

Spectral and Ligand-Binding Properties of an Unusual Hemoprotein, the Ferric Form of Soluble Guanylate Cyclase[†]

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ABSTRACT: The soluble form of guanylate cyclase (sGC) is a hemoprotein which serves as the only known receptor for the signaling agent nitric oxide (*NO). The enzyme is a heterodimer in which each subunit binds 1 equiv of 5-coordinate high-spin type *b* heme. *NO increases the V_{\max} of sGC up to 400-fold by binding to the heme to form a 5-coordinate ferrous nitrosyl complex. The electron paramagnetic resonance spectrum of the ferric form of the enzyme has been obtained. The spectrum displays rhombic symmetry and is indicative of a high-spin heme. Computer simulation of the EPR spectrum yields *g* values of 6.36, 5.16, and 2.0 with linewidths of 3.3, 4.1, and 3.3 mT, respectively. Using electronic absorption spectroscopy, it was observed that the ferric heme binds cyanide to form a 6-coordinate low-spin complex. The rate constants for association (k_{on}) and dissociation (k_{off}) of cyanide at 10 °C have been determined to be $(7.8 \pm 0.3) \times 10^{-2} \text{ M}^{-1} \text{ s}^{-1}$ and $(7.2 \pm 0.2) \times 10^{-5} \text{ s}^{-1}$, respectively. Unlike the ferrous form of the enzyme, which has a low affinity for ligands that form 6-coordinate complexes due to an unusually fast off-rate, the ferric form of the enzyme appears to have a low affinity for ligands due to a slow on-rate. The ferric heme binds azide with a K_d of $26 \pm 4 \text{ mM}$ to form a high-spin complex. The ferric form of the enzyme has a specific activity of ~57% that of the nonactivated ferrous form of the enzyme. However, in contrast to the mild activation of the ferrous enzyme by carbon monoxide, the ferric enzyme is not activated by cyanide. These results indicate that there may be a significant structural change in the protein upon the oxidation of the heme iron.

The guanylate cyclases are a family of enzymes that catalyze the formation of guanosine 3',5'-cyclic monophosphate (cGMP)¹ from guanosine 5'-triphosphate (GTP) (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 such as ANF, which bind to an extracellular receptor domain (Garbers & Lowe, 1994), while sGC serves as the predominant, if not the only, receptor for the intercellular signaling agent nitric oxide (*NO) [for recent reviews, see Marletta (1994, 1993), Nathan (1992), and Bredt and Snyder (1994)]. In the presence of *NO, the V_{\max} of sGC is increased up to 400-fold (Stone & Marletta, 1995a).

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 two subunits show significant homology to each other, and the enzyme is believed to bind up to 2 equiv of *b*-type heme (iron protoporphyrin IX) per heterodimer

(Stone & Marletta, 1995a). Spectrally there appears to be only one type of heme in the heterodimer, consistent with the conclusion that each subunit binds 1 equiv of heme at a conserved site in each of the two subunits. As isolated, the heme is ferrous with the unusual property of not binding oxygen. Studies employing electronic absorption spectroscopy indicate that the ferrous heme is 5-coordinate high spin with histidine as the sole axial ligand (Stone & Marletta, 1995a, 1994). The heme will bind *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). Correlation of electronic spectra with enzyme activity suggests that the 5-coordinate ferrous nitrosyl form of the enzyme is the activated form of the enzyme (Stone & Marletta, 1996). The ferrous heme of sGC binds carbon monoxide (CO) or nitrosomethane to form 6-coordinate low-spin complexes, in which the off-rate of the distal ligand is orders of magnitude faster than that for most other 5-coordinate ferrous hemoproteins (Stone & Marletta, 1995b). This fast off-rate results in a K_d for CO that is 3 orders of magnitude higher than that of CO for Hb or Mb. However, at high concentrations, CO is able to mildly (~4-fold) activate the enzyme by binding to the heme (Stone & Marletta, 1994, 1995b).

