Mechanism of Nitrosyl Transfer. Dissociation of Nitric Oxide from Cobalt Nitrosyls

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Received September 14, 1981

Abstract: Kinetic evaluation of nitrosyl transfer from $Co(NO)(DMGH)_2$ to hemoglobin has established that the mechanism of this transformation involves nitric oxide association with hemoglobin subsequent to dissociation of nitric oxide from $Co(NO)(DMGH)_2$. Specific association of the β93-cysteine sulfhydryl group with this nitrosyl complex is observed, but $Co(DMGH)_2$ is relatively unselective in its association with basic amino acids. Inverse kinetic dependence on the concentration of these bases is observed; six amino acids per hemoglobin tetramer, including the two β93-cysteine units, are capable of association with $Co(DMGH)_2$. Nitric oxide dissociation from metal nitrosyls also explains the production of nitrato complexes in reactions of these metal nitrosyls with molecular oxygen and the oxidation of oxymyoglobin by $Co(NO)(DMGH)_2$.

Intermolecular transfer of a metal-bound nitrosyl ligand to a coordinatively unsaturated transition-metal complex is currently understood to occur through formation of a μ-bridged nitrosyl (eq 1).[1,2] This mechanistic understanding is based on the presumed

$$M(NO) + M' \rightleftharpoons [M(\mu\text{-}NO)M'] \rightleftharpoons M + M'(NO) \quad (1)$$

nucleophilic character of the nitrosyl nitrogen in these bent nitrosyl complexes[3-5] and on their reported stability toward nitrosyl dissociation.[1,2] However, only product analyses have been employed to characterize the nature of nitrosyl-transfer reactions.

We have recently reported that cobalt(II) nitrosyl complexes undergo nitrosyl transfer to iron hemoproteins.[6] The black $[Co(NH_3)_5NO]Cl_2$, previously believed to be capable of direct intermolecular nitrosyl transfer,[7] was found to undergo irreversible liberation of nitric oxide in aqueous media[8] to form hemoprotein nitrosyl complexes with the same selectivity as free nitric oxide.[9-12] In contrast, $[CoCl(NO)(en)_2]ClO_4$ (en = ethylenediamine) and $[Co(NO)(DMGH)_2(ROH)]$ (DMGH = dimethylglyoximate(1-)) or $[Co(NO)(TMGH)_2(ROH)]$ (TMGH = tetramethyleneglyoximate(1-)) selectively transferred the nitrosyl ligand to iron(II) hemoproteins. Kinetic investigation of these latter reactions with deoxyhemoglobin as the nitrosyl acceptor, through which the rate law for nitrosyl transfer could be examined for the first time, described the formation of nitrosylhemoglobin as first order in the hemoprotein and first order in the cobalt nitrosyl complex.

The observed rate law for nitrosyl transfer to hemoglobin presents a substantial mechanistic conflict. On the one hand, the heme cavity of hemoglobin and related hemoproteins is sterically restrictive,[13-15] Nitric oxide, but not the cobalt nitrosyl reagents, should have access to the heme iron. On the other hand, nitric oxide dissociation from these cobalt nitrosyls has not been observed,[1,4] and there is convincing evidence in related transformations that electrophilic association occurs on the metal-bound nitrosyl group.[3-5,16] We have examined nitrosyl transfer from $[Co(NO)(DMGH)_2(ROH)]$ to deoxyhemoglobin in kinetic detail and now report the resolution of this mechanistic conflict.

Experimental Section

Reagents, Chemicals, and Stock Solutions. Human hemoglobin A (type IV), obtained from Sigma Chemical Co., was reduced with excess sodium dithionite and further purified by passing the resulting aqueous solution through a G-25 Sephadex column using 0.05 M phosphate buffer at pH 7.0. Concentrated solutions of oxyhemoglobin (approximately 1.0 mM heme) were degassed under reduced pressure (less than 0.5 torr), and the resultant deoxyhemoglobin was maintained in a sealed air-tight flask at atmospheric pressure under nitrogen. Reaction of β93-cysteine residues of the reactant hemoglobin was performed at pH 7.8 with oxyhemoglobin and a 10-fold molar excess of iodoacetamide, relative to heme, according to the procedure of Winterbourn and Carrell.[17] The β93-sulfhydryl-blocked hemoglobin was separated from excess iodoacetamide on a G-25 Sephadex column and deoxygenated as previously described. The absence of accessible sulfhydryl groups was determined with 5,5'-dithiobis(2-nitrobenzoic acid) by the method of Ellman.[18]

Nitrosyl-transfer agents $[CoCl(NO)(en)_2]ClO_4$,[19] $[Co(NO)(DMGH)_2(MeOH)]$,[1] $[Co(NO)(TMGH)_2(MeOH]$,[20] and $Co(DMGH)_2$[21] were prepared by standard procedures. Spectroscopic analyses were employed for structural identification, and elemental analyses (Galbraith Labs) provided additional structural confirmation for $[Co(NO)(TMGH)_2(MeOH)]$.

Anal. Calcd for $CoC_{13}H_{22}N_5O_6$: C, 38.72; H, 5.50; N, 17.37. Found: C, 38.55; H, 5.54; N, 17.34.

Stock solutions of cobalt nitrosyl reagents were prepared in 0.05 M phosphate buffer at pH 7.0, deoxygenated under reduced pressure (less than 0.5 torr) prior to their use, and maintained in sealed air-tight flasks at atmospheric pressure under nitrogen; such solutions were stable for at least 3 days at room temperature. However, in the presence of sodium dithionite, a well-known trapping agent for nitric oxide, rapid loss of nitric oxide from $Co(NO)(DMGH)_2$ was observed. Heme concentrations were calculated from the extinction coefficients of Banerjee and co-workers.[22] Hemoglobin, $Co(DMGH)_2$, and cysteine solutions, although stable over a minimum 3-h period in the absence of oxygen, were prepared immediately prior to their use for product or kinetic determinations.

