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Effect of Strain in the Proximal Ligand on the Binding of Nitric Oxide and Carbon Monoxide to Chelated Protoheme Complexes

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The binding of NO and CO to chelated protoheme-L-histidine methyl ester (HM-H), protoheme-glycyl-L-histidine methyl ester (HM-GH), and free protoheme (HM) has been studied in methanol–DMSO solution. In all cases, the NO adducts are five-coordinated, indicating that binding of NO occurs with displacement of the axial base, and confirms the strong negative trans effect exerted by NO in heme complexes, though it is found that the presence of strain in the iron–histidine bond of HM-H has a positive influence on NO binding, making it thermodynamically more favorable than for HM-GH. The equilibrium constants thus decrease in the series: HM > HM-H > HM-GH. In contrast to NO, CO has a positive trans effect, and therefore, an opposite trend is observed in the binding of this ligand to the heme complexes.

Introduction

The interaction of nitric oxide with the iron centers of heme proteins has primary importance in a number of in vivo activities.[1] For instance, NO acts as a physiological messenger by binding to the heme cofactor of soluble guanylate cyclase (sGC), thereby triggering a cascade of metabolic events that promotes smooth muscle relaxation.[2−4] A generally accepted mechanism of sGC activation involves displacement of the proximal axial histidine ligand of the resting enzyme by NO,[5] with the resulting five-coordinate Fe^{II}−NO complex activating the catalytic conversion of GTP to cGMP, although this has been questioned recently.[6] In other cases, e.g., with myoglobin, hemoglobin, and horseradish peroxidase,[7,8] nitric oxide binds to the heme without displacing the proximal ligand, except at low pH,[9−11] or in the presence of allosteric effectors.[12,13] Nitric oxide binds strongly to ferrous complexes of natural and synthetic porphyrins and less strongly also to the corresponding ferric complexes.[14−18] Normally, addition of NO to a heme complex results in the formation of the five-coordinate adduct, even in the presence of a large excess of base.[19] In order to get the six-coordinate NO adduct of protoheme, a concentration of 1-methylimidazole as high as 20% (v/v) had to be used in the solution.[20,21] Quantitative data on the binding of NO

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(1) Ignarro, L., Ed. *Nitric Oxide: Biology and Pathobiology*; Academic Press: San Diego, 2000.
(2) Moncada, S.; Palmer, R. M. J.; Higgs, E. A. *Pharmacol. Rev.* **1991**, *43*, 109−142.
(3) Ignarro, L. J. *Biosci. Rep.* **1999**, *19*, 51−71.
(4) Schlossmann, J.; Hofman, F. *Drug Discovery Today* **2005**, *10*, 627−634.
(5) (a) Denninger, J. W.; Marletta, M. A. *Biochim. Biophys. Acta* **1999**, *1411*, 334−350. (b) Dierks, E. A.; Hu, S.; Vogel, K. M.; Yu, A E.; Spiro, T. G.; Burstyn, J. N. *J. Am. Chem. Soc.* **1997**, *119*, 7316−7323.
(6) Cary, S. P.; Winger, J. A.; Derbyshire, E. R.; Marletta, M. A. *Trends Biochem. Sci.* **2006**, *31*, 231−239.
(7) Hoshino, M.; Laverman, L. E.; Ford, P. C. *Coord. Chem. Rev*. **1999**, *187*, 75−102.
(8) Møller, J. K. S.; Skibsted, L. H. *Chem. Rev*. **2002**, *102*, 1167−1178.
(9) Duprat, A. F.; Traylor, T. G.; Wu, G. Z.; Coletta, M.; Sharma, V. S.; Walda, K. N.; Magde, D. *Biochemistry* **1995**, *34*, 2634−2644.
(10) Coletta, M.; Ascenzi, P.; Smulevich, G.; Mantini, A. R.; Del Gaudio, R.; Piscopo, M.; Geraci, G. *FEBS Lett.* **1992**, *296*, 184−186.
(11) Coletta, M.; Ascenzi, P.; Polizio, F.; Smulevich, G.; Del Gaudio, R.; Piscopo, M.; Geraci, G. *Biochemistry* **1998**, *37*, 2873−2878.
(12) Ascenzi, P.; Coletta, M.; Santucci, R.; Polizio, F.; Desideri, A. *J. Inorg. Biochem*. **1994**, *53*, 273−280.
(13) Polizio, F.; De Sanctis, G.; Ascenzi, P.; Coletta, M. *J. Biol. Inorg. Chem*. **1998**, *3*, 458−462.
(14) Antonini, E.; Brunori, M. *Hemoglobin and Myoglobin in Their Reactions with Ligands*; North Holland: Amsterdam, 1971.
(15) Wyllie, G. R. A.; Scheidt, W. R. *Chem. Rev*. **2002**, *102*, 1067−1089.
(16) Lim, M. D.; Lorkovic, I. M.; Ford, P. C. *J. Inorg. Biochem*. **2005**, *99*, 151−165.
(17) Ford, P. C.; Lorkovic, I. M. *Chem. Rev.* **2002**, *102*, 993−1017.
(18) Scheidt, W. R.; Ellison, M. K. *Acc. Chem. Res.* **1999**, *32*, 350−359.
(19) Kharitonov, V. G.; Sharma, V. S.; Magde, D.; Koesling, D. *Biochemistry* **1997**, *36*, 6814−6818.
(20) Romberg, R. W.; Kassner, R. J. *Biochemistry* **1979**, *18*, 5387−5392.