Previously, it was demonstrated that the ferric form of the enzyme could be generated by oxidation of the ferrous enzyme with ferricyanide (Stone & Marletta, 1995a, 1994). Studies employing electronic absorption spectroscopy have indicated that the ferric heme is also 5-coordinate high-spin with histidine as the sole axial ligand and that this heme binds cyanide at millimolar concentrations to form a

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¹ Abbreviations: sGC, soluble guanylate cyclase; ANF, atrial natriuretic factor; *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; EPR, electron paramagnetic resonance.

6-coordinate low-spin complex (Stone & Marletta, 1995a, 1994). In this report, the electron paramagnetic resonance spectrum of the ferric enzyme is presented. This spectrum demonstrates conclusively that the ferric heme is high spin. Also, the kinetic constants for the binding of cyanide to the ferric heme have been determined using electronic absorption spectroscopy, as has the K_d for the binding of azide. These studies, along with activity measurements of the ferric enzyme, suggest that a significant structural change in the protein is associated with the oxidation of the heme iron.

MATERIALS AND METHODS

Materials. 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.

EPR Spectroscopy. Ferrous sGC (15 μ M heme) in 125 mM TEA, 250 mM NaCl, and 25% glycerol, pH 7.4, was oxidized with 810 μ M potassium ferricyanide. A buffer bank was prepared under identical conditions. The sample and buffer were placed in EPR tubes under nitrogen and stored in liquid nitrogen until the spectra were recorded. EPR spectra were obtained using a 9 GHz Varian Century-Line EPR spectrometer digitized on a TRACOR/Northern NS-900 signal averager. Cryogenic temperatures were monitored with a carbon composition resistor, magnetic field measurements were made with a Model 3193 Systron Donner digital NMR gaussmeter, and frequency measurements were made with a Model 5340A Hewlett Packard frequency counter. Conditions and details of spectra collection were as described in the figure legend. The EPR spectrum of the enzyme was corrected for background by subtraction of a spectrum of the buffer recorded under identical conditions. The final EPR spectrum was computer simulated using programs that were adapted from the "g-strain" EPR simulator programs that have been previously described (Hagen et al., 1985a,b; Hearshen et al., 1986). The computer simulation was optimized by nonlinear minimization methods (quadratic interpolation) using software written in FORTRAN.

Cyanide Binding Kinetics. Ferric sGC was prepared by applying ferrous enzyme to a 0.7×20 cm Bio-Gel P-6 DG gel-filtration column (Bio-Rad) equilibrated with 25 mM TEA and 50 mM NaCl, pH 7.4, to remove all DTT. The enzyme was then oxidized by titration with ferricyanide, with monitoring of the oxidation by electronic spectroscopy (300–800 nm). The sample was applied again to the gel-filtration column to remove ferricyanide. Experiments were performed on a Cary 3E spectrophotometer at 10 °C. Ferric sGC (0.3 μ M) was placed in a cuvette under air. Potassium cyanide (5–35 mM) was then added, and the sample was mixed thoroughly. Stock solutions by KCN were adjusted to pH 7.4 with concentrated HCl in a fume hood prior to addition to the cuvette. Electronic absorption spectra were then recorded at specific time intervals until the binding was complete. The absorbance at 391 nm was determined for each time point, and from these values was subtracted the absorbance at 391 nm of the final spectrum. The natural logarithm of these differences, $\ln[A_{391}(t) - A_{391}(f)]$, was then plotted versus time to determine the pseudo-first-order rate constant (k_{obs}) for the binding of cyanide to the ferric heme of sGC. The values for k_{obs} were then plotted versus the total cyanide concentration to determine the association rate constant (k_{on}) of cyanide.

To directly measure the dissociation rate constant (k_{off}) of cyanide, after oxidation of the enzyme with ferricyanide, 20 mM KCN was added, and the sample was allowed to equilibrate until complex formation was complete. The excess ferricyanide and unbound KCN were then removed by gel filtration as described above. The sample (0.35 μ M heme) was then placed in a cuvette at 10 °C. Electronic absorption spectra were recorded at time intervals over a period of 20 h. For each spectrum, the absorbances at 391 and 418 nm were determined, and the $\Delta\Delta\text{Abs}[(418 - 391)_t - (418 - 391)_f]$ were calculated, where "t" is the spectrum at a specific time, and "f" is the final spectrum. The natural logarithm of these values was then plotted versus time to determine the k_{off} for cyanide.

