

Electron Paramagnetic Resonance and Oxygen Binding Studies of α -Nitrosyl Hemoglobin

A NOVEL OXYGEN CARRIER HAVING NO-ASSISTED ALLOSTERIC FUNCTIONS*

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α -Nitrosyl hemoglobin, $\alpha(\text{Fe-NO})_2\beta(\text{Fe})_2$, which is frequently observed upon reaction of deoxy hemoglobin with limited quantities of NO *in vitro* as well as *in vivo*, has been synthetically prepared, and its reaction with O₂ has been investigated by EPR and thermodynamic equilibrium measurements. α -Nitrosyl hemoglobin is relatively stable under aerobic conditions and undergoes reversible O₂ binding at the heme sites of its β -subunits. Its O₂ binding is coupled to the structural/functional transition between T- (low affinity extreme) and R- (high affinity) states. This transition is linked to the reversible cleavage of the heme Fe-proximal His bonds in the $\alpha(\text{Fe-NO})$ subunits and is sensitive to allosteric effectors, such as protons, 2,3-bisphosphoglycerate, and inositol hexaphosphate. In fact, $\alpha(\text{Fe-NO})_2\beta(\text{Fe})_2$ is exceptionally sensitive to protons, as it exhibits a highly enhanced Bohr effect. The total Bohr effect of α -nitrosyl hemoglobin is comparable to that of normal hemoglobin, despite the fact that the oxygenation process involves only two ligation steps. All of these structural and functional evidences have been further confirmed by examining the reactivity of the sulfhydryl group of the Cys^{β93} toward 4,4'-dipyridyl disulfide of several α -nitrosyl hemoglobin derivatives over a wide pH range, as a probe for quaternary structure. Despite the halved O₂-carrying capacity, α -nitrosyl hemoglobin is fully functional (cooperative and allosterically sensitive) and could represent a versatile low affinity O₂ carrier with improved features that could deliver O₂ to tissues effectively even after NO is sequestered at the heme sites of the α -subunits. It is concluded that the NO bound to the heme sites of the α -subunits of hemoglobin acts as a negative allosteric effector of Hb and thus might play a role in O₂/CO₂ transport in the blood under physiological conditions.

chiometric amounts of NO ([NO]/[heme] \ll 0.5) in solution (1–3) and in the erythrocytes (4–6), the predominant species formed upon equilibrium are α -nitrosyl Hbs, *i.e.* $\alpha(\text{Fe-NO})\alpha(\text{Fe})\beta(\text{Fe})_2$ or $\alpha(\text{Fe-NO})_2\beta(\text{Fe})_2$. Such compounds were readily identified by their EPR spectra with a set of sharp triplet ¹⁴N hyperfine structures ($A_z = 17$ Gauss) around $g_z = 2.009$, which is derived from the 5-coordinate nitrosyl hemes in the α -subunits. When rats or mice had been exposed to doses of lipopolysaccharide, tumor necrosis factor, nitroglycerin, nitrite, or NO (7–12), their plasma concentration of NO was known to increase. Venous bloods from the treated animals invariably exhibited EPR spectra with distinct triplet hyperfine signals. Such EPR spectra cannot be expected from tetranitrosyl Hb, $\alpha(\text{Fe-NO})_2\beta(\text{Fe-NO})_2$, in the absence of IHP at a physiological pH of 7.4 (13). Therefore, it is obvious that the primary nitrosyl products formed upon reaction of deoxy Hb with NO under physiological conditions, where [NO] \ll [heme], are α -nitrosyl Hbs (6, 12, 14, 15). In order to assess physiological roles of such compounds, we have investigated its O₂ binding properties of $\alpha(\text{Fe-NO})_2\beta(\text{Fe})_2$ by EPR and O₂ binding measurements as well as the reactivity of Cys^{β93} toward 4-PDS as a probe for the quaternary structure. We have found that its oxygenation characteristics and allosteric functions make $\alpha(\text{Fe-NO})_2\beta(\text{Fe})_2$ a unique cooperative low affinity O₂ carrier with full allosteric sensitivity that could deliver O₂ to tissues efficiently under physiological conditions. This study has also provided a new insight into the molecular mechanism of cooperativity and allostery in Hb, particularly the major role of the α -heme Fe-F helix linkage in the quaternary structural transition, and the mode interaction of Hb with NO.

EXPERIMENTAL PROCEDURES

Reagents—2,3-Bisphosphoglycerate, IHP, *p*MB, 4-PDS, Tris, bis-Tris, bis-Tris propane, dithiothreitol, catalase, superoxide dismutase (Sigma), and argon (grade 5 gas; BOC gases, Murray Hill, NJ) were used without further purification. Nitric oxide (99.00% pure; MG Industries, Malvern, PA) was purified by passing through a series of gas-bubble washing bottles containing 1 M NaOH and deoxygenated distilled water and another bottle containing deoxygenated distilled water. Purified NO gas was used under strict anaerobic conditions. Anaerobic conditions were obtained by removing O₂ from the media with repeated evacuation and flushing with water-saturated argon gas or by continuous flushing with water-saturated argon gas over the surface of stirred reaction media. The use of dithionite as reductant was avoided as much as possible to prevent inducing unknown side reactions.