Product Identifications. Hemoprotein nitrosyl complexes formed by nitrosyl transfer from cobalt nitrosyls were identified as the sole hemoprotein reaction products by ultraviolet and visible spectral analyses through comparisons with the spectra of hemoprotein nitrosyls produced

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Scheme I

$$L_nCoNO \rightleftharpoons L_nCo + NO \quad (2)$$
$$NO + Hb \rightarrow HbNO \quad (3)$$

by direct combination with nitric oxide. In contrast to the outcome of reactions of hemoglobin with stoichiometric amounts of nitric oxide, in which methemoglobin is produced in addition to nitrosylhemoglobin,[23] comparable transformations with the cobalt nitrosyls produce nitrosylhemoglobin as the sole hemoprotein product. With the exception of the black $[Co(NH_3)_5NO]Cl_2$ which serves as a nitric oxide donor both to iron(II) and iron(III) hemoproteins, these nitrosyl-transfer agents reacted stoichiometrically with hemoglobin, myoglobin, and horseradish peroxidase (Fe^{2+})[24] to form their corresponding nitrosyl complexes.[6] The corresponding iron(III) hemoproteins were inert to nitrosyl transfer by these reagents, even when a 10- to 20-fold molar excess was employed, which indicates that the cobalt nitrosyls do not irreversibly dissociate nitric oxide in the presence of the hemoprotein.

Kinetic Measurements. Reactions were initiated with the injection, using a gas-tight syringe, of a concentrated solution of the cobalt nitrosyl reagent into the deoxyhemoglobin (usually 5–20 μM) contained in 0.05 M phosphate buffer at pH 7.0. Reaction rates were determined at 10.0 °C by monitoring the decrease in absorbance at 430 nm with time using a Pye Unicam SP8-200 spectrophotometer. In all kinetic experiments reactions were carried out under pseudo-first-order conditions where cobalt nitrosyl concentrations were in 10-fold excess with respect to the total heme groups present; the reaction rate order with respect to cobalt nitrosyl has been previously reported.[6] The resultant time courses were fitted to an integrated single exponential process from which the pseudo-first-order rate constants were calculated. Typically, from 3 to 5 replicate time courses were obtained for each kinetic determination, and the averaged rate constants are reported. With the exception of reactions that were performed in the presence of $Co(DMGH)_2$, which followed first-order kinetics through 2 half-lives, pseudo-first-order kinetics were exhibited through greater than 90% of the time courses of nitrosyl-transfer reactions.

Equilibrium Measurements. Equilibrium data for amino acid association with $Co(NO)(DMGH)_2$ and with $Co(DMGH)_2$ were obtained in deoxygenated 0.05 M phosphate-buffered aqueous solution at 25.0 °C, Uniform amounts of the amino acid stock solution were added by gas-tight syringe to a solution of the cobalt reagent, and the resultant spectrum was recorded from approximately 430–210 nm. The change in absorbance at or near the absorption maximum in this spectral region was calculated, and the equilibrium constant was determined from a plot of $1/\Delta A$ vs. 1/[amino acid]. For the combination of interactive amino acids with $Co(NO)(DMGH)_2$ and with $Co(DMGH)_2$ linear behavior was observed over a minimum fivefold change in the molar ratio of amino acid to cobalt reagent. Spectral changes were negligible for the combination of histidine or lysine with $Co(NO)(DMGH)_2$.

Results

First-order rate dependence on both hemoglobin and the cobalt nitrosyl reagent suggests either direct nitrosyl transfer from cobalt to the iron(II) centers of hemoglobin (eq 1) or nitric oxide dissociation from the cobalt nitrosyl followed by competitive association of nitric oxide with hemoglobin (Scheme I, Hb = hemoglobin). If the mechanism defined by eq 1 is operative, the rate for nitrosylhemoglobin formation will be independent of the concentration of L_nCo. However, if Scheme I is operative and nitric oxide dissociation is reversible, the rate for nitrosylhemoglobin formation will be inversely dependent on the concentration of L_nCo.[25] The kinetic results from investigations in which the rate for nitrosyl transfer from $Co(NO)(DMGH)_2$ was determined in the presence of added $Co(DMGH)_2$ are described in Figure 1. Linear inverse dependence on the initial concentration of $Co(DMGH)_2$ is observed but, as might be anticipated from the known electrophilicity of $Co(DMGH)_2$,[27] the rate for nitrosyl transfer also appears to be dependent on the molar ratio of $Co(DMGH)_2$ to hemoglobin. However, since the molar ratio of $Co(NO)(DMGH)_2$ to hemoglobin was maintained at 10.0 in these determinations, the rate for nitrosyl transfer could also be described as dependent on the molar ratio of $Co(DMGH)_2$ to Co(NO)-

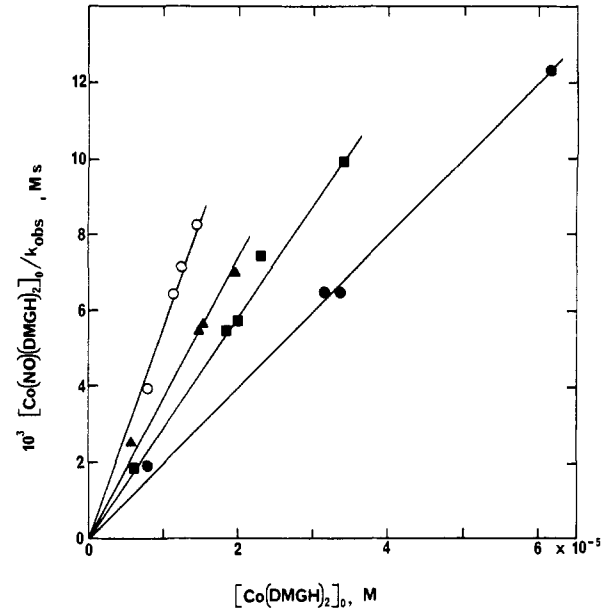

Figure 1. Plots of the reciprocal of $k_{obsd}/[Co(NO)(DMGH)_2]_0$ vs. $[Co(DMGH)_2]_0$ for the reaction of $Co(NO)(DMGH)_2$ with hemoglobin at 10.0 °C in deoxygenated 0.05 M phosphate buffer, pH 7.0. $[Co(DMGH)_2]_0/[Hb]_0$ = 1.0 (○), 2.0 (▲), 3.0 (■), and 5.0 (●), where $[Hb]_0$ = $[heme]_0$.

