Anesthetic-like Interactions of Nitric Oxide with Albumin and Heme Proteins

A MECHANISM FOR CONTROL OF PROTEIN FUNCTION*

Vijaya Sampath‡‡, Xiao-Jian Zhao‡¶, and Winslow S. Caughey‡‡***

From the ‡Department of Biochemistry and Molecular Biology, Colorado State University, Fort Collins, Colorado 80523 and the †Laboratory of Persistent Viral Diseases, Rocky Mountain Laboratories, NIAID, National Institutes of Health, Hamilton, Montana 59840

Noncovalent bonding interactions of nitric oxide (NO) with human serum albumin (HSA), human hemoglobin A, bovine myoglobin, and bovine cytochrome c oxidase (CcO) have been explored. The anesthetic nitrous oxide (NNO) occupies multiple sites within each protein, but does not bind to heme iron. Infrared (IR) spectra of NNO molecules sequestered within albumin, with NO present, support the binding of NO and NNO to the same sites with comparable affinities. Perturbations of IR spectra of the Cys-S thiols and/or the iron(II) N-O stretching region denote changes in protein structure. Experiments evaluating the relative affinities of binding of NO and carbon monoxide (CO) to iron(II) sites of the heme proteins led to evidence of NO binding to noniron, nonsulfur sites as well. With HbA, IR spectra of cysteine thiols and/or the iron(II) N-O stretching region denote changes in protein structure due to NO, NNO, or CO occupying noniron sites with an order of decreasing affinities of NO > NNO > CO. Loss of NO from some, not all, noniron sites in heme proteins is very slow (τ90 ~ hours). These findings provide examples in which NO and anesthetics alter the structure and properties of protein similarly, and support the hypothesis that some physiological effects of NO (and possibly CO) result from anesthetic-like noncovalent bonding to sites within protein or other tissue components. Such bonding may be involved in mechanisms for control of oxygen transport, mitochondrial respiration, and activation of soluble guanylate cyclase by NO.

The mechanisms of anesthesia and the regulatory functions of nitric oxide (NO) remain far from clear. Potential roles of anesthetic-protein interactions in anesthesia have become of increasing interest (1–5) as have the interactions of NO with hemeproteins. Reactions of NO with HbA that result in protein conformation changes (6–10). Nitrosations (i.e. attack of protein residues by NO−) on exposure of proteins to NO with a suitable oxidant present are the only reactions of NO that have been discussed other than the binding of NO to metal sites. Evidence of NO occupying sites within proteins as an uncharged diatomic free radical by means of anesthetic-like noncovalent bonding has not been reported.

The binding of volatile anesthetics to serum albumins and the effects of such binding on protein structure are considered in several recent reports. Specific, saturable binding of halothane and other anesthetics to bovine serum albumin and anesthetic-induced alterations in the structure or carrier function of this protein have been shown (11–16). The anesthetic nitrous oxide (NNO) was detected at sites within human serum albumin (HSA) (5). Infrared (IR) spectra of bound NNO molecules demonstrated sites of two types. The exposure of HSA to NNO enhanced absorbance in the S-H stretching region of the IR spectrum giving evidence of an NNO-induced change in protein conformation near Cys204 (5). In 1992, NO was proposed to circulate in mammalian plasma primarily in association with albumin, due to formation of the S-nitrosothiol derivative (17). However, the similarities in the structure and physical properties of NNO and NO suggest the possibility that NO may also interact at some sites within albumin in the manner shown for NNO.

IR evidence of NNO molecules at multiple sites within hemoglobin, myoglobin, and cytochrome c oxidase (CcO) has also been obtained (5, 18). NNO neither serves as a ligand to heme iron nor reacts with thiols. The ability of NO to alter heme protein structure and function was shown by shifts in the IR spectra of cysteine thiols of HbA (5) and by partial and reversible inhibitions of CcO (18). The well established ability of NO to ligate to heme iron in HbA, Mb, and CcO, and to copper B in CoO (19, 20), led to the assumption of metal binding in proposed mechanisms for physiologically important reactions of NO with hemeproteins. Reactions of NO with HbA that result in 5-nitrosothiol derivative formation have also received much attention (8). However, alternative mechanisms whereby NO alters heme protein structure and function by noncovalent anesthetic-like bonding to sites that involve neither metal nor cysteine sulfur remain unexplored.

Much interest in possible physiological roles of carbon monoxide (CO), including a messenger role in the nervous system and the activation of soluble guanylate cyclase, has developed recently (21–23). The biochemical mechanisms related to these...
Interactions of NO, NNO, and CO with Proteins

Effects of CO have not been elucidated, but those proposed thus far have considered the ability of CO to bind to iron(II) of hemeproteins. The occupancy of nonmetal protein sites by CO, as with NO, remains unstudied.