Preparation of $\alpha(\text{Fe-NO})_2\beta(\text{Fe-O}_2)_2$ —All preparation procedures were carried out at 4 °C. Freshly outdated adult human Hb was obtained from a local branch of the American Red Cross; it was purified according to the method of Drabkin (16) and stripped from organic phosphates by the method of Berman *et al.* (17). The Hb solution was stored in the CO form, and no further attempt was made to strip Hb from its minor components.

α - and β -chains from Hb in the CO form were separated according to the method of Bucci and Fronticelli (18) using the *p*MB treatment with modifications. After overnight incubation, the *p*MB-treated Hb solution

When deoxy hemoglobin (Hb)¹ is exposed to less-than-stoi-

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¹ The abbreviations used are: Hb, hemoglobin; IHP, inositol hexaphosphate; BPG, 2,3-bisphosphoglycerate; *p*MB, *p*-hydroxymercuribenzoate; 4-PDS, 4,4'-dipyridyl disulfide; α , α -subunit; β , β -subunits; (Fe), deoxy heme; (porphyrin), protoporphyrin IX; (Ni), nickel protoporphyrin IX; (Fe-NO), nitrosyl heme; (Fe-O₂), oxy heme, and (Fe-CO), carbonmonoxy heme; EPR, electron paramagnetic resonance.

was passed through a Sephadex G-25 with 20 mM Tris buffer, pH 8.5, and the eluent was loaded onto a Macro-Prep HighQ (Bio-Rad) column equilibrated with the same buffer. Elution of isolated chains was achieved by applying a salt gradient using a ConSep LC 100 liquid chromatography system (Millipore Corp., Bedford, MA). The pMB-treated α -chains were eluted first, followed by unreacted Hb and finally by the pMB-treated β -chains. The α - and β -chains were reconstituted to their native sulfhydryl forms after incubation for 2 h with dithiothreitol in a concentration of 3 mg per ml of chain solution, in the presence of 5 μ M of catalase. Subsequently, reconstituted Hb chains were passed through Sephadex G-25 fine in 10 mM bis-Tris buffer, pH 7.4, flushed with CO, concentrated by ultrafiltration through a disc membrane, Omega 10K (Filtron, Clinton, MA) if necessary, and kept on ice for further use. The quality of chains was checked by spectrophotometry, cellulose acetate electrophoresis, and the ability to reconstitute tetrameric Hb.

α -Nitrosyl chains were prepared according to Henry and Banerjee (14) with some modifications. About 3 ml of 3 to ~4 mM heme of α -subunits were mixed with the same amount of 0.2 M bis-Tris buffer, 0.4 M Cl⁻, pH 7.4, and were converted to the oxy form by illumination under a stream of pure O₂. The α -chains in oxy form were then transferred to a flask sealed with a rubber stopper. Pure argon gas, washed in water, was flushed over the surface of the continuous stirred solution in the flask. After approximately 40 min, about 0.5 ml of a solution of sodium dithionite (3 mg in 1 ml of deoxygenated distilled water) was injected with a syringe through the stopper to assure complete deoxygenation of the sample. Then, NO gas was injected into the flask. Once the reaction was completed (within a few minutes), all NO in excess was removed from the flask by purging thoroughly with argon again. The α -nitrosyl chain solution was then transferred anaerobically to a Sephadex G-25 column in 10 mM bis-Tris propane, pH 7.4, and eluted with deoxygenated buffer. From this point, the nitrosyl derivative can be exposed to air without any immediate decomposition. However, some met Hb formation was observed in samples stored at temperatures above 5 °C for prolonged periods of time. Therefore, it was used as soon as possible. The concentration of α (Fe-NO) was calculated using extinction coefficients of 13.57 and 13.83 mm⁻¹ cm⁻¹ at 572 and 544 nm, respectively, at pH 7.0. β (Fe-O₂) chains were obtained from the CO derivative in the same manner as for the α chains, as indicated above. The α (Fe-NO)₂ β (Fe-O₂)₂ hybrid was prepared by simply mixing α (Fe-NO) with an equimolar amount of β (Fe-O₂). Tetranitrosyl Hb was prepared in the same manner as α (Fe-NO) chains. The integrity of preparations was examined by spectrophotometry, acetate cellulose electrophoresis, and EPR spectroscopy. Partially nitrosylated hybrid tetramers (α (Fe-NO)₂ β (Fe-O₂)₂ and α (Fe-O₂)₂ β (Fe-NO)₂) were stored at 0 °C for short storage and at liquid nitrogen temperature for long storage. Tetranitrosyl Hb (α (Fe-NO)₂ β (Fe-NO)₂) was stored anaerobically at low temperatures. No detectable alteration of the compounds such as heme oxidation, ligand exchange, loss of ligands, and subunit exchange occurred under such conditions of storage.