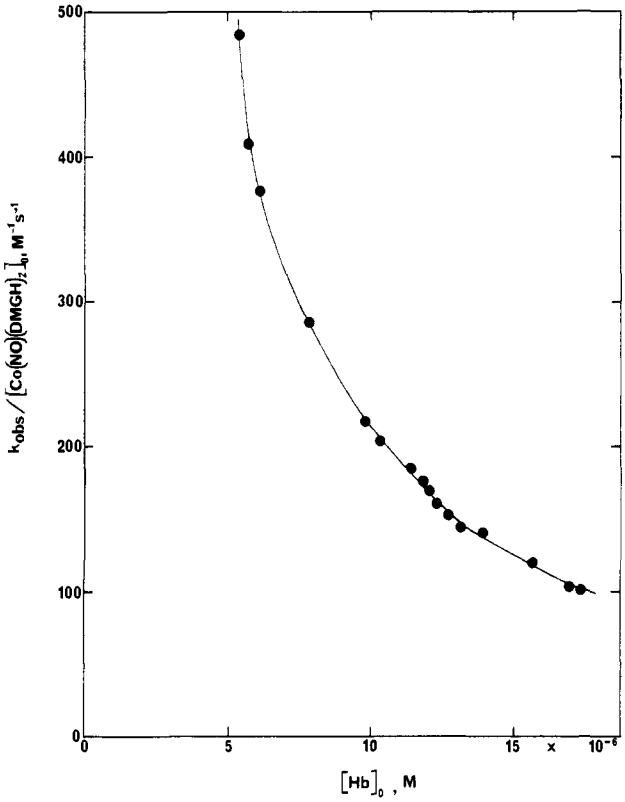

Figure 2. Dependence of the rates for nitrosyl transfer on the initial concentration of hemoglobin. Reactions were performed in deoxygenated 0.05 M phosphate buffer at 10.0 °C with $[Co(NO)(DMGH)_2]_0/[Hb]_0$ = 10.0, pH 7.0.

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(24) Our previous report[6] that (ethylenediamine)- and (glyoximato)cobalt nitrosyl complexes do not undergo nitrosyl transfer to iron(II) horseradish peroxidase is erroneous. This hemoprotein nitrosyl complex is rapidly formed in the absence of excess sodium dithionite.

(25) Cooperativity is not observed in nitric oxide association with hemoglobin. The rates for nitric oxide association with the α and β chains of hemoglobin are experimentally identical.[26] The same kinetic identity would not be anticipated in direct nitrosyl transfer.

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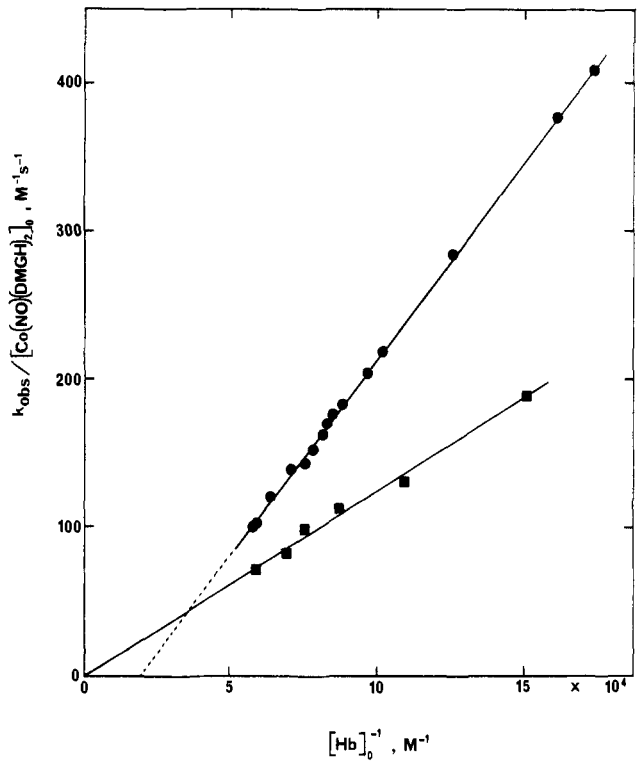

Figure 3. Plots of $k_{obsd}/[Co(NO)(DMGH)_2]_0$ vs. the reciprocal of the initial hemoglobin concentration (a) for unmodified hemoglobin (●) and (b) for iodoacetamide-modified hemoglobin (■). Reactions were performed in deoxygenated 0.05 M phosphate buffer (pH 7.0) at 10.0 °C with an exact 10-fold molar excess of $Co(NO)(DMGH)_2$, based on the concentration of heme iron(II).

$(DMGH)_2$ or could reflect the ability of this electrophilic reagent to inhibit nitrosyl transfer through association with hemoglobin and, therefore, a more detailed determination of the nature of this nitrosyl-transfer reaction was undertaken.

Hemoglobin as a Cobalt Ligand. Nucleophilic sites on the globin chains of hemoglobin represent associative ligands for $Co(DMGH)_2$ and $Co(NO)(DMGH)_2$ that could alter their effective solution concentrations. In order to determine the effect of hemoglobin as a cobalt ligand, the rates for nitrosyl transfer from $Co(NO)(DMGH)_2$ were determined over a fourfold range in hemoglobin concentration. Pseudo-first-order kinetics were observed through 4 half-lives, and a plot of the calculated second-order rate constant ($k_{obsd}/[Co(NO)(DMGH)_2]_0$) vs. initial hemoglobin concentration $[Hb]_0$ is seen (Figure 2) to describe an inverse dependence of the rate on the hemoglobin concentration. When the same rate data are plotted as a function of $1/[Hb]_0$ (Figure 3a), linear inverse dependence is observed with a negative intercept for the extended line.