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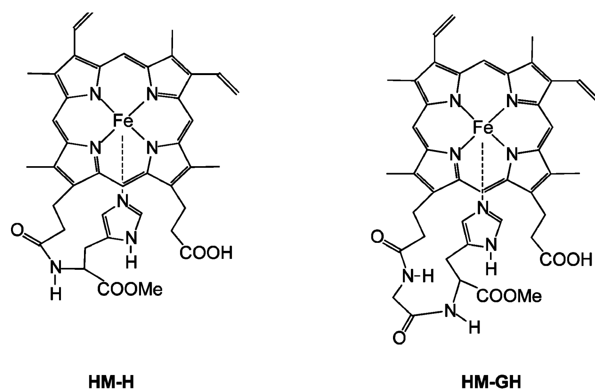
Chart 1



to low-molecular-weight heme complexes are scarce, particularly in the case of natural porphyrins. They basically refer to the six-coordinate protoheme(1-MeIm)NO complex (1-MeIm = 1-methylimidazole) in 20% (v/v) 1-MeIm/aqueous buffer, studied by Romberg and Kassner[20] and Rose and Hoffman[21] and to the five-coordinate chelated protoheme−NO complex (with unbound imidazole tether).[22,23] In contrast to NO, binding of CO to heme complexes has been extensively studied.[24] We thought that to obtain reliable data on the negative trans effect of NO on heme complexes it could be useful to employ chelated heme complexes with variable strength in the axially bound ligand. As we have shown previously by computational methods,[25] axial coordination of imidazole to iron(III) in the chelated deuterohemin−histidine complex is strained due to the limited size of the chelate ring carrying the imidazole donor, but this strain is removed when the chelate ring is enlarged with introduction of a second amino acid in the chelating arm. Therefore, we report here comparative NO and CO binding data on the covalently modified heme complexes protoheme-L-histidine methyl ester (HM-H) and protoheme-glycyl-L-histidine methyl ester (HM-GH), containing an axially bound histidine (Chart 1),[26] and on protoheme (HM).

Experimental Section

Compounds and solvents accessible from commercial sources were of highest purity available and used as received. Anhydrous methanol and DMSO were obtained by treatment with activated 3 Å molecular sieves. Nitric oxide (SIAD, Bergamo, Italy) was passed through a degassed column of NaOH pellets to remove higher nitrogen oxides before use. Optical absorption spectra were recorded with HP 8452A or HP 8453 diode-array spectrophotometers. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker AC 300 spectrometer operating at 7.03 T. Elemental analyses were performed at the microanalytical laboratory of the Chemistry Department in Milano. ESI-MS and FAB-MS spectra were obtained with a Thermo-Finnigan LCQ Advantage and a VG 7070 EQ spectrometer, respectively.