Temperature Dependence of the Aerobic met Hb Formation of Nitrosyl Hb Derivatives—To determine the optimal temperature for quantitative O₂ equilibrium and EPR studies, the rates of aerobic formation of met Hb for α -nitrosyl Hb, tetranitrosyl Hb, and oxy Hb samples were measured. The reaction was followed by the absorbance increase at 630 nm of met Hb over time with a Hewlett-Packard 8452A diode array spectrophotometer (Hewlett-Packard, Palo Alto, CA). To avoid artifacts due to turbidity or water vapor condensation at low temperatures, readings were corrected with a second wavelength at 800 nm. Sample concentration was 60 μ M heme in 50 mM bis-Tris-propane containing 0.1 M Cl⁻, pH 7.4. Data were collected every second for 20 min and analyzed according to a first-order kinetic scheme.

Oxygen Equilibrium Measurements—Oxygen equilibrium curves were measured by an improved version of Imai's automatic method (19) with the following modifications. Absorbance was monitored using a computer-controlled Olis-Cary 118 spectrophotometer (Olis, Bogart, GA). Oxygen concentrations were monitored with a low noise, high response electrode (O₂ Sensors, Gadwyne, PA), using a custom-made amplifier (Biomedical Instrumentation Shop, University of Pennsylvania Medical Center, Philadelphia, PA). The signal was then digitized using a 12-bit A/D converter. Absorption changes were monitored at 560 nm. Sample concentration was 120 μ M heme in 50 mM bis-Tris-propane buffer, containing 0.1 M Cl⁻, and small amounts of catalase and superoxide dismutase. Measurements were carried out at 15 °C. Analyses of oxygenation data were performed according to a two-step model, corresponding to the third and fourth O₂ bindings in Hb, as reported previously (20).

Kinetic Studies of the Sulfhydryl Reactivity of Cys⁶⁹³ of α -nitrosyl Hb Derivatives toward 4-PDS—This method (21) was carried out as described previously (22, 23), with the following modifications for a quantitative measurement. A standard solution of 4-PDS was prepared by dissolving approximately 10 mg in 10 ml of deoxygenated distilled water at 60 °C. The concentration of 4-PDS was calculated using an extinction coefficient of 16.3 mm⁻¹ cm⁻¹ at 247 nm and pH 7.0.

For oxy derivatives, 2 ml of the derivative (40 μ M heme) in 50 mM bis-Tris propane buffer, containing 0.1 M Cl⁻, was placed in a quartz cuvette thermostatted at 15 °C and containing a small stirring bar. An amount of 4-PDS equivalent to a final concentration of 160 μ M was added to the solution and the reaction was monitored with a Hewlett-Packard 8452A diode array spectrophotometer by measuring the absorbance increase at 324 nm. Data points were collected at a rate of one per s. The dead time of the reaction was 2 s. For deoxy derivatives, a long-neck anaerobic quartz cuvette sealed with a stopper was used. Concentrations of Hb samples and 4-PDS were the same as for oxy derivatives. Deoxygenation was carried out at 4 °C by flushing pure argon into the cuvette. Once the deoxygenation of the sample was confirmed by spectrophotometry, the anaerobic cuvette was then transferred to the temperature-controlled cell holder of the spectrophotometer. As soon as the solutions in the cuvette reached 15 °C, the reaction was initiated by injecting with a gas-tight syringe through the stopper an aliquot of a deoxygenated 4-PDS solution. The dead time in this case was approximately 5 s.

EPR Measurements—Buffers used for EPR measurements were 0.1 M sodium acetate buffers, pH 4.8–5.8, and 0.1 M bis-Tris propane buffers, pH 6.0–pH 9.0, containing 0.1 M Cl⁻. Oxygen binding equilibria for EPR samples were obtained at 15 °C in a modified Imai cell (19), in which O₂ concentrations of samples were continuously monitored by a sensitive O₂ electrode (O₂ Sensors). Aliquots of sample were taken at determined partial pressures of O₂ and anaerobically transferred into EPR tubes and immediately frozen by immersion into liquid nitrogen. EPR measurements were carried out with a Varian X-band EPR spectrometer, model E109 (Varian Associates, Palo Alto, CA), integrated with the data-acquisition system (Scientific Software Services, Normal, IL). EPR samples (300 μ l of 500 μ M heme) in quartz EPR tubes (3-mm precision bore) were frozen by immersion into liquid nitrogen and measured at liquid nitrogen temperature. The spectrometer was operated at a microwave frequency of 9.11GHz, microwave power of 20mW, modulation frequency of 100kHz, modulation amplitude of 2.0 Gauss, magnetic field scan rate of 125 Gauss/min, and time constant of 0.2. Recorded EPR data were manipulated with the EPR software (Scientific Software Services) for quantitative analyses and plotted using Origin for Windows, Version 5.0 (Microcal, Northampton, MA).

RESULTS

Temperature Dependence of the Met Hb Formation of Nitrosyl Hb Derivatives—Native oxy Hb, α (Fe-NO)₂ β (Fe-O₂)₂, and tetranitrosyl Hb were slowly oxidized to respective met forms under aerobic conditions. Both nitrosyl Hb derivatives were less stable than native oxy Hb, and tetranitrosyl Hb was the least stable of the species. Half-life times at 37 °C were 15 h, 2 h, and 41 min for oxy Hb, α (Fe-NO)₂ β (Fe-O₂)₂, and tetranitrosyl Hb, respectively. However, these values increased to 42 h, 22 h, and 16 h, respectively, at 15 °C. Accordingly, all experiments were conducted at this temperature.