Binding of Azide to sGC. Ferric sGC was prepared as described above for the cyanide binding experiments. Ferric sGC (0.8 μ M) in 25 mM TEA and 50 mM NaCl, pH 7.4, was placed in a cuvette under air. Several additions of sodium azide were made such that the concentration of azide was varied between 2 and 200 mM. Azide at a concentration of 200 mM had no appreciable effect on the pH of the sample. After each addition of azide the electronic absorption spectrum was recorded. The initial spectrum of ferric sGC was subtracted from each of these spectra to generate azide difference binding spectra. The Δ absorbances at 391 and 417 nm were determined, and the $\Delta\Delta\text{Abs}(417 - 391)$ was calculated and plotted versus the concentration of azide. The plot was then fit to a standard saturation equation, $\Delta\Delta\text{Abs} = \{\Delta\Delta\text{Abs}(\text{max}) \times [\text{N}_3^-]\} / \{K_d + [\text{N}_3^-]\}$, for determination of the K_d of azide.

Guanylate Cyclase Activity Measurements. Ferric enzyme and ferrous enzyme lacking DTT were prepared as described above. Both were assayed in the absence of DTT under argon. Ferric enzyme was also preincubated with either 2 mM DTT or 20 mM KCN (2 h on ice) and then assayed in the presence of the preincubation reagent. For the assays, buffer containing 50 mM TEA pH 7.4, 4.5 mM MgCl_2 , and 1.5 mM GTP (with or without 2 mM DTT or 20 mM KCN) was placed in a gas-tight vial under 1 atm of argon. The vials were incubated at 37 °C for 10 min. The reaction was initiated by the injection of sGC with a gas-tight syringe to give a final reaction volume of 1.0 mL with an enzyme concentration of 3 $\mu\text{g/mL}$. At 2 min time intervals for 10 min, 100 μL aliquots were removed with a gas-tight syringe and then quenched with 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 by radioimmunoassay (Amersham) following the procedure provided by the manufacturer.

RESULTS

EPR Spectroscopy. The EPR spectrum of the ferric form of sGC is shown in Figure 1. Computer simulation of the rhombic spectrum yielded g values of 6.36, 5.16, and 2.0 with line widths of 3.3, 4.1, and 3.3 mT, respectively. Integration of the simulation yielded a spin concentration of 15 μM , in agreement with the heme concentration determined by electronic absorption spectroscopy (data not shown) using an extinction coefficient of $110 \text{ mM}^{-1} \text{ cm}^{-1}$ at 431 nm for the ferrous enzyme (Stone & Marletta, 1995a).

Cyanide Binding Kinetics. Upon the addition of 4 mM KCN to the ferric enzyme, the Soret shifted from 391 to

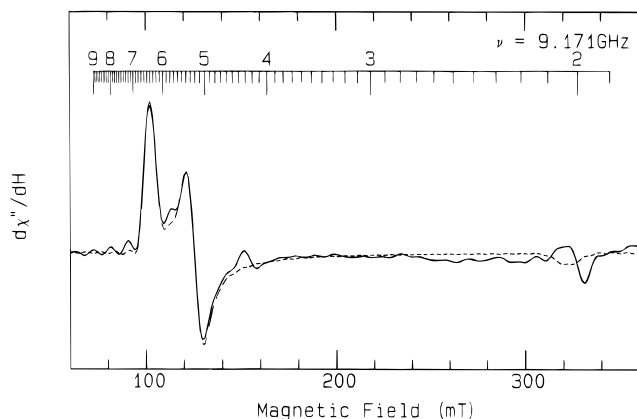


FIGURE 1: Electron paramagnetic resonance spectrum of ferric sGC. Ferric sGC (15 μ M heme), under nitrogen, $T = 20$ K, modulation amplitude = 10 mT, power = 20 mW, 16 1-min scans. (—) Original spectrum, (---) computer simulation.