Synthesis of Glycyl-L-Histidine Methyl Ester (GH). The peptide was prepared with a modification of the procedure previously used.[27] *Z*-Glycyl-L-histidine (1 g) was esterified according to a general procedure, thus obtaining *Z*-glycyl-L-histidine methyl ester dihydrochloride. Then, the *N*-benzyloxycarbonyl group (*Z*-group) was removed from the protected peptide by dissolving the peptide in 80% acetic acid (30 mL) and adding palladium−charcoal (10% palladium content, 1 g). The mixture was hydrogenated at atmospheric pressure with stirring until carbon dioxide evolution ceased. The catalyst was removed by filtration on Celite, and the filtrate was evaporated to dryness under vacuum. The residue, suspended in diethyl ether, was stirred for 1 h, and then the product was filtered off and washed several times with diethyl ether and dried under vacuum over potassium hydroxide. The dihydrochloride salt H-Gly-L-HisOMe•2HCl (0.7 mmol) was suspended in dry, cooled (0 °C) dichloromethane (30 mL). Then, gaseous NH_3 (Aldrich) was bubbled into the mixture under stirring. The resulting precipitate was filtered off and the filtrate was concentrated. The precipitate was submitted twice to the same treatment and the filtrate, after evaporation of the solvent, afforded the free peptide (0.57 mmol). Anal. Calcd for $C_9H_{14}N_4O_3$ (226.24): C 47.77; H 6.24; N 24.77. Found: C 47.24; H 6.33; N 24.14. ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C, TMS): δ 3.00−3.31 (m, 2H, CH_2-imidazole), 3.54 (s, 2H, CH_2-NH_2), 3.67 (s, 3H, OCH_3), 4.81 (t, 1H, α-CH), 6.80 (m, 1H, imidazole-4CH), 7.44 (m, 1H, imidazole-2CH). ^{13}C NMR (75.5 MHz, $CDCl_3$, 25 °C, TMS): δ 29.4, 42.9, 51.1, 52.7, 119.6, 133.5, 135.9, 170.4, 171.6. ESI-MS: m/z (%) = 226 (100) $[M]^{\cdot+}$.

Synthesis of HM-H and HM-GH. The ferric forms of HM-H and HM-GH were prepared as described in the literature.[26,28,29] The FAB-MS spectra of HM-H and HM-GH exhibit cluster of peaks centered at m/z = 768 and 824 uma, respectively, for the cationic ferric complexes, in perfect agreement with the simulated spectra.

Binding Experiments. The binding experiments were carried out anaerobically in methanol−DMSO (9:1, v/v) using optical cells of 1 cm pathlength fitted with Schlenk connections. Solutions of the ferric forms of HM, HM-H, and HM-GH in the cuvette were prepared by adding to the methanol−DMSO (9:1, v/v) solvent a few microliters of a concentrated solution of the complexes in DMSO. The spectra of the iron(III) complexes turn from sharp Soret peaks, observed immediately after dilution, to slightly broader peaks during a few minutes, indicating a moderate aggregation of the complexes in the methanol−DMSO solvent mixture. After removal of molecular oxygen through several vacuum−argon cycles, the iron(II) species were obtained by reduction of iron(III) with the minimum amount of $NaBH_4$. Saturated NO and CO solutions (1 atm) were prepared in degassed methanol, assuming solubilities of 1.4×10^{-2} M atm^{-1} for NO[30] and 4.2×10^{-4} M atm^{-1} for CO[31]