Coordination States of Nitrosyl Hemes as Measured by EPR—Isolated α (Fe-NO) and β (Fe-NO) subunits exhibited EPR spectra around g = 2.0 of the 6-coordinate nitrosyl hemes at pH 7.4, as shown in Fig. 1A, in agreement with previous reports (14, 15, 24). However, the overall line shape of their EPR spectra was distinctly different from one to the other: the isolated α (Fe-NO) subunits (Fig. 1A, *broken line*) showed a more rhombically distorted line shape than the isolated β (Fe-NO) subunits (*dotted line*). These spectra were independent of pH in a range from pH 6.0 to pH 9.0. The EPR spectrum of tetranitrosyl Hb (*solid line*) was essentially a sum of those of the isolated α (Fe-NO) and β (Fe-NO) subunits, as previously reported (14, 15, 24) and did not change significantly over a pH range from 7.0 to 9.0. The EPR spectrum of the α -deoxy, β -nitrosyl hybrid, α (Fe)₂ β (Fe-NO)₂ was reported to be practically identical with that of isolated β (Fe-NO) subunits (spectrum B

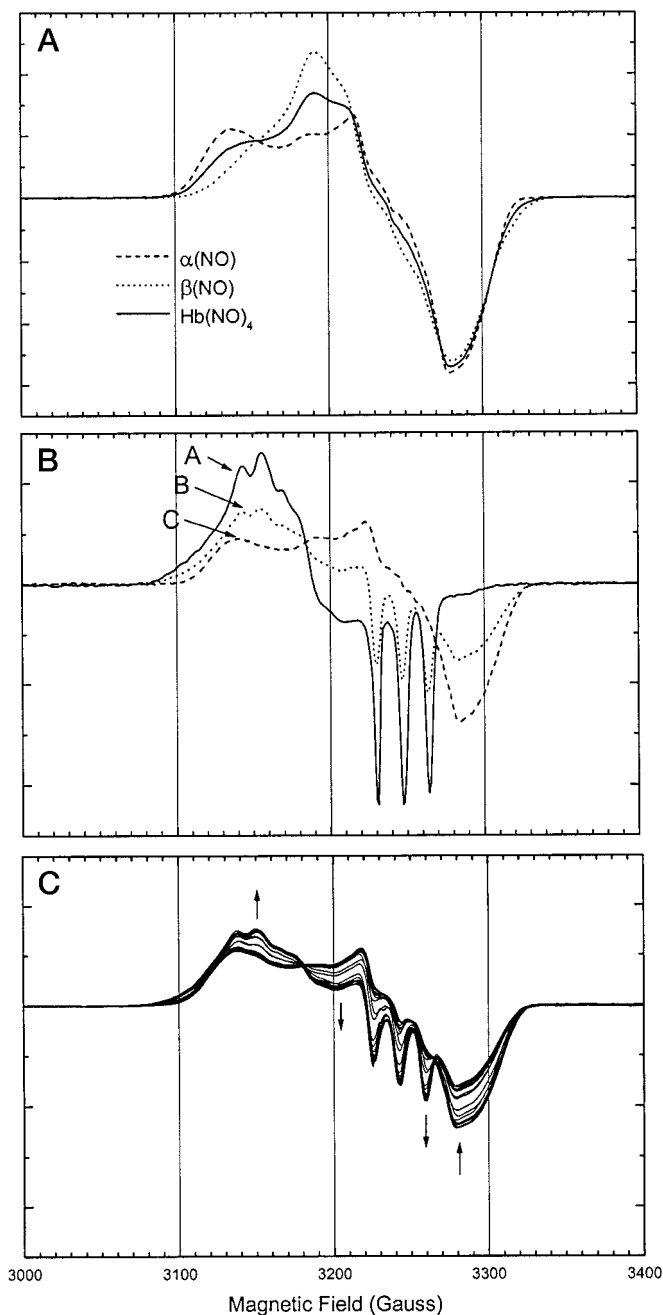


FIG. 1. A, EPR spectra of isolated $\alpha(\text{Fe-NO})$ and $\beta(\text{Fe-NO})$ subunits and tetranitrosyl Hb, $\alpha(\text{Fe-NO})_2\beta(\text{Fe-NO})_2$. Samples ($500 \mu\text{M}$ heme) were dissolved in 0.1 M bis-Tris propane buffer, pH 7.4, at 15°C prior to freezing at 77K for EPR measurements. B, EPR spectra of α -nitrosyl Hb at different pH and in the presence and absence of O_2 and IHP. Spectrum A (solid line), at pH 4.8 in the absence of O_2 and the presence of 3 mM IHP; spectrum B (dotted line), at pH 7.4 in the absence of O_2 and IHP; spectrum C (broken line), at pH 9.0 in the presence of O_2 and the absence of IHP. C, changes in EPR spectra of α -nitrosyl Hb during a stepwise deoxygenation process at pH 7.4 and 15°C . Arrows indicate the direction of spectral changes during deoxygenation. The midpoint of the EPR spectra transition was attained at $p\text{O}_2$ of 1.65 mm Hg at 15°C .

in Fig. 1A) (14, 24) and was pH-independent over a pH range from 6.0 to 9.0.