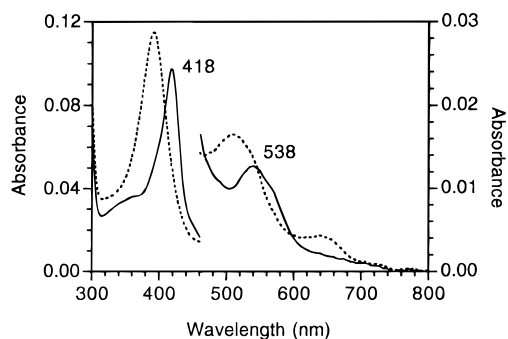


FIGURE 2: Electronic absorption spectrum of the cyanide complex of ferric sGC. Ferric sGC (1.0 μ M heme) in 25 mM TEA, pH 7.4, and 50 mM NaCl, under air in the absence (···) and presence (—) of 4 mM potassium cyanide. The scale on the left refers to the Soret region, and the scale on the right refers to the α/β region.

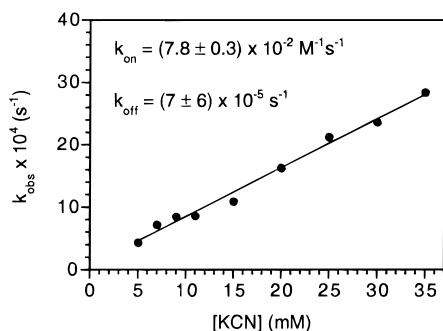


FIGURE 3: Plot of k_{obs} versus the concentration of cyanide. The pseudo-first-order rate constants (k_{obs}) for the binding of cyanide at 10 °C were plotted versus the concentration of potassium cyanide. The plot was fit to a line by linear regression. The slope is the rate constant for association (k_{on}), and the y-intercept is the rate constant for dissociation (k_{off}) of cyanide.

418 nm and there appeared a broad band at 538 nm in the α/β region (Figure 2). The transition was slow, requiring about 2 h to reach completion. For each concentration of KCN employed, the plot of $\ln[A_{391}(t) - A_{391}(f)]$ versus time was linear, indicating monophasic binding (data not shown). For each plot, the slope of the line is the pseudo-first-order rate constant (k_{obs}) for the binding of cyanide to the ferric heme of sGC. Figure 3 depicts a plot of the k_{obs} for cyanide binding versus the concentration of cyanide. The plot is linear, resulting in a k_{on} (slope) of $(7.8 \pm 0.3) \times 10^{-2} \text{ M}^{-1} \text{ s}^{-1}$ and a k_{off} (y-intercept) of $(7 \pm 6) \times 10^{-5} \text{ s}^{-1}$. Direct measurement of the k_{off} yielded a value of $(7.2 \pm 0.2) \times$

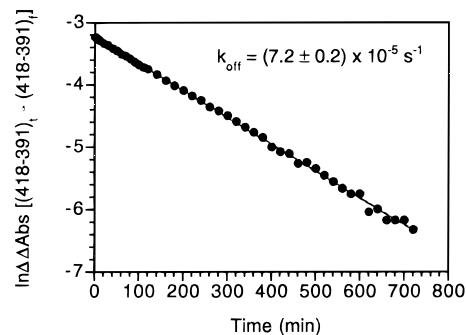


FIGURE 4: Determination of the k_{off} for cyanide. The ferric-cyano complex of sGC was applied to a gel-filtration column equilibrated with 25 mM TEA and 50 mM NaCl, pH 7.4, to remove unbound cyanide. The sample was then incubated at 10 °C for 20 h, with the intermittent recording of electronic absorption spectra. The spectra were used to calculate the $\ln\{\Delta\text{Abs}[(418 - 391)_t - (418 - 391)_f]\}$. The plot of this parameter versus time is a straight line, the slope of which is the k_{off} for cyanide.

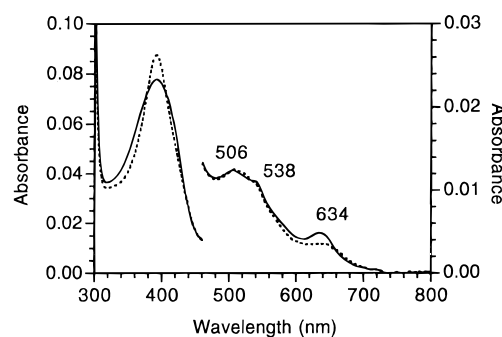


FIGURE 5: Electronic absorption spectrum of the azide complex of ferric sGC. Ferric sGC (0.8 μ M heme) in 25 mM TEA, pH 7.4, and 50 mM NaCl, under air, in the absence (···) and presence (—) of 150 mM sodium azide. The scale on the left refers to the Soret region, and the scale on the right refers to the α/β region.