(21) Rose, E. J.; Hoffman, B. M. *J. Am. Chem. Soc.* **1983**, *105*, 2866−2873.
(22) Traylor, T. G.; Sharma, V. S. *Biochemistry* **1992**, *31*, 2847−2849.
(23) Traylor, T. G.; Duprat, A. F.; Sharma, V. S. *J. Am. Chem. Soc.* **1993**, *115*, 810−811.
(24) (a) Traylor, T. G. *Acc. Chem. Res.* **1981**, *14*, 102−109. (b) Jameson, G. B.; Ibers, J. A. *Comments Inorg. Chem.* **1983**, *2*, 97−126. (c) Collman, J. P.; Fu, L. *Acc. Chem. Res.* **1999**, *32*, 455−463. (d) Collmann, J. P.; Boulatov, R.; Sunderland, C. J.; Fu, L. *Chem. Rev.* **2004**, *104*, 561−588. (e) Spiro, T. G.; Wasbotten, I. H. *J. Inorg. Biochem.* **2005**, *99*, 34−44. (f) Silvemail, N. J.; Roth, A.; Schulz, C. E.; Noll, B. C.; Scheidt, W. R. *J. Am. Chem. Soc.* **2005**, *127*, 14422−14433.
(25) Casella, L.; Monzani, E.; Fantucci, P.; Gullotti, M.; De Gioia, L.; Strini, A.; Chillemi, F. *Inorg. Chem.* **1996**, *35*, 439−444.
(26) De Sanctis, G.; Fasciglione, G. F.; Marini, S.; Sinibaldi, F.; Santucci, R.; Monzani, E.; Dallacosta, C.; Casella, L.; Coletta, M. *J. Biol. Inorg. Chem.* **2006**, *11*, 153−167.
(27) Monzani, E.; Linati, L.; Casella, L.; De Gioia, L.; Favretto, M.; Gullotti, M.; Chillemi, F. *Inorg. Chim. Acta* **1998**, *273*, 339−345.
(28) Roncone, R.; Monzani, E.; Murtas, M.; Battaini, G.; Pennati, A.; Sanangelantoni, A. M.; Zuccotti, S.; Bolognesi, M.; Casella, L. *Biochem. J.* **2004**, *377*, 717−724.
(29) Ryabova, E. S.; Dikiy, A.; Hesslein, A. E.; Bjerrum, M. J.; Ciurli, S.; Nordlander, E. *J. Biol. Inorg. Chem.* **2004**, *9*, 385−395.
(30) Tran, D.; Skelton, B. W.; White, A. H.; Laverman, L. E.; Ford, P. C. *Inorg. Chem.* **1998**, *37*, 2505−2511.

at 25 °C. For the titration experiments, small aliquots of ligand solutions were transferred to the cuvette containing the heme solution (typically 0.5−1 μM) using argon-purged airtight syringes. Spectra were taken after each addition and corrected for dilution. The equilibrium constants were determined by fitting the plots of Δabsorbance between the wavelength maxima of the adduct and the starting iron(II) complex vs ligand concentration using the hyperbolic portion of the curve.

The binding of imidazole to HM was studied anaerobically in methanol−DMSO (9:1, v/v) solution by titrating the heme (∼1.2 μM) with a concentrated solution of imidazole (52 mM) in degassed methanol; the spectra were recorded with a custom-designed immersible fiber-optic quartz probe (HELLMA), with 0.5 cm pathlength, fitted to a Schlenk vessel and connected with the diode-array spectrophotometer. The imidazole binding constant to HM was obtained as reported above for NO and CO.

Results and Discussion

The complexes HM-H and HM-GH are obtained as an isomeric 1:1 mixture containing the histidine substituent arm bound to one of the carboxylate chains at positions 2 or 18 of the porphyrin ring. The pattern of substitution on the porphyrin ring does not influence the binding of ligands at the iron center. The NO and CO binding studies to HM, HM-H, and HM-GH were performed in methanol−DMSO 9:1 (v/v) solutions of the heme complexes, the use of DMSO being dictated by the necessity to minimize porphyrin aggregation. This problem is severe for free HM, while the presence of the chelating arm in HM-H and HM-GH reduces the extent of aggregation. Natural porphyrins and their metal complexes exhibit a strong tendency to aggregation, but solutions of these compounds in aqueous mixtures with solvents like DMSO and DMF, both with large Gutman donor numbers,[32] reduce the extent of aggregation.[33] We previously found that protohemin is essentially monomeric in a DMSO−aqueous buffer solution 1:10 (v/v) at 0.1 μM concentration.[34] The concentration needed to perform the ligand binding experiments here are larger, but we can assume that the monomeric heme complexes are the major forms present in the solutions.