Similar plots with the observed first-order rate constant k_{obsd} exhibit the same apparent dependencies on hemoglobin concentration. The slope of the line from a plot of k_{obsd} vs. $1/[Hb]_0$ is 2.24 M s^{-1}, and a positive intercept (0.0033 s^{-1}) is obtained. However, in this and subsequent kinetic descriptions, results for nitrosyl transfer are described as second order to maintain consistency in kinetic comparisons when the apparent first-order rate constant fails in comparative correlations.

Whereas heme iron(II) units of hemoglobin serve as nitrosyl acceptors, the globin proteins function to inhibit nitrosyl transfer. Basic amino acids such as lysine and histidine, as well as the cysteine sulfhydryl group, represent suitable binding sites on hemoglobin for association with $Co(NO)(DMGH)_2$ or $Co(DMGH)_2$, and of these three amino acids the accessible β93-cysteine sulfhydryl group can be effectively blocked by its conversion to sulfide derivatives.[17] When hemoglobin, modified by treatment with iodoacetamide, is employed for kinetic determinations identical with those described in Figures 2 and 3a, the resulting rate data also exhibit linear inverse dependence on

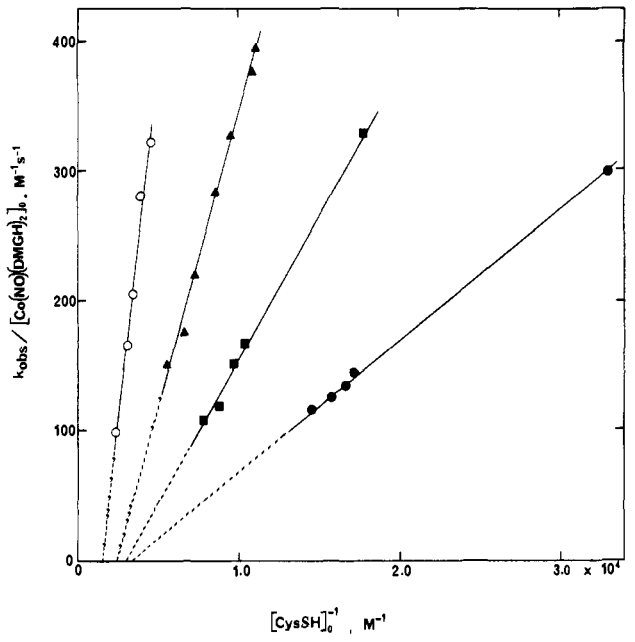

Figure 4. Plots of $k_{obsd}/[Co(NO)(DMGH)_2]_0$ vs. the reciprocal of the added cysteine concentration under reaction conditions identical with those described in Figure 3. $[CysSH]_0/[Hb]_0$ = 20.0 (○), 10.0 (▲), 5.0 (■), and 3.0 (●).

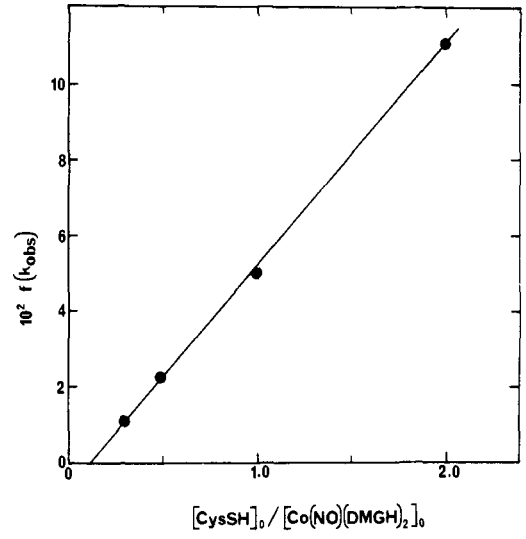

Figure 5. Plot of the slopes of the lines from Figure 4, $f(k_{obsd}) = k_{obsd}$-$[CysSH]_0/[Co(NO)(DMGH)_2]_0$, vs. $[CysSH]_0/[Co(NO)(DMGH)_2]_0$. The intersection at the x axis occurs at $[CysSH]_0/[Co(NO)(DMGH)_2]_0$ equal to 0.11.

hemoglobin concentration (Figure 3b).[28] The slope of the line describes a substantially greater rate inhibition for the cysteine-blocked hemoglobin, but this line has an intercept at the origin. Linear inverse dependence on the hemoglobin concentration is also observed with k_{obsd}, and the resultant line also intercepts the origin.

If blocking the β93-cysteine sulfhydryl group of hemoglobin effectively reduces the rate for nitrosyl transfer, then increasing the relative concentration of this functional group should lead to rate acceleration. Consequently, rates for nitrosyl transfer were determined as a function of the concentration of added cysteine at molar ratios of cysteine to hemoglobin of 20.0, 10.0, 5.0, and 3.0. As anticipated, the calculated second-order rate constants exhibit linear inverse dependence on the initial concentration of cysteine at each constant molar ratio of cysteine to hemoglobin

(28) Blocking of the β93-cysteine sulfhydryl groups of hemoglobin through reaction with *N*-ethylmaleimide[17] provided kinetic results identical with those obtained with iodoacetamide modified hemoglobin.

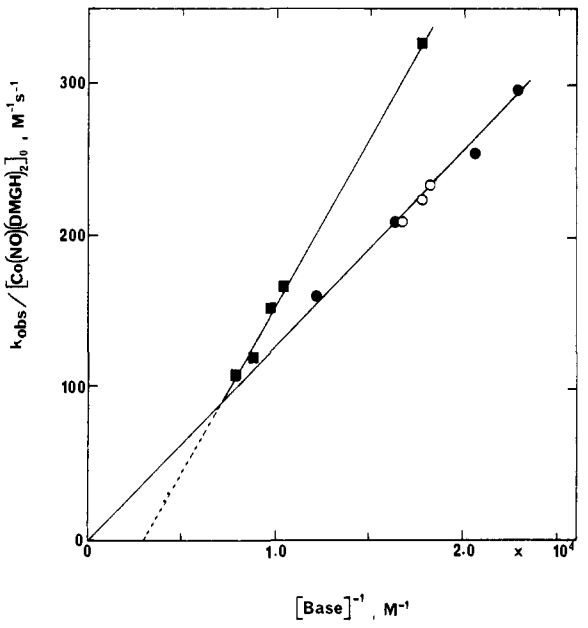

Figure 6. Plots of $k_{obsd}/[Co(NO)(DMGH)_2]_0$ vs. the reciprocal of the added amino acid concentration at $[base]_0/[Hb]_0 = 5.0$ under reaction conditions identical with those described in Figure 3: CysSH (■); lys (●); His (○).