We report here experimental results that indicate NO can reversibly occupy sites within HSA and hemeproteins in the manner of NNO (and other anesthetics) and, in so doing, can induce changes in the properties and structures of the proteins. These findings provide support for considering NO-protein interactions of this type as potentially important in at least some of the physiological roles of NO. Limited evidence of the ability of CO to occupy noniron as well as iron(III) sites in hemoglobin is also presented.

**EXPERIMENTAL PROCEDURES**

Materials—Fatty acid- and globulin-free human serum albumin (99% from Sigma) was used as received. Recrystallized heart cytochrome c oxidase and myoglobin were isolated and purified from fresh bovine heart as was hemoglobin A from human blood via methods described earlier (24–26). Nitric oxide was obtained as 15N16O (99% from General Air Services and Supply), CO (99.5% from General Air Services and Supply), N2 (99.9% from General Air Services and Supply), halothane (99% from Halocarbon Laboratories), and as 5% 14N16O, 95% N2 (technical grade, Air Products and Chemicals). All solutions were made anaerobic by evacuation and flushing with nitrogen.

**Albumin Studies**—Solutions of HSA in 200 mM Tris-Cl buffer, pH 7.2, were made anaerobic by evacuation and flushing with nitrogen. HSA solutions were exposed to varying amounts of 15N16O, NNO, and/or nitrogen to give a total pressure of 1 atm. Halothane and chloroform were added to HSA solutions as liquids. In each case the treated solution was allowed to stand for at least 1 h prior to recording IR spectra. All operations were carried out at 20 °C in both albumin and hemeprotein studies.

**Hemeprotein Studies**—Hemeprotein solutions were prepared in 200 mM sodium phosphate buffer, pH 7.2, under strictly anaerobic conditions as described earlier (19, 28). Several methods were used to detect the relative affinities of the proteins for NO and CO and the slow release of NO. In one method a solution (2.5 ml) of deoxy-Mb (or deoxy-Hb), 5 μM in heme and CO (unliganded and fully-reduced) at 10 μM in heme A was prepared in a sealed 1-cm path length spectrophotometer cell. A NO/N2 gas mixture or CO was gradually added incrementally via a gas-tight syringe under atmospheric pressure. The visible/Soret spectrum was measured after each addition and second-derivative analysis of the Soret spectrum carried out. In a second procedure, CcONO was prepared initially from a solution (2.5 ml) of CcO (10 μM in heme A) by slowly adding, at atmospheric pressure, an amount of NO/N2 (−0.3 ml) that was just sufficient for the complete ligation of heme a, iron(II) with NO as observed in visible/Soret spectra. N2 was then passed over the solution for 15 min to remove the dissolved free NO, as well as the NO present above the solution, without decreasing metal-bound NO. A solution of deoxy-Mb or deoxy-Hb at 8–10 mM in heme was introduced into the CcONO solution with a gas-tight syringe to give a solution containing 5 μM Mb or Hb. Changes in visible/Soret spectra were monitored over 24 h. The Mb or Hb level was then increased to 15 μM and the visible/Soret spectra followed for another 24 h. An analogous procedure was used to measure the relative affinities of these hemeproteins for CO. In a third method the order of protein addition was reversed. A solution (2.5 ml) of 5 μM deoxy-Mb or deoxy-Hb was exposed to 0.3 ml of NO/N2, which was just sufficient to saturate the heme iron with NO as shown in visible/Soret spectra, followed by exposure to N2 for 15 min. Changes in visible/Soret spectra were monitored for 48 h after the addition of 10 μM CcO. Spectra were observed for an additional 24 h after raising the Mb or Hb levels to 15 μM.

To evaluate the number of nonmetal sites for NO in Hb, a solution

**Fig. 1. S-H infrared bands for HSA generated by NNO and NO.** An anaerobic solution of 2 mM HSA was exposed to NNO or NO at the partial pressures indicated, and allowed to stand for 1 h prior to recording the spectrum. Total pressure was brought to 1 atm with N2. The reference solution used was 2 mM HSA in buffer. Dashed lines C and F represent bands of 100% Gaussian profile from curve-fitting into two bands with maxima at 2563 cm⁻¹ and 2579 cm⁻¹ and widths at one-half maximum absorbance of 22 cm⁻¹ and 13 cm⁻¹, respectively. For C, and maxima at 2563 cm⁻¹ and 2548 cm⁻¹ and widths at one-half maximum absorbance of 25 cm⁻¹ and 22 cm⁻¹, respectively, for F, insets show plots of maximum absorbance versus variations in NNO or NO partial pressure. One-half maximum absorbance was achieved at 0.35 atm with NNO and 0.47 atm with NO.