10^{-5} s^{-1} (Figure 4). Using this value and that for the k_{on} yields a calculated value for the K_d of $0.92 \pm 0.04 \text{ mM}$.

Binding of Azide to sGC. Upon addition of 150 mM sodium azide to ferric sGC, the Soret decreased in extinction and broadened, and the porphyrin $\rightarrow \text{Fe}^{+3}$ charge-transfer band increased in extinction and shifted from 642 to 634 nm (Figure 5). In contrast, fluoride failed to perturb the spectrum of ferric sGC at concentrations as high as 500 mM (data not shown). Unlike the binding of cyanide, the binding of azide was rapid, with complete binding within 30 s. The K_d for azide was determined by recording the electronic absorption spectrum of the enzyme in the presence of varying concentrations of azide. The initial spectrum of ferric sGC was subtracted from each of these spectra to generate azide difference binding spectra. In the difference spectra there were peaks at 357 and 417 nm and a trough at 391 nm (data not shown). The Δ absorbances at 391 and 417 nm were determined, and the $\Delta\Delta\text{Abs}(417 - 391)$ was calculated and plotted versus the concentration of azide (Figure 6). The plot was fit to a standard saturation equation, yielding a K_d for azide of $26 \pm 4 \text{ mM}$.

Guanylate Cyclase Activity Measurements. For all incubations of sGC, the enzyme activity was linear for the duration of the experiment (10 min). The specific activities obtained are listed in Table 1. The ferric enzyme has a specific activity of 57% that of the ferrous enzyme. Preincubation of the ferric enzyme with DTT resulted in a specific activity that was identical to that of the ferrous enzyme.

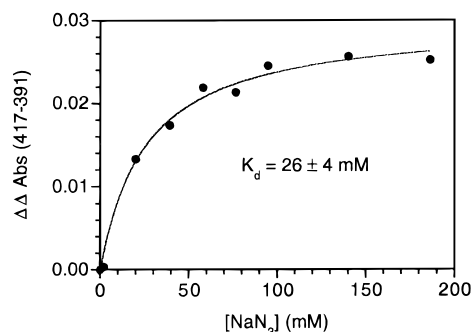


FIGURE 6: Determination of the azide dissociation constant. The values of $\Delta\Delta\text{Abs}(417 - 391)$ were determined as described in the text and plotted versus the concentration of sodium azide. The data were fit to a standard saturation equation, $\Delta\Delta\text{Abs} = \{\Delta\Delta\text{Abs}(\text{max}) \times [\text{N}_3^-]\} / \{K_d + [\text{N}_3^-]\}$, for determination of the K_d of azide.

Table 1: Specific Activity of Ferric sGC

initial oxidation state	preincubation ^a	sp act. ^b (nmol of cGMP/min·mg)
ferrous	none	69 ± 9
ferric	none	39 ± 5
ferric	2 mM DTT	70 ± 4
ferric	20 mM KCN	39 ± 6

^a Preincubation was carried out for 2 h on ice, and the reagent in the preincubation mixture was included in the assay mixture. ^b Enzyme activity was determined by radioimmunoassay as described in the Materials and Methods section.

Preincubation (2 h) and assaying in the presence of 20 mM KCN failed to alter the specific activity of the ferric enzyme.

DISCUSSION

Previous studies employing electronic absorption spectroscopy suggested that the ferric heme in sGC is 5-coordinate high spin (Stone & Marletta, 1995a, 1994). The EPR spectrum of ferric sGC reported here displays rhombic symmetry (Figure 1). The g values obtained from the simulation of the spectrum are indicative of high-spin iron (Cammack & Cooper, 1993; Smith & Pilbrow, 1980). The EPR spectra of 6-coordinate high-spin heme complexes, such as metHb, tend to display axial symmetry, with a single feature near $g = 6$, whereas the EPR spectra of 5-coordinate high-spin heme complexes usually show a rhombic distortion with two features near $g = 6$ (Goodin & McRee, 1993). However, it is as yet unclear how strong the correlation is between the EPR anisotropy and the coordination state. Nonetheless, the EPR spectrum demonstrates that the ferric heme of sGC is definitely high spin and probably 5-coordinate, in agreement with the electronic absorption spectra.