Protoheme is monomeric and six-coordinate in pure DMSO solution (with λ_{max} = 424, 524, and 552 nm), with two solvent molecules bound in the axial positions.[35] When a DMSO solution of HM is diluted in methanol−DMSO 9:1, the complex partially aggregates and likely distributes into different species; the electronic spectrum (with λ_{max} = 416, 522, and 548 nm) suggests that a species with mixed DMSO and methanol coordination becomes dominant in these conditions. An indication of the relative strength of DMSO binding to the heme could be obtained by the measurement of the binding constant of imidazole to HM in the mixed solvent of methanol−DMSO 9:1 (v/v). Upon adding imidazole to HM, the spectral maxima change to 422, 524, and 554 nm, with maintenance of several isosbestic points (Figure 1). The titration clearly indicates the binding of a single imidazole molecule (Hill coefficient of 1.01 ± 0.01) with a constant of 680 ± 10 M^{-1}. The final spectrum of the HM−imidazole complex resembles those of HM-H and HM-GH in the same solvent and that of the HM−imidazole complex in pure DMSO,[35] indicating the presence of a six-coordinated heme with a bound DMSO molecule. The binding constant of imidazole to HM obtained here is 1−2 orders of magnitude smaller than those obtained for reduced hemes in solvents of weaker donor strength,[36] showing that DMSO is a relatively strong ligand for the reduced heme. Generally, data on the binding of a donor base to heme complexes are difficult to obtain because the binding of a second base molecule, to form a six-coordinated bis(base)−heme complex, occurs with a greater equilibrium constant.[36]

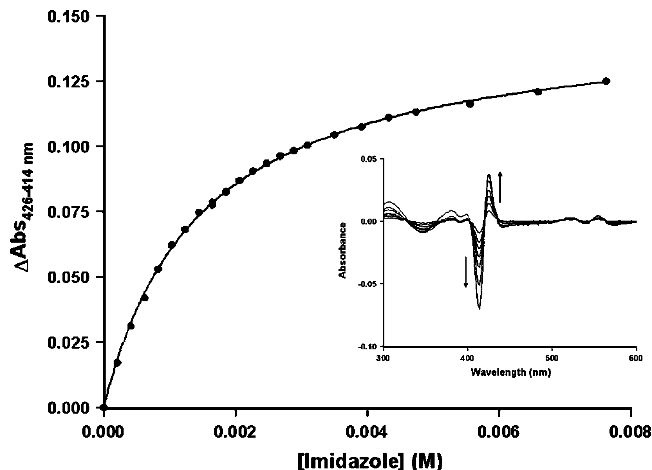


Figure 1. Plot of $\Delta A_{426-414}$ vs imidazole concentration for the titration of a 7.2 × 10^{-6} M solution of HM in methanol−DMSO (9:1, v/v) with imidazole. The pathlength of the immersible fiber-optic quartz probe (HELLMA) used for this experiment was 0.5 cm. The inset shows the difference spectra with the isosbestic point observed in the titration.

Addition of excess NO to solutions of HM, HM-H, and HM-GH leads to an adduct with optical features (Soret band at 392 nm, a weak shoulder near 480 nm, and β and α bands at 532 and 572 nm) which are characteristic of five-coordinated protoheme−NO species,[37,38] and similar to those of the sGC−NO complex.[39] The weakening effect exerted by NO on the trans axial ligand bound to the heme is well known.[18,40] This results, for instance, in a very small binding constant for imidazole to heme−NO complexes.[41] However, for the three complexes studied here, spectral titrations with NO show that the ease with which the trans axial ligand to