![Graph showing absorbance vs. wavenumber for HSA](image-url)
Interactions of NO, NNO, and CO with Proteins

FIG. 2. S-H infrared bands for HSA generated by mixtures of NNO with halothane, chloroform, and NO. Conditions as in Fig. 1. A solution of HSA was treated as indicated. A, exposure to 0.74 atm NNO, 0.26 atm N₂. B, saturated with chloroform (−62 mm). C, solution B exposed to 0.74 atm NNO, 0.26 atm N₂. D, saturated with halothane (−17 mm). E, solution D exposed to 0.74 atm NNO, 0.26 atm N₂. F, 1 atm NO. G, 0.8 atm NO, 0.2 atm NNO. H, 0.5 atm NO, 0.5 atm NNO. I, 0.2 atm NO, 0.8 atm NNO. K, 1 atm NNO.

RESULTS

Albumin Experiments

Perturbations of the SH-IR Spectrum of Cys^{34} by NO, NNO, Halothane, and Chloroform—The increases in absorbance near 2560 cm⁻¹ in the IR spectrum of an anaerobic solution of HSA at pH 7.2 that resulted from exposure of the solution to NNO or NO are shown in Fig. 1. Curve-fitting of the absorbance induced by NNO with 100% Gaussian profiles, as in spectrum C, gives two bands: a major band at 2563 cm⁻¹ with about 85% of total intensity and a minor band at 2579 cm⁻¹. Assignment of these bands to S-H stretch vibrations was discussed earlier (5, 19, 29–31). With NO, as in spectrum F, curve-fitting yields one band at 2563 cm⁻¹, the same wavenumber as found for the major band with NNO, and a second less intense band at 2548 cm⁻¹. The insets of Fig. 1 show the dependence of band intensities on partial pressures of NNO and NO. Raising the partial pressure beyond 0.6 atm did not increase S-H band intensity with either gas. One-half maximum absorbance was achieved at 0.35 atm for NNO and 0.47 atm for NO.

Saturation of a solution of HSA with either halothane or chloroform resulted in a S-H band (Fig. 2) that was broader and at higher wavenumber (−2576 cm⁻¹) than occurred with either NNO or NO (Fig. 2). The spectra obtained with NNO/halothane and NNO/chloroform mixtures reflected a combination of the spectra exhibited by the individual anesthetics. Mixtures of NO and NNO resulted in NO-type and NNO-type S-H bands with their relative contributions varying as the ratio of their partial pressures were changed.

Perturbation of IR Spectra of NNO Molecules within HSA—The NNO-IR spectrum obtained when HSA was exposed only to NNO is shown in Fig. 3A. As reported earlier (5), curve-fitting reveals two major bands at 2220 cm⁻¹ and 2225 cm⁻¹ and minor “hot bands” at lower wavenumbers. Spectra B, C, and D of Fig. 3 exhibit a reduction in the intensity of the 2225 cm⁻¹ band relative to the 2220 cm⁻¹ band as the partial pressure of NNO relative to NO decreases. Spectra A and B are nearly identical indicating that the NNO sites are essentially as fully occupied by NNO at 0.8 atm NNO and 0.2 atm NO as when
NNO is 1 atm with NO absent. However, spectra C and D show that at lower NNO/NO pressure ratios, the intensity of the 2225 cm\(^{-1}\) band relative to the 2220 cm\(^{-1}\) band decreases, as does the combined band intensity. Thus, at NO partial pressures greater than 0.2 atm, occupancy by NNO at both NNO sites decreases, but more so at the more polar 2225 cm\(^{-1}\) site.

Hemeprotein Experiments

Relative Affinities of Iron(II) Sites in Hb, Mb, and CcO for NO and CO—The visible/Soret spectra for unliganded fully reduced Hb, Mb, and CcO and their iron(II) nitrosyl complexes (Fig. 4) served as reference spectra for determining the binding of NO to heme iron(II) in these proteins. For example, spectra of a solution of deoxy-Mb and unliganded fully reduced CcO were recorded as the solution was exposed to increasing volumes of a 5% NO, 95% N\(_2\) gas mixture (Fig. 5). With NO absent, the second-derivative minimum at 441 nm represents the combination of unresolved bands for deoxy-Mb at 435 nm and unliganded CcO at 444 nm. Exposure to 0.2 ml of gas mixture gave spectra consistent with only partial conversion of deoxy-Mb to MbNO. Exposure to 0.4 ml resulted in second-derivative minima at 421 and 444 nm, as expected for Mb being present as mainly MbNO and CcO remaining mostly unliganded. Increasing the volume of gas to 0.6 ml resulted in bands due to both MbNO and CcONO. The addition of deoxy-Mb to a solution of CcONO resulted in the rapid transfer of NO from heme a\(_3\) of CcONO to Mb heme iron(II) (Fig. 6B). Similar experiments with CO demonstrated the greater affinity of Mb for CO (data not shown).