The α -nitrosyl, β -deoxy hybrid, $\alpha(\text{Fe-NO})_2\beta(\text{Fe})_2$, on the other hand, exhibited an EPR spectrum of mixed 5- and 6-coordinate nitrosyl hemes at pH 7.4, as indicated by the appearance of a sharp triplet hyperfine structure ($A_z = 17 \text{ Gauss}$ at $g_z = 2.009$) of the 5-coordinate nitrosyl heme (spectrum B in Fig. 1B). This implied that the α -heme Fe-His (F8) bonds were

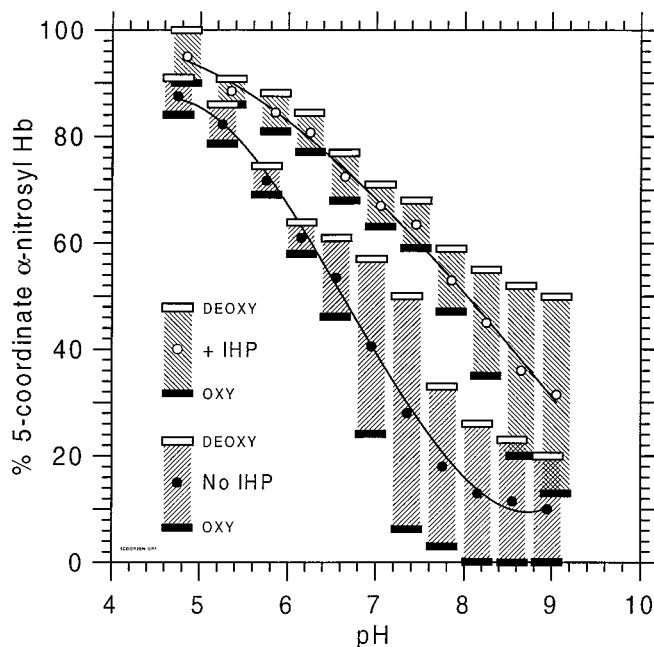


FIG. 2. The 5- \leftrightarrow 6-coordination equilibrium of $\alpha(\text{Fe-NO})$ subunits of α -nitrosyl Hb as a function of pH and O_2 saturation at 15°C in the presence and absence of IHP. The percentage of the 5-coordinate $\alpha(\text{Fe-NO})$ subunits was calculated using spectra A and C of Fig. 1B as standards for 100% and 0% 5-coordinate $\alpha(\text{Fe-NO})$, respectively. The pH dependence of midpoints (open and closed circles) of the coordination transition at fixed pH in the presence and absence of 3 mM IHP, respectively, is related to the Bohr effect of O_2 equilibrium of α -nitrosyl Hb.

partially cleaved in $\alpha(\text{Fe-NO})_2\beta(\text{Fe})_2$ under these conditions (15, 25–27). The EPR spectrum of $\alpha(\text{Fe-NO})_2\beta(\text{Fe})_2$ was pH-dependent. The 6-coordinate state was favored at higher pH, whereas the proportion of the 5-coordinate state increased at acidic pH (Fig. 2, open rectangles). Upon addition of stoichiometric amounts of CO or saturating concentrations of O_2 , the 5- \leftrightarrow 6-coordination equilibrium of the α -nitrosyl hemes of $\alpha(\text{Fe-NO})_2\beta(\text{Fe})_2$ shifted in favor of the 6-coordinate state as a function of pH (Fig. 2, closed rectangles). The α -nitrosyl hemes of $\alpha(\text{Fe-NO})_2\beta(\text{Fe-CO})_2$ (not shown) and $\alpha(\text{Fe-NO})_2\beta(\text{Fe-O}_2)_2$ (Fig. 1B, spectrum C, and Fig. 2, closed rectangle at pH 9.0) were estimated to be essentially 100% 6-coordinate at pH 9.0. The EPR spectral changes, which were induced by the oxygenation at the β -hemes, were smaller at acidic extremes and most pronounced at around pH 7.4. Inositol hexaphosphate shifted the 5- \leftrightarrow 6-coordination equilibrium of the α -nitrosyl hemes of $\alpha(\text{Fe-NO})_2\beta(\text{Fe})_2$ in favor of the 5-coordinate state (Fig. 2). The EPR spectral changes, induced by the oxygenation at the β -hemes in the presence of IHP, were larger at higher pH values. Its EPR spectrum indicated that its α -nitrosyl hemes were essentially 100% 5-coordinate at and below pH 5.0 in the presence of IHP and the absence of O_2 (Fig. 1B, spectrum A, and Fig. 2, open rectangle at pH 4.9). The effect of BPG on the coordination equilibrium of the α -nitrosyl hemes of $\alpha(\text{Fe-NO})_2\beta(\text{Fe})_2$ and $\alpha(\text{Fe-NO})_2\beta(\text{Fe-O}_2)_2$, as measured by EPR (not shown), were also in favor of the 5-coordinate state, but less pronounced than that observed with IHP (Fig. 2). The degree of saturation with O_2 of the β -hemes in $\alpha(\text{Fe-NO})_2\beta(\text{Fe})_2$ was readily controlled by adjusting the concentration of O_2 in the medium, which was in turn regulated by the atmospheric partial pressure of O_2 ($p\text{O}_2$). A series of EPR spectra of $\alpha(\text{Fe-NO})_2\beta(\text{Fe})_2$ at numbers of fixed $p\text{O}_2$ values at 15°C during the course of deoxygenation at pH 7.4 (Fig. 1C) shows reasonable isosbestic points, indicating that the observed spectral changes were derived from the two-component system of the 5- \leftrightarrow 6-co-