(Figure 4). The slopes of these lines increase with the increasing molar ratio of $[CysSH]_0/[Hb]_0$ or $[CysSH]_0/[Co(NO)(DMGH)_2]_0$ and, in fact, are directly related to the molar ratio of these reactants (Figure 5). The hypothetical negative intercepts of the extended lines from Figure 4 do not exhibit a definable linear dependence on $[CysSH]_0/[Hb]_0$ or $[CysSH]_0/[Co(NO)(DMGH)_2]_0$ but are observed to approach zero as the ratio of these reactants is decreased. There is no apparent linear correlation when k_{obsd} is plotted against $1/[CysSH]_0$.

Histidine and lysine also increase the rate for nitrosyl transfer from $Co(NO)(DMGH)_2$ to hemoglobin but, as is evident from the data presented in Figure 6, the kinetic characteristics of nitrosyl transfer with added histidine and lysine are quite different from those observed with added cysteine. These results do, however, parallel those obtained with β93-cysteine-blocked hemoglobin (Figure 3b). The slope of the line obtained from a plot of $k_{obsd}[Co(NO)(DMGH)_2]_0$ vs. $1/[Lys]_0$ is identical with that obtained with histidine and is only half the value obtained with cysteine under identical conditions. Furthermore, this line intersects at the origin rather than at a hypothetical negative value of $k_{obsd}/[Co(NO)(DMGH)_2]_0$ which is observed upon extension of the line that describes the data obtained with added cysteine.

Specificity of Amino Acid Association with $Co(NO)(DMGH)_2$. Clarkson and Basolo have estimated equilibrium constants for the association of a variety of amine and phosphine bases with several cobalt nitrosyl complexes (eq 4) in acetone,[3] and they observe a

$$L_4CoNO + B \rightleftharpoons L_4Co(NO)B \quad (4)$$

distinct relationship between the basicity constants for these basic substrates and the equilibrium constants for their association with nitrosyl complexes. Similar information for $Co(NO)(DMGH)_2$ has not been obtained although the association constant of this nitrosyl complex with pyridine in acetone has been reported (25 M^{-1} at 25 °C) to be comparatively high in relation to association constants with Schiff base cobalt nitrosyl complexes.[29] Consequently, we have examined the susceptibility of this glyoximate complex for association with representative amino acids. The negative intercepts from kinetic results obtained in reactions of $Co(NO)(DMGH)_2$ with hemoglobin in the presence and absence of added cysteine (Figures 3 and 4) suggest a considerable specificity for the association of cysteine with this cobalt nitrosyl complex.

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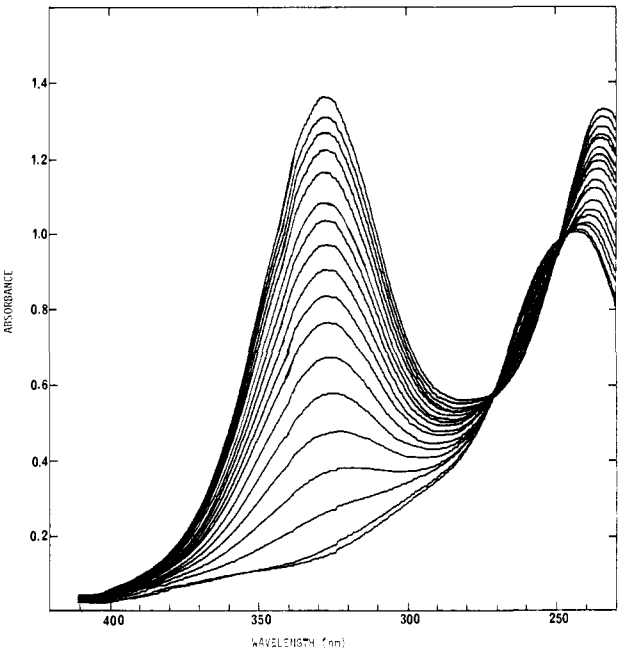

Figure 7. Spectral changes in the UV–visible region for sequential additions of cysteine (2.40×10^{-5} M per unit addition) to $Co(NO)(DMGH)_2$ (5.05×10^{-5} M) at constant volume in deoxygenated 0.05 M phosphate buffer, pH 7.0, at 25.0 °C. The initial spectrum of $Co(NO)(DMGH)_2$ exhibits a maximum at 244 nm.

Figure 7 describes the spectral changes obtained when uniform amounts of cysteine are added sequentially to $Co(NO)(DMGH)_2$ in phosphate-buffered deoxygenated aqueous solution. Spectral maxima are observed at 327 and 234 nm with isosbestic points at 272 and 248 nm. The equilibrium constant calculated from the observed increase in absorbance at 327 nm is 80.7 M^{-1} (25.0 °C). Neither histidine nor lysine effect any change in the absorption characteristics of $Co(NO)(DMGH)_2$, even when these amino acids were employed in 20-fold molar excess over the cobalt nitrosyl complex. Thus, of all of the amino acids that constitute hemoglobin, only cysteine effectively associates with $Co(NO)(DMGH)_2$ and, as is suggested from kinetic results with iodoacetamide-treated hemoglobin (Figure 3), only the β93-cysteine residue is accessible for association with the cobalt nitrosyl complex.