Visible/Soret spectra of a solution of deoxy-Hb and unliganded CcO, upon exposure to increasing volumes of NO, demonstrated that HbNO formed first leaving CcO unliganded (data not shown). Similar experiments with CO indicated HbCO formed first. Following the addition of deoxy-Hb to a solution containing CcONO a slow transfer of NO from heme a\(_3\) of CcONO to Hb occurred (Fig. 7, A-D). Experiments with CcOCO and deoxy-Hb revealed a fast transfer of CO from CcOCO to Mb, which was complete within 5 min.

Discrimination between Hb and Mb as iron(II) nitrosyl or carbonyl species was more readily achieved by use of ligand IR spectra than visible/Soret spectra (19, 20). Both NO and CO were shown by N-O or C-O stretching bands to bind preferentially to the iron(II) of Mb in an anaerobic solution of deoxy-Mb and deoxy-Hb, equimolar in heme (data not shown). However, based on C-O stretching band parameters (32), when CO was added to an aerobic solution of oxy-Mb and oxy-Hb, equimolar...
in heme, the order of affinities for CO was shown to be Hb β subunits > Hb α subunits > Mb.

The visible/Soret spectrum of a solution of CcCO or HbCO upon exposure to NO underwent immediate changes that were consistent with the conversion of the carbonyl species to the nitrosyl species. NO was less effective in displacing CO from MbCO. If a solution of MbCO had been prepared by exposing deoxy-Mb only to the amount of CO needed to completely saturate iron(II) sites, the displacement of CO by NO was much slower than in a similar experiment with HbCO. Furthermore, CO at high levels slowly displaced the heme-bound NO of MbNO, if the MbNO solution had been prepared with the minimum amount of NO needed to saturate all iron(II) sites with NO. Thus, the order of affinities of NO > CO is more pronounced for CcO and Hb than for Mb.

**Evidence of Slow Dissociation of NO from Noniron Sites in CcO and Hb**—The visible/Soret spectrum 35 min after the addition of deoxy-Mb to a solution of CcONO (Fig. 6C), which indicated only MbNO and ligand-free CcO were present, changed upon standing. The spectrum recorded after 120 min

---

**Fig. 5.** Nitric oxide-induced changes in visible/Soret spectra of a mixture of cytochrome c oxidase and myoglobin. The solution (2.5 ml) contained cytochrome c oxidase (10 μM heme A, i.e. 5 μM heme a₃) and 5 μM Mb. Direct spectra, - - -. Second-derivative spectra, ---. A, anaerobic solution after degassing with N₂. B, after exposure of solution A to 0.2 ml of NO/N₂. C, after exposure of solution A to 0.4 ml of NO/N₂. D, after exposure of solution A to 0.6 ml of NO/N₂. Exposure of the solution to greater volumes of NO/N₂ than in D gave the same spectrum as in D. Minima in second-derivative spectra: 441 and 605 nm for A; 421, 442, and 605 nm for B; 421, 444, and 605 nm for C; 421, 443, 451, and 604 nm for D. The NO/N₂ gas mixture was 5% NO, 95% N₂.

**Fig. 6.** Time-dependent changes in visible/Soret spectra following addition of deoxymyoglobin to a solution of cytochrome c oxidase iron(II) nitrosyl. Direct spectra, - - -. Second-derivative spectra, ---. A, solution of CcO 10 μM in heme A (5 μM heme a₃) after exposure to 5% NO, 95% N₂ until nearly all heme a₃ sites were converted to iron(II) nitrosyl, followed by surface flushing with N₂ for 15 min. B, immediately after addition of deoxy-Mb to a level 5 μM in heme B. C, solution B after standing 35 min. D, solution B after standing 120 min. Minima in second-derivative spectra: 428, 443, 451, and 604 nm for A; 421, 444, and 604 nm for B; 421, 444, and 605 nm for C; 422, 443, 451, and 604 nm for D.
(Fig. 6D) revealed that now all the heme $a_3$ of CeO, as well as all Mb heme, contained iron(II)-bound NO. Analogous experiments, except for the substitution of Hb for Mb, resulted in similar, but slower, spectral changes (Fig. 7).