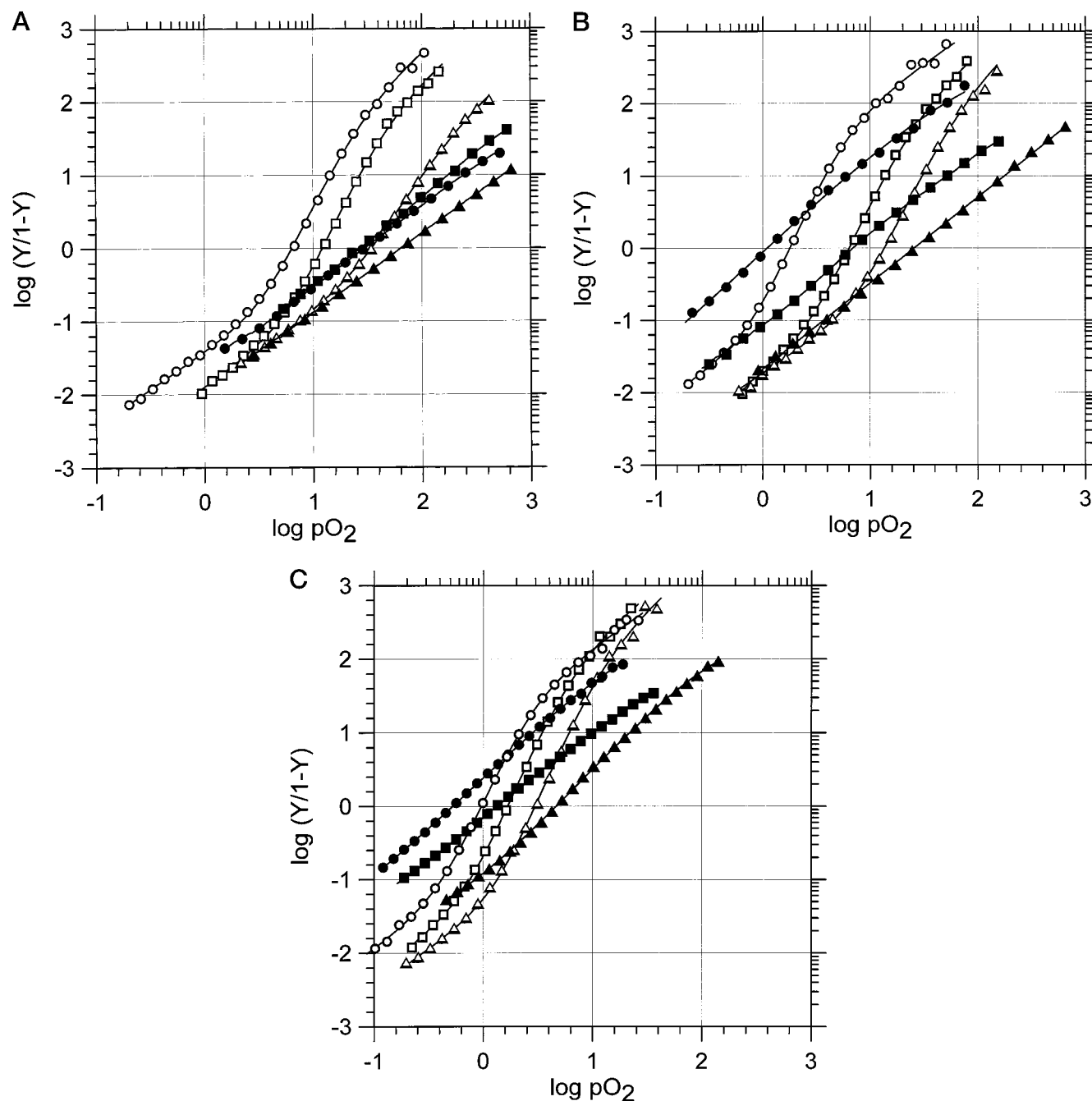


FIG. 3. Oxygen equilibrium curves for α -nitrosyl Hb (closed symbols), compared with those for native HbA (open symbols), at pH 5.8 (A), 7.4 (B), and 8.2 (C). Circles, no organic phosphates; squares, + 2 mM BDPG; triangles, + 2 mM IHP. Plotted points correspond to one every three data points. Experimental conditions: sample concentration was 120 μ M heme in 50 mM bis-Tris-propane buffer, containing 0.1 M Cl^- and small amounts of catalase and superoxide dismutase. Measurements were carried out at 15 $^{\circ}\text{C}$.