(31) Liu, Q.; Takemura, F.; Yabe, A. *J. Chem. Eng. Data* **1996**, *41*, 589−592.
(32) Gutman, V. *The Donor-Acceptor Approach to Molecular Interactions*; Plenum Press: New York, 1978.
(33) White, W. J. In *The Porphyrins*; Chapter 7, Academic Press: New York, 1979; Vol. V.
(34) Monzani, E.; Bonafè, B.; Fallarini, A.; Redaelli, C.; Casella, L.; Minchiotti, L.; Galliano, M. *Biochim. Biophys. Acta* **2001**, *1547*, 302−312.
(35) Mincey, T.; Traylor, T. G. *J. Am. Chem. Soc.* **1979**, *101*, 765−766.
(36) (a) James, B. R. In *The Porphyrins*; Chapter 6, Academic Press: New York, 1979; Vol. V. (b) Momenteau, M.; Reed, C. A. *Chem. Rev.* **1994**, *94*, 659−698.
(37) Yoshimura, T. In *Bioradicals Detected by ESR Spectroscopy*; Ohya-Nishiguchi, H., Parker, L., Eds.; Birkhäuser Verlag: Basel, 1995; pp 217−235.
(38) Yoshimura, T.; Osaki, T. *Arch. Biochem. Biophys.* **1984**, *229*, 126−135.
(39) Stone, J. R.; Marletta, M. A. *Biochemistry* **1994**, *33*, 5636−5640.
(40) McCleverty, J. A. *Chem. Rev.* **2004**, *104*, 403−418.
(41) Praneeth, V. K. K.; Näther, C.; Peters, G.; Lehnert, N. *Inorg. Chem.* **2005**, *45*, 2795−2811.

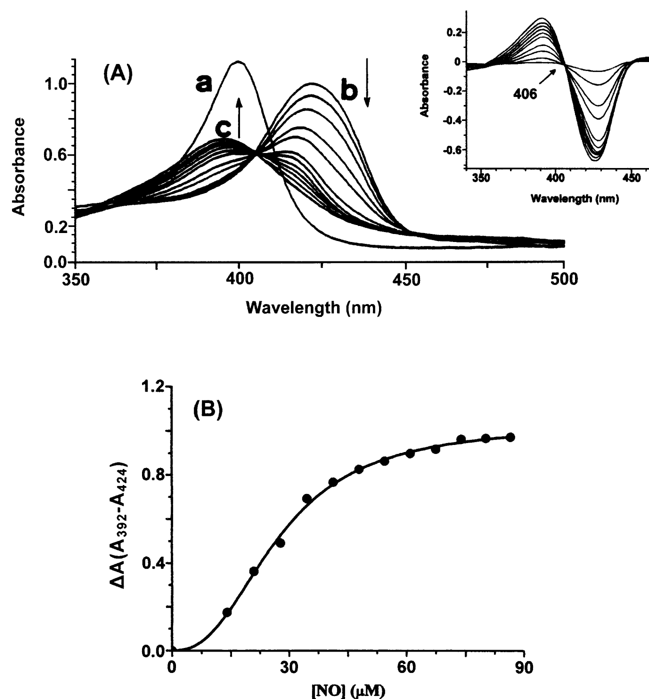


Figure 2. (A) Titration of 1.37 × 10^{-5} M solution of HM-GH in methanol−DMSO (9:1, v/v) with a saturated NO solution in methanol. The representative spectra refer to (a) the ferric protoheme complex (λ_{max} = 398 nm), (b) the ferrous protoheme complex (λ_{max} = 424 nm), and (c) the formation of the five-coordinate HM-GH−NO adduct (λ_{max} = 392 nm) upon addition of NO solutions from 7 up to 87 μM. The inset shows the difference spectra with isosbestic point at 406 nm, excluding the spectra corresponding to the initial part of the titration. (B) The corresponding plot of $\Delta A_{392-424}$ vs NO concentration (μM).