The same specificity for association is not observed with $Co(DMGH)_2$, although the spectral changes obtained from the combination of cysteine, histidine, or lysine with $Co(DMGH)_2$ suggest that two cysteine entities become sequentially ligated to cobalt whereas only one histidine or lysine are associated. Spectral maxima are observed at 248 and 242 nm for histidine and lysine, respectively, in their association with $Co(DMGH)_2$, with isosbestic points at 240 and 238 nm, respectively. Equilibrium constants calculated from the observed increases in absorbance at 250 nm are 4.1×10^4 M^{-1} for histidine and 26×10^4 M^{-1} for lysine, both determined at 25.0 °C, and are reflective of the relative basicities of these amino acids. Association of cysteine with $Co(DMGH)_2$ appears to occur in two stages. Initial association (Figure 8a) produces a spectral maximum at 250 nm with an associated isosbestic point at 218 nm. The equilibrium constant calculated from this data is 6.4×10^4 M^{-1}. Further addition of cysteine to the cobalt(II) glyoximate complex produces a new spectral band at 319 nm (Figure 8b), in addition to that at 250 nm, from which the association constant for the combination of $(CysSH)Co(DMGH)_2$ with a second cysteine is estimated to be less than 2×10^3 M^{-1}.

Discussion

Kinetic studies of nitrosyl transfer from $Co(NO)(DMGH)_2$ to hemoglobin suggest first-order dependence on the concentration of the heme iron(II) unit of hemoglobin and on the concentration of $Co(NO)(DMGH)_2$. Inverse dependence on the concentration

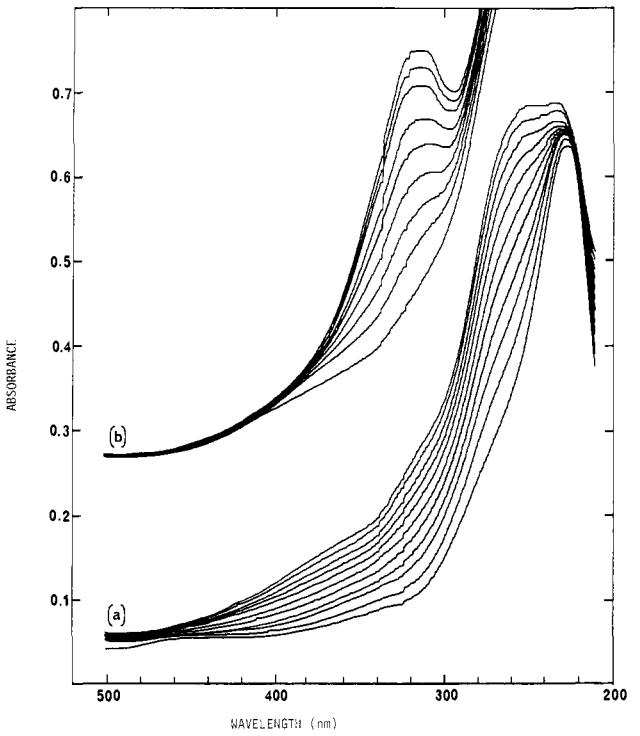

Figure 8. (a) Spectral changes in the UV–visible region for sequential additions of cysteine (0.102×10^{-5} M per unit addition) to $Co(DMGH)_2$ (2.03×10^{-5} M) at constant volume in deoxygenated 0.05 M phosphate buffer, pH 7.0, at 25.0 °C. The initial spectrum of $Co(DMGH)_2$ exhibits a maximum at 226 nm. (b) Spectral changes upon addition of 0.25 molar equiv portions of cysteine (0.510×10^{-5} M per unit addition) commencing with the solution containing 0.50 molar equiv of cysteine, based on $Co(DMGH)_2$. Spectrum is vertically displaced by 0.20 absorbance units.

Scheme II

$$Co(NO)(DMGH)_2 + L \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} LCo(NO)(DMGH)_2 \quad (5)$$

$$Co(NO)(DMGH)_2 \underset{k_{-6}}{\overset{k_6}{\rightleftharpoons}} Co(DMGH)_2 + NO \quad (6)$$

$$Co(DMGH)_2 + B \underset{k_{-7}}{\overset{k_7}{\rightleftharpoons}} BCo(DMGH)_2 \quad (7)$$

$$Hb + NO \xrightarrow{k_8} HbNO \quad (8)$$

of hemoglobin is related to the associative capabilities of $Co(NO)(DMGH)_2$ and $Co(DMGH)_2$ with the nucleophilic amino acid residues that constitute the globin proteins. The nitrosyl transfer reagent exhibits exceptional specificity in its association with the β93-cysteine sulfhydryl groups of hemoglobin whereas $Co(DMGH)_2$, the anticipated product from nitrosyl transfer, is capable of relatively indiscriminant association with nucleophilic amino acid residues. The data obtained appear to be consistent with the sequence of reactions described in Scheme II. The cobalt nitrosyl reagent is proposed to undergo reversible dissociation of nitric oxide (eq 6) which is capable of subsequent association with hemoglobin (eq 8). Nucleophilic substrates (L), which are identified in this investigation as accessible cysteine sulfhydryl groups, associate with the cobalt nitrosyl reagent to form the corresponding ligated cobalt nitrosyl complex, which is considered to be less susceptible to nitric oxide dissociation than the unassociated complex.[30] The electrophilic $Co(DMGH)_2$ is captured by basic substrates (B), which includes accessible cysteine residues of hemoglobin as well as histidine and lysine, to form the corresponding ligated adducts.

By applying the steady-state approximation to eq 5–8, and assuming that $k_{-6}[Co(DMGH)_2] >> k_8[Hb]$, $k_6[Co(NO)(DMGH)_2] >> k_{-7}[BCo(DMGH)_2]$, and $k_7[B] >> k_{-6}[NO]$, the following rate expression can be derived:

$$\text{rate} = \frac{d[HbNO]}{dt} = \frac{k_7 k_8[Hb][B]}{k_{-6}(k_5[L] + k_6)}\left(\frac{k_{-5}[Co(NO)(DMGH)_2]_0}{[Co(NO)(DMGH)_2]} - k_{-5} + k_{-6}\right) \quad (9)$$

The term $[Co(NO)(DMGH)_2]_0$ is the total concentration of added cobalt nitrosyl reagent, and $[Co(NO)(DMGH)_2]$ is the actual concentration of this reagent in the presence of nucleophilic substrates such as cysteine, This rate expression describes the observed first-order dependence of the rate for nitrosyl transfer on the concentrations of hemoglobin and $Co(NO)(DMGH)_2$, the inverse dependence on [L] (Figures 3a and 4), which in the absence of added cysteine is equal to half the concentration of reactant hemoglobin, and a relationship between the rate of reaction and the $[B]/[Co(NO)(DMGH)_2]$ ratio (Figure 5). The distinctly linear relationship observed for plots of $k_{obsd}/[Co(NO)(DMGH)_2]_0$ vs. $1/[L]$ in Figures 3a and 4 suggest that $k_5[L] >> k_6$ in the concentration ranges that have been employed.

