stamler, J. S. (1994) *Cell 78*, 931−936.
Stone, J. R., & Marletta, M. A. (1994) *Biochemistry 33*, 5636−5640.
Stone, J. R., & Marletta, M. A. (1995) *Biochemistry* (in press).
Stone, J. R., Sands, R. H., Dunham, W. R., & Marletta, M. A. (1995) *Biochem. Biophys. Res. Commun. 207*, 572−577.
Traylor, T. G., & Sharma, V. S. (1992) *Biochemistry 31*, 2847−2849.
Verma, A., Hirsch, D. J., Glatt, C. E., Ronnett, G. V., & Snyder, S. H. (1993) *Science 259*, 381−384.
Waldman, S. A., & Murad, F. (1987) *Pharmacol. Rev. 39*, 163−196.

BI951564W