A solution (2.5 ml) of 5 $\mu$m deoxy-Hb was exposed to 0.3 ml of NO/N2 to saturate all heme iron(II) sites with NO and then surface-flushed with N2 for 15 min to completely remove all the NO in buffer. Following the addition of another 5 $\mu$m deoxy-Hb, the second-derivative Soret minimum at 433 nm due to deoxy-Hb decreased slowly as a minimum at 418 nm (HbNO) increased. After 3 h, only HbNO was present. Similar changes in spectra occurred over 4 h following the addition of a second 5 $\mu$m deoxy-Hb, and, over 5 h, after a third 5 $\mu$m addition of deoxy-Hb. However, no further spectral changes occurred over 24 h following a fourth addition of 5 $\mu$m deoxy-Hb. These findings support the slow release of NO from noniron sites in both CeO and Hb with the released NO subsequently binding to heme iron(II) of added Mb or Hb.

**Perturbations of IR Spectra of MbNO and HbNO Solutions in the N-O Stretching Region by NO, NNO, CO, and O2**—The S-H stretching bands due to $\beta$93, $\beta$112, and a104 cysteines were perturbed very little when a CO-saturated solution of HbCO or a NO-saturated solution of HbNO was flushed with N2 (Fig. 9, A-D). Incubations of solutions of HbCO, HbNO, and HbO2 saturated with CO, NO, and O2, respectively, under a small volume of NNO also had only small effects on the S-H bands (Fig. 9, E, G, and I). However, incubations under a larger volume of NNO resulted in substantial shifts in spectra for the $\beta$93 and $\beta$112 cysteines (Fig. 9, F, H, and K). NNO-IR spectra recorded for solutions E to K of Fig. 8 (data not shown) demonstrated that exposure to the larger volume of NNO resulted in a 5-fold larger concentration of NNO molecules at sites within protein than when the smaller volume of NNO was used. Furthermore, the solutions exposed to the larger volume of NNO exhibited a band near 2226 cm$^{-1}$ (the band for NNO molecules at the most polar sites) that was more intense than the bands for NNO at less polar sites. The solutions of HbCO, HbNO, and HbO2 that had been exposed to the larger volume of NNO had only small effects on the S-H bands (Fig. 9, B, D, and F). NNO-IR spectroscopy to determine the extent of NO bound to iron(II).

**Perturbations of Thiol IR Spectra of Hb by NO, NNO, CO, and O2**—The S-H stretching bands due to $\beta$93, $\beta$112, and a104 cysteines were perturbed very little when a CO-saturated solution of HbCO or a NO-saturated solution of HbNO was flushed with N2 (Fig. 9, A-D). Incubations of solutions of HbCO, HbNO, and HbO2 saturated with CO, NO, and O2, respectively, under a small volume of NNO also had only small effects on the S-H bands (Fig. 9, E, G, and I). However, incubations under a larger volume of NNO resulted in substantial shifts in spectra for the $\beta$93 and $\beta$112 cysteines (Fig. 9, F, H, and K). NNO-IR spectra recorded for solutions E to K of Fig. 8 (data not shown) demonstrated that exposure to the larger volume of NNO resulted in a 5-fold larger concentration of NNO molecules at sites within protein than when the smaller volume of NNO was used. Furthermore, the solutions exposed to the larger volume of NNO exhibited a band near 2226 cm$^{-1}$ (the band for NNO molecules at the most polar sites) that was more intense than the bands for NNO at less polar sites. The solutions of HbCO, HbNO, and HbO2 that had been exposed to the larger volume of NNO had only small effects on the S-H bands (Fig. 9, B, D, and F). NNO-IR spectroscopy to determine the extent of NO bound to iron(II).

**Discussion**

**Albumin Studies**—The IR spectra reported here provide a means for detecting alterations in HSA conformation in solu-
tion in areas of the Cys34 thiol and NNO binding. HSA is a single polypeptide with 585 residues containing 34 cysteines paired in disulfide bridges and one free cysteine (Cys34) (33). Crystal structure determinations reveal a heart-shaped molecule containing three domains: I (residues 1–195), II (196–383), and III (384–585), with similar three-dimensional structures (34, 35). Each domain can be divided into two subdomains A and B that contain six and four α-helices, respectively. The subdomains are linked by an extended loop. Cys34 is located in a short loop linking helices h2 and h3 in subdomain IA. The recent structure at 2.5-Å resolution places Cys34 on the surface of the protein (35). The thiol side chain is positioned toward the interior of the protein surrounded by side chains of Pro35, His39, Val77, and Tyr34 that appear to isolate the thiol from external reactants. However, the difference electron density map did not provide detailed information on the immediate environment of the thiol. Furthermore, the structure in solution may differ from the crystal structure in the steric relationships among the amino acid side chains near Cys34.