When the β93-cysteine sulfhydryl group is blocked, $[Co(NO)(DMGH)_2] = [Co(NO)(DMGH)_2]_0$ and $[L] = 0$. The rate expression (eq 9) reduces to rate $= k_7 k_8[Hb][B]/k_{-6}$ and a plot of k_{obsd} vs. [B] is anticipated to exhibit a linear relationship with a slope equal to nk_7k_8/k_{-6}, where n is the number of basic amino acid residues that are capable of interaction with $Co(DMGH)_2$. However, inverse dependence on hemoglobin concentration is observed (Figure 3b), which suggests that nitrosyl transfer is inhibited by basic amino acid residues of hemoglobin. This inverse dependence implicates ligated cobalt(II) glyoximate as a nitric oxide acceptor in a transformation that, at least with histidine and lysine as ligands, results in the displacement of these bases (eq 10). When eq 10 is included with eq 6–8 in Scheme II, the

$$BCo(DMGH)_2 + NO \xrightarrow{k_{10}} Co(NO)(DMGH)_2 + B \quad (10)$$

$$\text{rate} = \frac{d[HbNO]}{dt} = \frac{k_6 k_8[Hb][Co(NO)(DMGH)_2]}{(k_{-6} + k_{10}K_7[B])[Co(DMGH)_2]} \quad (11)$$

corresponding rate expression becomes eq 11, where $[Co(DMGH)_2] = (k_6[Co(NO)(DMGH)_2] + k_{-7}[BCo(DMGH)_2]/(k_{-6}[NO] + k_7[B])$, a constant dependent on the ratio $[Co(NO)(DMGH)_2]/[B]$ when $k_{-7}[BCo(DMGH)_2] >> k_6[Co(NO)(DMGH)_2]$ and $k_{-6}[NO] >> k_7[B]$. The kinetic data described in Figures 3b and 6 correspond with the rate law of eq 11 and suggest that $k_{-6} >> k_{10}K_7[B]$. Similarly, when $[Co(NO)(DMGH)_2] = (k_{-5}[Co(NO)(DMGH)_2] + (k_6 - k_{-5})[Co(NO)(DMGH)_2])/(k_5[L] + k_6)$ is substituted into eq 11, the resulting equation adequately describes the relationships that are observed in Figures 3a, 4, and 5.

In the absence of the β93-cysteine sulfhydryl group eq 11 adequately represents the governing rate law for nitrosyl transfer. In the presence of $Co(DMGH)_2$ whose concentration is in excess of the concentration of the β93-cysteine sulfhydryl group, association of $Co(DMGH)_2$ with cysteine effectively prevents competitive association with $Co(NO)(DMGH)_2$ ($K_7/K_5 \simeq 800$), and eq 11 is also operative under these conditions. When $[Co(DMGH)_2]_0 < [B]$, $[Co(DMGH)_2]_0 \simeq [BCo(DMGH)_2]$ and nitric oxide association occurs competitively with $BCo(DMGH)_2$ (eq 10) and hemoglobin (eq 8). However, when $[Co(DMGH)_2]_0 > [B]$, $[Co(DMGH)_2]_0 = [Co(DMGH)_2] + [BCo(DMGH)_2]$ and $[BCo(DMGH)_2] \simeq [B]_0$. So long as $[Co(DMGH)_2]_0 < [B]$ inhibition of nitrosyl transfer by added $Co(DMGH)_2$ will be governed principally by eq 10; when $[Co(DMGH)_2]_0 > [B]$, both $BCo(DMGH)_2$ and the aquated $Co(DMGH)_2$ compete for nitric oxide association. Thus the slopes of the lines obtained from plots of $[Co(NO)(DMGH)_2]_0/k_{obsd}$ vs. $[Co(DMGH)_2]_0$ (Figure 1) should reflect the number of basic amino acid groups of hemo-

(30) By analogy to the effect of axial bases on the stability of cobalt dioxygen complexes: (a) Carter, M. J.; Rillema, D. P.; Basolo, F. *J. Am. Chem. Soc.* **1974**, *96*, 392. (b) Basolo, F.; Hoffman, B. M.; Ibers, J. A. *Acc. Chem. Res.* **1975**, *8*, 384.

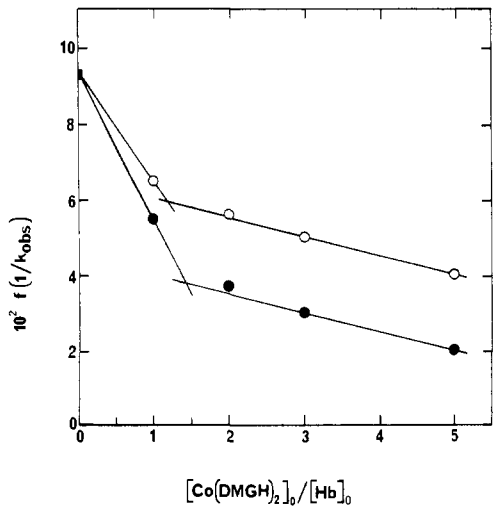

Figure 9. Plot of the slopes of the lines from Figure 1 (●) and from the corresponding data for iodoacetamide-modified hemoglobin (○), $f(1/k_{obsd}) = [Co(NO)(DMGH)_2]_0/k_{obsd}[Co(DMGH)_2]_0$, vs. $[Co(DMGH)_2]_0/[Hb]_0$. Intersections occur at $[Co(DMGH)_2]_0/[Hb]_0$ values of 1.42 for unmodified hemoglobin and at 1.15 for iodoacetamide-modified hemoglobin.