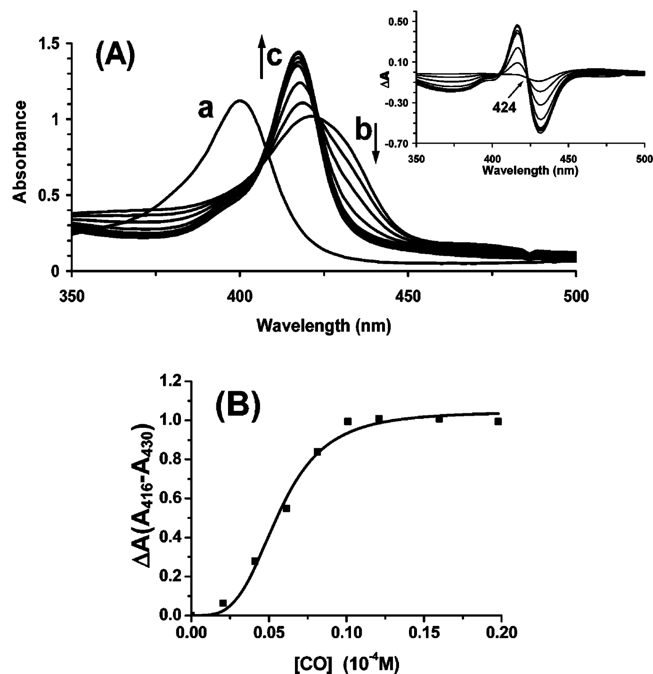
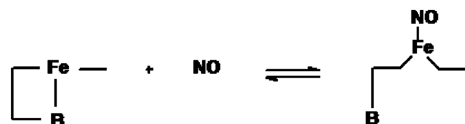


Figure 3. (A) Titration of 9.8 × 10^{-6} M solution of HM-GH in methanol−DMSO (9:1, v/v) with a saturated CO solution in methanol. The representative spectra refer to (a) the ferric protoheme complex (λ_{max} = 398 nm), (b) the ferrous protoheme complex (λ_{max} = 424 nm), and (c) the formation of the six-coordinate HM-GH−CO adduct (λ_{max} = 416 nm) upon addition of CO solutions from 0.004 up to 0.02 mM. The inset shows the difference spectra with isosbestic point at 424 nm, excluding the spectra corresponding to the initial part of the titration. (B) The corresponding plot of $\Delta A_{416-430}$ vs CO concentration.

Scheme 1



the Fe^{II} center is released is very different. Figure 2 shows the optical spectral changes observed in the titration of HM-GH with NO. Except for the initial phase, the spectra display isosbestic points, indicating that a simple binding equilibrium occurs with concomitant displacement of the trans-axial imidazole, as shown schematically in Scheme 1.

As shown by the $\Delta A_{392-424}$ vs [NO] plot, in the initial phase NO binding occurs to monomeric and oligomeric heme complexes and favors disaggregation.[42] The binding constant was therefore estimated by fitting the hyperbolic portion of the curve, discarding the initial part. The spectral behavior upon NO binding to the other heme complexes is similar, though the equilibrium constants reported in Table 1 show that a marked reduction in affinity occurs in the series HM > HM-H > HM-GH depending on the strength of the bond between the iron and the trans-axial ligand (DMSO for HM, imidazole for HM-H and HM-GH). The absolute values of the heme−NO binding constants that we found here are lower than those reported previously,[20−23] particularly for five-coordinated heme−NO complexes, for which, though,

Table 1. Binding Constants Obtained by Spectral Titration of Chelated Protohemin Complexes with NO and CO in MeOH−DMSO (9:1, v/v) Solutions at 25 °C

NO adduct	K_{NO} (M^{-1})	CO adduct	K_{CO} (M^{-1})	K_{NO}/K_{CO}
HM−NO	1.05 × 10^6	HM−CO	4.98 × 10^4	21.1
HM-H−NO	3.05 × 10^5	HM-H−CO	5.68 × 10^4	5.4
HM-GH−NO	3.66 × 10^4	HM-GH−CO	6.81 × 10^4	0.5

the data were reported with no experimental detail.[22,23] Therefore, at least compared with the data for six-coordinate imidazole−heme−NO complexes,[20,21] the binding constants found here are lower. This depends on the different solvent and in particular on the use of DMSO, which binds more strongly than water to the heme iron, as the NO binding process actually involves a ligand displacement reaction.