The direct measurement of the stretching vibration of the S-H bond in IR spectra provides S-H band parameters that characterize the S-H bond and reflect interactions of the thiol with its environs. At the neutral pH used here, the S-H stretching band is expected to be very weak or undetectable as a result of extensive ionization of this unusually acidic thiol (36). Therefore, the detection of S-H IR bands reflected a change in protein conformation that reduced the acidity of the thiol.

The frequency of an S-H stretching band increases with

---

**FIG. 8.** Infrared spectra in the N-O stretching region for myoglobin and hemoglobin iron(II) nitrosyls. Anaerobic solutions of deoxy-Mb (9 mM) and deoxy-Hb (9 mM in heme) were exposed to sufficient 5% NO, 95% N2 to bind NO at all iron(II) sites. Further exposure caused no changes in IR and visible/Soret spectra. A, MbNO solution saturated with NO. B, solution A after exposure to N2 flushing for 30 min. C, HbNO solution saturated with NO. D, solution C after flushing with N2 for 30 min. F, solution C after flushing with CO for 30 min. F, solution C after flushing with NNO for 30 min. Band widths at 10% maximum absorbance: A, 16 cm⁻¹; B, 24 cm⁻¹; C, 20 cm⁻¹; D, 18 cm⁻¹. CO-saturated solution of MbCO or HbCO 9 mM in heme was used as a reference solution.

**FIG. 9.** Effects of flushing with N2 or NNO on S-H infrared spectra of solutions of HbCO, HbNO, and HbO2. Reduced hemoglobin concentration: 8 mM in heme. A, CO-saturated solution. B, solution A after N2-flushing for 30 min. C, NO-saturated solution. D, solution C after N2 flushing for 30 min. E, CO-saturated solution (0.1 ml) exposed to 1 ml of NNO for 30 min. F, as in E, with exposure to 16 ml of NNO for 30 min. G, NO-saturated solution (0.1 ml) exposed to 1 ml of NNO for 30 min. H, as in G, with exposure to 16 ml of NNO for 30 min. I, O2-saturated solution (0.1 ml) exposed to 1 ml of NNO for 30 min. K, as in I, with exposure to 16 ml of NNO for 30 min.
increasing bond strength and the intensity increases as the strength of the dipole associated with the S-H bond increases (5, 30). The Cys$^{34}$ S-H stretching band at 2563 cm$^{-1}$, as induced by both NNO and NO, suggests a nonpolar environment and hydrogen bonding between the thiol and an adjacent proton acceptor such as His$^{39}$ or Tyr$^{34}$. The slight asymmetry of the NNO-induced band of Fig. 1C is consistent with one major protein conformation and one minor conformation that exhibit maxima at 2563 cm$^{-1}$ and 2579 cm$^{-1}$, respectively. The wavenumber difference indicates that the S-H bond strength is greater in the latter than the former. The absorbance induced by NO also supports the generation of two significantly different thiol environments, one of which, with a band at 2563 cm$^{-1}$, is identical to the major environment induced by NNO. The second environment, in which the S-H bond is weaker, since the wavenumber (2548 cm$^{-1}$) is lower, is not generated by NNO. The affinities of NNO and NO for the sites that give rise to the S-H bands are nearly identical, although bands are first detected at lower pressures with NNO than NO (Fig. 1, insets).

Spectra for mixtures of NNO and NO (Fig. 2) are consistent with mixtures of NNO-type and NO-type protein conformations. The higher wavenumbers for the S-H bands generated by halothane and chloroform support stronger S-H bonds and weaker hydrogen bonding than are found with either NNO or NO. Our findings indicate that NO and anesthetics may also affect the bonding interactions involved and the frequencies of NNO-IR bands make the wavenumber (2548 cm$^{-1}$) is lower, is not generated by NNO. The affinities of NNO and NO for the sites that give rise to the S-H bands are nearly identical, although bands are first detected at lower pressures with NNO than NO (Fig. 1, insets).

Spectra for mixtures of NNO and NO (Fig. 2) are consistent with mixtures of NNO-type and NO-type protein conformations. The higher wavenumbers for the S-H bands generated by halothane and chloroform support stronger S-H bonds and weaker hydrogen bonding than are found with either NNO or NO. Our findings indicate that NO and anesthetics may also affect the bonding interactions involved and the frequencies of NNO-IR bands make the wavenumber (2548 cm$^{-1}$) is lower, is not generated by NNO. The affinities of NNO and NO for the sites that give rise to the S-H bands are nearly identical, although bands are first detected at lower pressures with NNO than NO (Fig. 1, insets).