Scheme III

$$Co(NO)(DMGH)_2 \rightleftharpoons Co(DMGH)_2 + NO \quad (12)$$
$$+B \downarrow\uparrow -B \qquad +B \downarrow\uparrow -B$$
$$BCo(NO)(DMGH)_2 \rightleftharpoons BCO(DMGH)_2 + NO \quad (13)$$
$$NO + O_2 \rightleftharpoons ONOO \quad (14)$$
$$ONOO + Co(DMGH)_2 \rightarrow Co(NO_3)(DMGH)_2 \quad (15)$$
$$2BCo(NO)(DMGH)_2 + O_2 \rightarrow 2BCo(NO_2)(DMGH)_2 \quad (16)$$

globin that are available for association with $Co(DMGH)_2$: slope $\simeq k'[B]$ when $[Co(DMGH)_2]_0 < [B]$ and slope $\simeq k''$ when $[Co(DMGH)_2]_0 >> [B]$. Figure 9 describes the results obtained from a plot of the values for the slopes of the lines from Figure 1 vs. $[Co(DMGH)_2]_0/[Hb]_0$ with the estimated zero point obtained from nitrosyl-transfer reactions with β93-cysteine blocked hemoglobin. Similar data for the iodoacetamide-modified hemoglobin, analogous to that derived from Figure 1, is also reported in Figure 9. If the points of intersection in this figure can be interpreted as defining the relative number of amino acid residues of hemoglobin that freely associate with $Co(DMGH)_2$, then four amino acids per hemoglobin tetramer in cysteine-blocked hemoglobin and six amino acids in the unmodified hemoglobin tetramer are operative. The same number of hemoglobin amino acids, including the two β93-cysteine residues, effectively bind copper(II).[17]

Equations 6, 7, and 10 describe a closed chemical system, consistent with the known stabilities of cobaloxime nitrosyls toward nitric oxide dissociation, that maintains nitric oxide in an estimated low concentration.[31] However, in the presence of a nitric oxide acceptor such as hemoglobin, $Co(DMGH)_2$ and the nitric oxide acceptor compete for unassociated nitric oxide. The occurrence

Scheme IV

$$Co(NO)(DMGH)_2 \rightleftharpoons Co(DMGH)_2 + NO \quad (17)$$
$$NO + MbO_2 \rightarrow Mb^+ + ONOO^- \quad (18)$$
$$ONOO^- \rightarrow NO_3^- \quad (19)$$

of nitrosyl transfer is thus a function of the relative donor–acceptor capabilities of $Co(DMGH)_2$ and the added nitric oxide acceptor. By analogy,[6] $Co(NO)(TMGH)_2$ and $[CoCl(NO)(en)_2]ClO_4$ exhibit the same characteristics in nitrosyl transfer reactions.

Nitric oxide dissociation by $Co(NO)(DMGH)_2$ also explains the production of $Co(NO_3)(DMGH)_2$ in the reaction of $Co(NO)(DMGH)_2$ with molecular oxygen[29] and accounts for the relative insensitivity of the nitrato/nitro product ratio to the nature and concentration of added bases (Scheme III). In this case molecular oxygen is the nitric oxide acceptor (eq 14).[32] Similar behavior by $[Ru(NO)_2(PPh_3)_2]$, nitrosyl transfer[1] and oxidation by molecular oxygen to the nitrato complex $[Ru(NO)(NO_3)(O_2)(PPh_3)_2]$,[33] suggests that nitric oxide dissociation also occurs from this nitrosyl complex. Indeed, nitric oxide dissociation from metal nitrosyls may be generally responsible for the occurrence of nitrosyl transfer[1,2] and of nitrosyl → nitrato complex formation,[29,33,34]

Further clarification of the mechanism for nitrosyl transfer has been obtained from reactions of oxymyoglobin with $Co(NO)(DMGH)_2$ in deoxygenated phosphate-buffered media. Stoichiometric conversion of oxymyoglobin to metmyoglobin occurs with the addition of an equivalent amount of this cobalt nitrosyl reagent without intermediate formation of deoxymyoglobin. Like the nitrosyl-transfer process, the rate for this oxidative transformation is first order $[MbO_2]$ and first order in $[Co(NO)(DMGH)_2]$. The stoichiometry and rate law for oxidation portray the mechanism described in Scheme IV, where eq 18 and 19 have been previously established by direct observation of the oxidation of oxymyoglobin with nitric oxide ($k_{18} = 37 \times 10^6$ M^{-1} s^{-1}).[35] If eq 17 is operative both in this transformation and in the nitrosyl-transfer process, the ratio of the rate constants for oxidation of oxymyoglobin by $Co(NO)(DMGH)_2$ and nitrosyl transfer to hemoglobin, obtained at the same concentration of hemoprotein, should be approximately equal to the ratio of the rate constants for nitric oxide oxidation of oxymyoglobin[35] and nitric oxide association with hemoglobin.[26a] The experimentally determined ratio for $Co(NO)(DMGH)_2$ induced transformations is 2.00, whereas the corresponding ratio for direct nitric oxide induced reactions is 1.85.[36] This agreement further confirms the validity of the nitric oxide dissociative mechanism for $Co(NO)(DMGH)_2$.

Acknowledgment. Support of this work by the United States Public Health Service (Grant No. ES 01673) is gratefully acknowledged. We wish to thank F. J. Van Doornik and C. L. Funckes for their initial efforts in this research and the National Science Foundation for their URP award to B.R.C. for the summer of 1980.

Registry No. $Co(NO)(DMGH)_2$, 36509-25-8; Hb, 9034-51-9; $Co(DMGH)_2$, 36451-49-7; CysSH, 52-90-4; Lys, 56-87-1; His, 71-00-1.

(31) In these investigations the concentration of nitric oxide is maintained at a constant level, $k_{obsd} = k_8[NO]_0$. The concentration of nitric oxide can be estimated from the measured values for k_{obsd} (this study) and k_8 (2×10^7 M^{-1} s^{-1}).[26a]

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(36) Full details of the oxidation of oxymyoglobin will be the subject of a forthcoming publication.