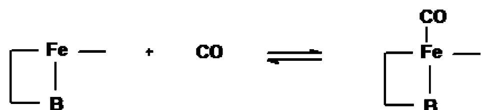
In contrast to NO, CO has a positive trans effect, as shown for instance by the increase in CO affinity by a variety of synthetic heme complexes for solvents of increased polarity,[24b,43] though the effect is difficult to rationalize in terms of donor strength of the axial base because σ and π bonding tend to counterbalance and the contributions of solvent polarity and solvation energy changes upon ligand binding are difficult to assess. The effect of strain in the chelating arm of substituted mesoheme complexes on the binding of O_2 and CO has been studied previously in micellar aqueous buffer to model the R and T states of hemoglobin.[44] We thus performed the CO binding experiments with HM, HM-H,

(42) As pointed out by a referee, we cannot exclude that traces of oxygen during transfer of the gas solution affect the initial data due to the extreme oxygen sensitivity of reduced heme.

(43) Rougee, M.; Brault, D. *Biochemistry* **1975**, *14*, 4100−4106.

(44) Geibel, J.; Cannon, J.; Campbell, D.; Traylor, T. G. *J. Am. Chem. Soc.* **1978**, *100*, 3575−3585.

Scheme 2



and HM-GH in methanol−DMSO 9:1 (v/v) expecting an opposite trend with respect to NO. Figure 3 shows a spectrophotometric titration of HM-GH with CO, which produces a six-coordinate adduct (Scheme 2). Also in this case the initial part of the spectral titration is affected by the presence of oligomeric species, which are progressively disaggregated on CO binding, as shown by the maintenance of isosbestic points after the initial addition of CO.[42] Similar binding experiments performed on HM and HM-H gave the equilibrium constants collected in Table 1. The spectra of the iron(II)−carbonyl species are different, reflecting the bonding type of the trans ligand. Therefore, the Soret band is at 414 nm for the CO adduct of HM, indicating a DMSO−HM−CO species,[35] and 416 nm for the corresponding adducts of HM-H and HM-GH. As for NO, binding of CO occurs as a displacement reaction with respect to the heme-bound DMSO; therefore, also the association constants of CO obtained in the methanol−DMSO 9:1 solvent mixture are orders of magnitude smaller than those obtained for reduced hemes in solvents of weaker donor strength,[24a−c,44] though it is interesting that CO binding is less sensitive to the strain in the chelating arm carrying the trans-imidazole ligand, and as a consequence, the ratio of K_{NO}/K_{CO} undergoes a marked reduction in the series HM, HM-H, HM-GH. The low sensitivity to strain in the imidazole coordination of the CO binding constant may also be connected to some compensation effect in the replacement of the heme-bound DMSO ligand, the binding strength of which is also likely affected by the trans ligand coordination.

Carbon monoxide activates sGC weakly and also binds relatively weakly to it ($\sim 10^4$ M^{-1}),[45,46] compared to, e.g., myoglobin ($\sim 10^7$ M^{-1}).[14] Thus, the CO binding strength to sGC must be strongly depressed, by some mechanism, to reduce the association constant to a value comparable to that found here for heme complexes in methanol−DMSO solution, while the CO affinity for myoglobin is in the range typically associated with CO binding to intermolecular imidazole−heme complexes in aqueous buffer.[21,22] In contrast, the affinity of sGC for NO must be very high, as the enzyme acts as a sensor of NO.[47,48] The affinity is likely regulated by other effector molecules in vivo,[48] but as shown here, the introduction of strain in the proximal imidazole−Fe^{II} bond provides by itself a significant contribution to the increase in the heme−NO binding strength.

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(45) Kharitonov, V. G.; Sharma, V. S.; Magde, D.; Koesling, D. *Biochemistry* **1999**, *38*, 10699−10706.
(46) Both the enzyme activity and CO binding to sGC are strongly increased in the presence of a benzylindazole derivative YC-1.[43]
(47) Bredt, D. S.; Snyder, S. H. *Annu. Rev. Biochem.* **1994**, *63*, 175−195.
(48) Lucas, K. A.; Pitari, G. M.; Kazerounian, S.; Ruiz-Stewart, I.; Park, J.; Schulz, S.; Chepenik, K. P.; Waldman, S. A. *Pharmacol. Rev.* **2000**, *52*, 375−414.