Available evidence does not permit identification of the precise sites within HSA that, when occupied by NNO, NO, halothane, or chloroform, result in an altered Cys$^{34}$ thiol environment. However, NNO-IR spectra do give insight into the polar nature of NNO sites. The wavenumber values of 2225 cm$^{-1}$ and 2220 cm$^{-1}$ for NNO-IR bands reflect environments with polarities between those in highly polar water and in a nonpolar alkane, wherein bands are found at 2230 cm$^{-1}$ and 2215 cm$^{-1}$, respectively (5, 40–42). Based on known effects of solvents on NNO-IR spectra, we conclude that the more polar 2225 cm$^{-1}$ site may involve a peptide bond carbonyl, a carbonyl plus an aromatic ring, or two aromatic rings. One aromatic ring from phenylalanine, tyrosine, or tryptophan may account for the limited polarity of the 2220 cm$^{-1}$ site (5). The aromatic structures of these amino acids are polar, yet hydrophobic (43, 44). The finding that NO competes with NNO more effectively at the more polar 2225 cm$^{-1}$ site is consistent with the expectation that NO bonding may have stronger contributions from dipole-dipole and/or dipole-induced dipole interactions (10).

Recent studies provide support for interactions of both halothane and chloroform with tryptophans of HSA and bovine serum albumin (13, 14). Considerations of the bonding interactions involved and the frequencies of NNO-IR bands make tryptophan an attractive binding site for both NO and NNO. The single tryptophan of HSA (Trp$^{214}$), conserved in mammals, is found between helices h2 and h3 of domain IIA and has a key structural role in formation of the subdomain IIA-binding site (33, 35). A potentially important factor in the ability of a compound, by binding at this site, to alter the environment of Cys$^{34}$ thiol located in subdomain IA, is the attachment of the tail of subdomain IIA to the interface region between subdomains IA and IB by hydrophobic interactions and hydrogen bonds. Future studies of effects, if any, of NO and NNO on Trp$^{214}$ fluorescence may clarify the nature of the interactions involved.

The findings reported here demonstrate the ability of each of the anesthetics, NNO, halothane, and chloroform, to alter HSA structure in the environment of the free thiol of Cys$^{34}$. NO closely mimics NNO in its effects on protein structure, and in binding to protein sites. It is noteworthy that NO can modify HSA structure without formation of the S-nitrosothiol derivative. However, consideration of the possible in vivo significance of such reactions of NO with HSA must be made with an appreciation of the much higher levels of NO used here than are currently expected to occur in vivo.

**Hemeprotein Studies**—The possibility that NO can bind to nonheme iron(II) sites in heme proteins that are similar to the multiple sites shown earlier to be occupied by NNO (5) was explored. The binding of NO and CO to iron(II) sites of the heme proteins was established by the visible/Soret spectra of the heme and IR spectra of NO and CO bound as ligands to iron(III). Under anaerobic conditions, the relative affinities of both NO and CO for heme iron(II) sites decreased in the order Mb $>$ Hb $>$ CeO. However, when oxygen as well as CO was present, Hb had a greater affinity for CO than did Mb. Although each protein has a greater affinity for NO than CO, the difference in the affinities is much less with Mb than with Hb or CeO.

The spectra of Figs. 6 and 7 demonstrate that CeO seques- tered NO reversibly at sites other than iron(II). The noniron sites became occupied by NO rapidly, and retained NO when the solution was flushed with sufficient N$_2$ to remove all NO from protein-free buffer. However, several hours were required for the loss of NO from all these sites as shown by the binding of the released NO to iron(II) sites of Mb or Hb. NO was also retained at noniron sites within Hb from which dissociation of NO was very slow. The number of “slow release” noniron sites approximates the number of NNO sites estimated earlier (5), a finding consistent with, but not proof of, NO and NNO binding at the same sites.