ordination equilibrium in the α -nitrosyl hemes. This further indicated that undesirable side reactions, such as formation of met heme, transfer of NO from α - to β -subunits, and release of NO from Hb, were practically negligible during the course of measurements. It should be pointed out that these spectra changes were reversible upon reoxygenation. The 5- \leftrightarrow 6-coordination equilibrium of the α -nitrosyl hemes of $\alpha(\text{Fe-NO})_2\beta(\text{Fe})_2$ was shifted in a predictable manner toward the 5-coordinated state upon deoxygenation as a function of pH (Fig. 2). The apparent pH dependence of the midpoints of the EPR spectral transition (Fig. 2, open and closed circles) is a measure of the Bohr effect of the O_2 binding in $\alpha(\text{Fe-NO})_2\beta(\text{Fe})_2$. Its O_2 affinity decreases (or its P_{50} value increases) continuously at lower pHs, even below neutral pH, where the

Bohr effect of O_2 binding of native Hb is leveled off. However, because EPR data obtained at 77 K may not represent true pH and O_2 equilibrium values of $\alpha(\text{Fe-NO})_2\beta(\text{Fe})_2$ measured at 15 $^{\circ}\text{C}$, caution is warranted in interpreting the EPR data too quantitatively.

Oxygen Binding Characteristics of $\alpha(\text{Fe-NO})_2\beta(\text{Fe-O}_2)_2$ —Results obtained from spectrophotometric O_2 equilibrium measurements are shown in Fig. 3. At pH 5.8 (Fig. 3A), α -nitrosyl Hb showed a strikingly diminished O_2 affinity, virtually absent cooperativity, and decreased effect of BPG and IHP. In the absence of organic phosphates, the lower asymptote of the Hill plot for α -nitrosyl Hb matched that for Hb, indicating that despite α -nitrosyl Hb having both the α -subunits ligated with NO, the O_2 affinity of the complementary β -subunits remained

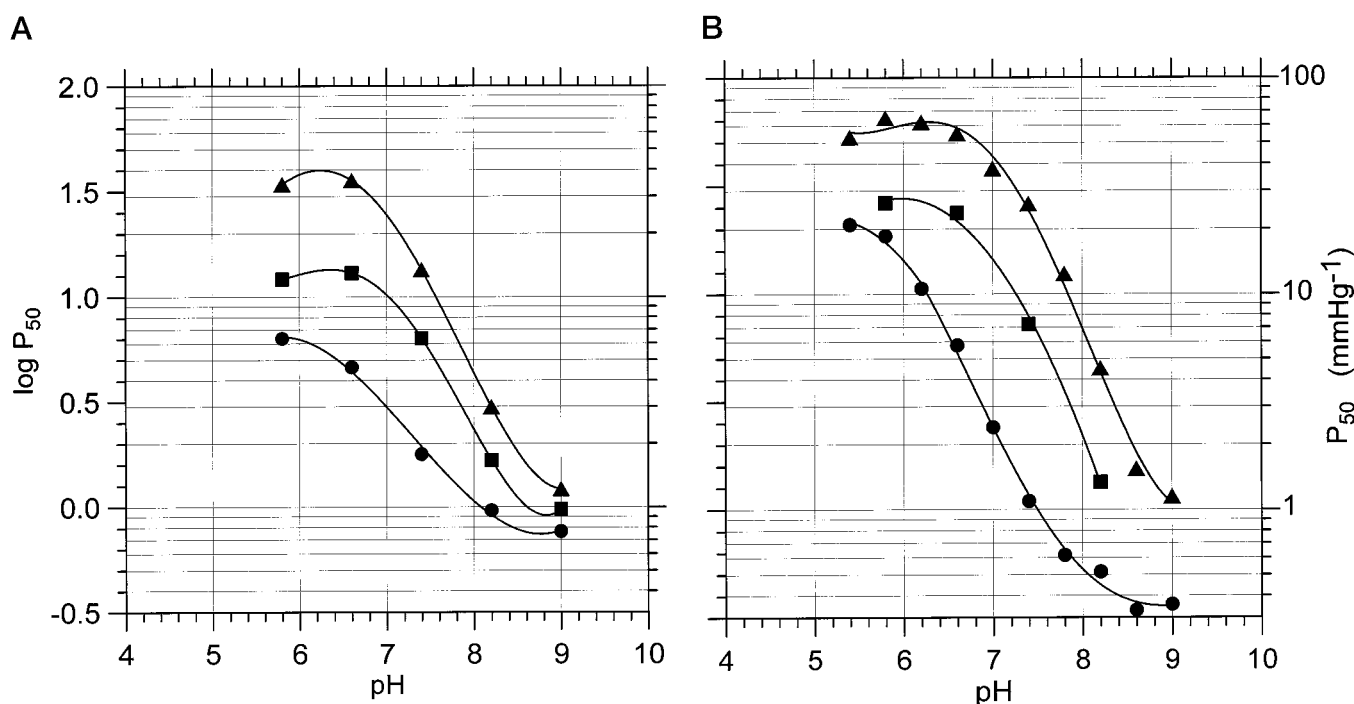


FIG. 4. Bohr effect on O_2 equilibria of native HbA (A) and α -nitrosyl Hb (B), expressed as $\log P_{50}$ versus pH. Circles, no organic phosphate; squares, + 2 mM BPG; triangles, + 2 mM IHP. Experimental conditions were the same as for Fig. 3.