As described above, the addition of KCN to the ferric enzyme results in a shift of the Soret from 391 to 418 nm and in the appearance of a broad band at 538 nm in the α/β region of the spectrum (Figure 2). The final spectrum is almost identical to the spectra of the cyanide complexes of metHb and metMb (Antonini & Brunori, 1971). Thus cyanide is able to bind to the ferric heme of sGC to form a low-spin 6-coordinate complex, where cyanide is one axial ligand and histidine the other. For the kinetics of cyanide binding, the linear plot of k_{obs} versus [KCN] is consistent with a simple one-step binding process (Figure 3). However, the on-rate of cyanide for sGC (10 °C) is 4 orders of magnitude slower than for metHb and metMb at 20 °C (Table 2). The direct measurement of the k_{off} for cyanide yielded

Table 2: Comparison of Equilibrium and Kinetic Constants^a

ligand	protein	k_{on} ($\text{M}^{-1} \text{s}^{-1}$)	k_{off} (s^{-1})	K_d
CN^-	sGC	7.8×10^{-2}	7.2×10^{-5}	0.9 mM
	Hb(R), Mb	$(1.1-1.7) \times 10^2$	$(5-12) \times 10^{-5}$	$0.4-0.7 \mu\text{M}$
	cytochrome c'	2.3×10^{-3}	1.8×10^{-5}	7.7 mM
N_3^-	sGC			26 mM
	Hb(R), Mb			4–80 μM
F^-	sGC			>500 mM
	Hb(R), Mb			13–27 mM

^a Values for sGC were determined at 10 °C. Values for R-state Hb, Mb, and cytochrome c' were determined at 20–25 °C. For cyanide, the k_{on} and K_d correspond to the total cyanide concentration at pH ~ 7 (Antonini & Brunori, 1971; Kassner, 1991).

a value of $(7.2 \pm 0.2) \times 10^{-5} \text{ s}^{-1}$ at 10 °C (Figure 4), in excellent agreement with the value determined from the y-intercept of the plot of k_{obs} versus [KCN] (Figure 3). The calculated values for k_{off} for the cyanide complexes of metHb and metMb are in the range of $(5-12) \times 10^{-5} \text{ s}^{-1}$ at 20 °C, similar to the rate constant obtained for sGC at 10 °C (Table 2). The ferric enzyme, therefore, has a much lower affinity for cyanide compared to metHb and metMb, due primarily to an unusually slow on-rate. This is in contrast to the binding of CO to ferrous sGC, where the low affinity was attributed primarily to a fast off-rate (Stone & Marletta, 1995b). Thus for sGC, the kinetic parameter responsible for the reduced ligand affinity depends on the oxidation state of the heme iron. This is in contrast to cytochrome c' , for which low ligand affinities are due to slow on-rates regardless of the oxidation state of the heme (Kassner, 1991). Experiments from a number of laboratories led to the conclusion that the slow on-rates were an outcome of steric hindrance at the sixth coordination site, a conclusion that has been supported by the reported crystal structures (Kassner, 1991). The alteration of the kinetic parameter responsible for the low ligand affinities for sGC might indicate that there is a significant conformational change in this protein upon oxidation of the heme.

Azide typically forms 6-coordinate low-spin complexes with hemoproteins, although the affinity of azide for hemoproteins is usually less than that of cyanide (Antonini & Brunori, 1971). Upon addition of 150 mM sodium azide to ferric sGC, the Soret decreased in extinction and broadened, and the porphyrin $\rightarrow \text{Fe}^{+3}$ charge-transfer band increased in extinction and shifted from 642 to 634 nm (Figure 5). Unlike the binding of cyanide, the binding of azide was rapid, with complete binding within 30 s. Furthermore, the final complex was not low spin as expected but high spin. However, it is unclear whether this high-spin complex with azide is 5-coordinate or 6-coordinate.