The IR spectra in the N-O stretching regions for solutions of MbNO and HbNO are altered by exposure to NO, CO, or N$_2$ (Fig. 8). The changes can be attributed to shifts in the Amide I spectrum of the protein rather than to perturbations of the true N-O stretching vibration. The absorbance recorded represents the sum of the true N-O stretching vibration plus the difference between the Amide I spectrum of the sample solution and the Amide I spectrum of the reference solution when a CO-saturated solution of the respective protein carbonyl was used as reference. In each case, curve-fitting revealed one fitted band that was consistent with a N-O stretching band with parameters identical to those found earlier when the reference solution used contained $^{15}$N$^{18}$O (1611 cm$^{-1}$ for MbNO and 1617 cm$^{-1}$ for HbNO) (19, 28). The problems associated with isolating the true N-O stretching band from the protein Amide I bands that appear in the same region of the IR spectrum have been discussed (10, 19, 28). Effects of N$_2$-flushing on the spectrum can be attributed to changes in protein secondary structure as the result of the removal of NO from noniron sites from which NO dissociates more rapidly than from the “slow release” sites discussed above. The smaller effects of flushing with NNO, than with N$_2$, suggests that NNO replaces NO at sites, and thereby causes only a small change in protein structure. A shift in the protein Amide I spectrum without displacing NO at iron(II), that occurs upon flushing with CO, can also result from loss of NO from noniron sites with partial or complete replacement with CO. The spectral shifts of Fig. 8 are consistent with NO occupying noniron protein sites from which NO can be dissociated, or replaced.

The IR spectra of thiols and sequestered NNO molecules of Fig. 9 provide evidence for the occupancy of noniron protein sites in Hb by NO, NNO, CO, or O$_2$. The Hb cysteine S-H stretching bands, although relatively weak, have the advantage of being in a region of the spectrum where other protein
bands are absent (5, 30). NNO-IR spectra provide direct evidence that when solutions of HbCO, HbNO, and HbO2, which had been saturated with NNO, were subsequently exposed to CO, NO, or O2, respectively, the loss of NNO from the more polar sites was greater than from the less polar sites. The effectiveness in displacing NNO decreased in the order NO > CO > O2. These findings suggest that each of these gases may be able to influence Hb function by occupying noniron sites.

Conclusions—The broader implications of these findings include the expectation that NO, volatile anesthetics, and probably CO may occupy similar sites within many proteins and, thereby, alter the structure and function of the protein. Such sites may also provide safe havens for NO by limiting its accessibility to external reactants. The precise nature of such sites remains elusive, but aromatic residues are attractive possible components. The ability of aromatic ring structures to bind NO, tightly and reversibly, is shown unequivocally by the NO binding between the cofacial aromatic groups of so-called “Venus fly trap” organic compounds recently reported by Kochi and co-workers (45, 46). The ability to detect NNO at different sites within protein by IR spectroscopy, coupled with the evidence found in support of NO and NNO binding at the same sites, utilize the utility of NO-IR spectroscopy for detecting potential nonmetal sites for NO binding via noncovalent bonding.

Physiologically important areas in which control by NO via noncovalent interactions of NO with protein may occur include oxygen transport, mitochondrial respiration, and the activation of soluble guanylate cyclase. Failure to invoke such interactions with hemoglobin may be the reason why considerations of only the reactions of NO with the thios and irons of hemoglobin have failed to provide a satisfactory mechanism for control of oxygen transport by NO (47). NO has been suggested to regulate CaO activity by forming metal nitrosyls at heme iron and/or copper (20, 48). Both NO and NNO inhibit CO reversibly (18, 48). Since NO does not serve as a ligand to these metals, the partial, reversible inhibition by NNO appears due to the noncovalent binding of NNO molecules to one or more of the nonmetal sites that are detected in IR spectra (5, 18, 28). Noncovalent bonding of NO to NNO sites, and possibly other sites, provides new mechanisms for the control of CO activity by NO.

NO binding to protein sites via noncovalent bonding, as well as to heme iron(II), may be particularly important in the activation of guanylate cyclase by NO and provides an explanation for findings recently reported by Marletta and co-workers (49). Activation was shown not to occur upon NO binding to heme iron, as had been widely assumed (49). Activation requires a subsequent step, the rate of which is dependent on NO concentration. The second step involves conversion of six-coordinate heme iron to a five-coordinate species, a conversion apparently induced by NO binding to an unknown site that is not heme iron. This loss of the ligand trans to NO is a process analogous to the change in heme coordination in HbA nitrosyl that is induced by inositol hexaphosphate as discovered in early studies of protein allosteric effects (50, 51). The porphyrin π-electron system of the heme and the aromatic amino acids widely found adjacent to the heme in hemeprotein structures (52) provide potential sites for noncovalent bonding of NO in hemeproteins. The availability of such sites, and the slow dissociation of NO from them, suggests that hemeproteins are likely to bind NO via noncovalent bonding much more avidly than occurs with HSA. Furthermore, the activation of soluble guanylate cyclase and some of the other effects of CO (21–23) may also involve noncovalent interactions of CO with protein.