If the complex is 5-coordinate with histidyl ligation, then the spectral change is due mostly to a change in the character of the Fe–His bond. Interestingly, this spectral transition is similar to that seen upon the pH titration of cytochrome c' (Horio & Kamen, 1961). With that protein, as the pH is increased from 5 to 9, the Soret band broadens and decreases in extinction due to the formation of a hyperporphyrin. Also, the charge-transfer band at 643 nm increases in extinction but does not shift position. There is good evidence that the spectral changes in cytochrome c' are due to the deprotonation of the histidine that is ligated to the heme iron and the resultant increase in the strength of Fe–histidine bond (Yoshimura et al., 1985). Therefore, for sGC, it is possible

that azide is binding to a site other than the heme. This binding might then cause a conformational change in the protein which results in an increase in the strength of the Fe—histidine bond either through deprotonation of the histidine or through realignment of the histidine such that is better able to bind to the iron. The location of this alternative binding site is unclear. However, a model for the binding of •NO to the heme of sGC derived from stopped-flow kinetics suggests that •NO is able to interact with a non-heme site on the protein (Stone & Marletta, 1996). Azide is unlikely to be causing the spectral change by binding to the GTP site, as GTP in the presence or absence of Mg^{2+} failed to bring about the spectral change (data not shown). Alternatively, it is also possible that the complex is 5-coordinate with an axial iron—azido bond.

Although the lack of a shift in the λ_{max} of the Soret band (391 nm) upon azide binding suggests that the final complex is 5-coordinate, the small shift in the charge-transfer band from 642 to 634 nm suggests the formation of a 6-coordinate high-spin complex (Stone & Marletta, 1995a). There are two ways in which a 6-coordinate high-spin complex could be formed. Azide may be directly binding to the heme iron to form an unusual 6-coordinate complex. All other ferric hemoproteins which have been studied thus far form low-spin complexes with azide. However, certain heme model compounds with sterically hindered imidazole axial ligands form 6-coordinate complexes with azide that are a mixture of high and low spin (Neya et al., 1985). It is possible that sGC is the first hemoprotein known to form a purely high-spin complex with azide. Another possibility is that azide is binding elsewhere on the protein (as described above) to cause a conformational change that allows some weak-field ligand to bind to the heme to form a 6-coordinate complex. Clearly, further experiments are required to definitively assign the coordination number of this complex as well as the site of azide binding.

The specific activity of the fully oxidized ferric enzyme is 57% that of the ferrous enzyme. Reduction of the iron by treatment with DTT leads to complete recovery in specific activity (Table 1) and also reverses the spectral change caused by ferricyanide (data not shown). Thus the ferric form of the enzyme is catalytically competent, although this basal activity is less than that of the ferrous enzyme. Preincubation with cyanide failed to activate the ferric enzyme even though this ligand forms a 6-coordinate complex with the heme. This is in contrast to the 4-fold activation of the ferrous enzyme achieved with CO which also forms a 6-coordinate complex with the heme (Stone & Marletta, 1994). The failure of cyanide to activate ferric sGC and the values for the kinetic constants for cyanide binding indicate that there may be a significant conformational change in the protein upon the oxidation of the heme iron. This conformational change might alter the kinetic parameter responsible for the low ligand affinities and might also prevent heme-mediated activation of the enzyme.

In summary, using electron paramagnetic resonance spectroscopy, the ferric heme of sGC has been determined to be high spin. The ferric heme binds cyanide to form a 6-coordinate low-spin complex. Unlike the ferrous form of the enzyme, which has a low affinity for ligands that form 6-coordinate complexes due to an unusually fast off-rate, the ferric form of the enzyme appears to have a low affinity for ligands due to a slow on-rate. The ferric form of the enzyme binds azide to form a high-spin complex. The shift in the position of the charge-transfer band indicates that this complex is 6-coordinate, although a 5-coordinate complex cannot be ruled out. The ferric form of the enzyme has a specific activity of ~57% that of the ferrous form of the enzyme. However, in contrast to the mild activation of the ferrous enzyme by carbon monoxide, the ferric enzyme is not activated by cyanide. These results indicate that there is a structural change in the protein upon the oxidation of the heme; the nature of this structural change is currently under investigation.

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