as low as at initial ligation stages of native Hb. In the presence of IHP, the lower asymptote for the α -nitrosyl Hb was also similar to that of Hb under the same conditions. On the other hand, at pH 8.2 (Fig. 3C), the upper asymptote of the curve for α -nitrosyl Hb approached that for Hb. This indicates that the affinity for O_2 of this derivative increased with pH, while showing a trend of being comparable but not completely equaling the O_2 affinity at the last oxygenation steps of Hb under similar conditions. In other words, α -nitrosyl Hb at this pH exhibited characteristics of a high affinity species. Inositol hexaphosphate, as well as BPG, to a lesser degree, had the effect on this derivative of shifting the curve to the right. Cooperativity was present and comparable to that for a Hb species with two binding sites. At pH 7.4 (Fig. 3B), the oxygenation curve for α -nitrosyl Hb shifted toward the upper asymptote of that for Hb, that is, the high affinity side. However, BPG and IHP decreased its O_2 affinity by shifting the curve toward the low affinity side.

A comparative view of the effect of organic phosphates on the O_2 affinities of Hb and α -nitrosyl Hb at different pH values can be clearly visualized in their Bohr effects (Fig. 4, A and B, respectively). In the presence of organic phosphates, α -nitrosyl Hb (Fig. 4B) showed a greatly enhanced Bohr effect. Around pH 7, the Bohr coefficients, estimated as $\Delta P_{50}/\Delta pH$, were -0.5 and -0.9 Bohr protons for Hb and α -nitrosyl Hb, respectively. In the presence of BPG and IHP, the Bohr effect increased in the case of Hb due to the major effect these phosphates had on lowering the O_2 affinity on the acidic side, in agreement with previous works (28). Bohr coefficients were -0.7 and -0.8 in presence of BPG and IHP, respectively. However, in the case of α -nitrosyl Hb, organic phosphates reduced the O_2 -affinity on both acidic and alkaline regions. Bohr coefficients in the presence of BPG or IHP were approximately -0.9 and did not differ from the condition without organic phosphates. The magnitude of the effects of BPG and IHP on lowering the O_2 affinity can be expressed as the ratio of partial pressure of O_2 at 50% saturation (P_{50}) in the presence and absence of the organic phosphate (i.e. $P_{50}^{+PHOSPHATE}/P_{50}^{NONE}$). For Hb, the enhanced effect of

organic phosphates on the acidic side was reflected as an increased ratio of 1.9 and 5.4 at pH 5.8 versus ratios 1.2 and 1.6 at pH 9, for BPG and IHP, respectively. The maximum effects of BPG and IHP occurred between pH 7 and 7.4, and the values were 3.6 and 7.6 for BPG and IHP, respectively. In the case of α -nitrosyl Hb, these organic phosphates exerted a dramatic effect on its O_2 affinity. The maximum effect was registered at pH 7.4, and the ratios were 6.6 and 23.9 for BPG and IHP, respectively. The equally effective ability of BPG and IHP to lower the O_2 affinity of α -nitrosyl Hb over both sides of the pH range is reflected in a rather symmetric bell-shape curve (not shown). The pH dependence of the O_2 equilibrium constants for the third and fourth binding steps, K_3 and K_4 , in the absence and presence of IHP, is shown in Fig. 5A. Curves were smoothed by fixing only the upper and lower asymptotes. The lowest K_3 and highest K_4 values were obtained by extrapolating curves for $\log K_3$ in the presence of IHP at low pH values and $\log K_4$ in the absence of IHP at high pH values, respectively. Values for lowest K_3 and highest K_4 thus obtained were 0.011 and 7.23 mm Hg $^{-1}$, respectively. The number of protons released at the i th oxygenation step, ΔH^+_i ($i = 3, 4$), were then obtained from the slope of the curves in the absence and presence of IHP and are shown in Fig. 5, B and C, respectively. The total Bohr effect, expressed as ΔH^+_{total} , was calculated as the sum of ΔH^+_3 and ΔH^+_4 and is equal to the total Bohr protons released upon oxygenation of one molecule of α -nitrosyl Hb. ΔH^+_{total} values were 1.67 at pH 6.6 in the absence of IHP and 1.71 at pH 8.6 in the presence of IHP. The average number of released protons, ΔH^+_{AV} , can be obtained by dividing ΔH^+_{total} by the number of ligation steps (2 in this case), which yielded values of -0.84 and -0.86 protons per ligation. This is in agreement with the overall values of approximately -0.9 , estimated from Fig. 4B. Cooperativity for α -nitrosyl Hb, as expressed by n_{max} , was found to vary considerably with pH. In either the presence or absence of IHP, cooperativity was minimal under acidic conditions, approaching values of 1 (noncooperative) as pH was decreased. In the absence of IHP, n_{max} reached values around 1.4 above pH 6.6. In the presence of

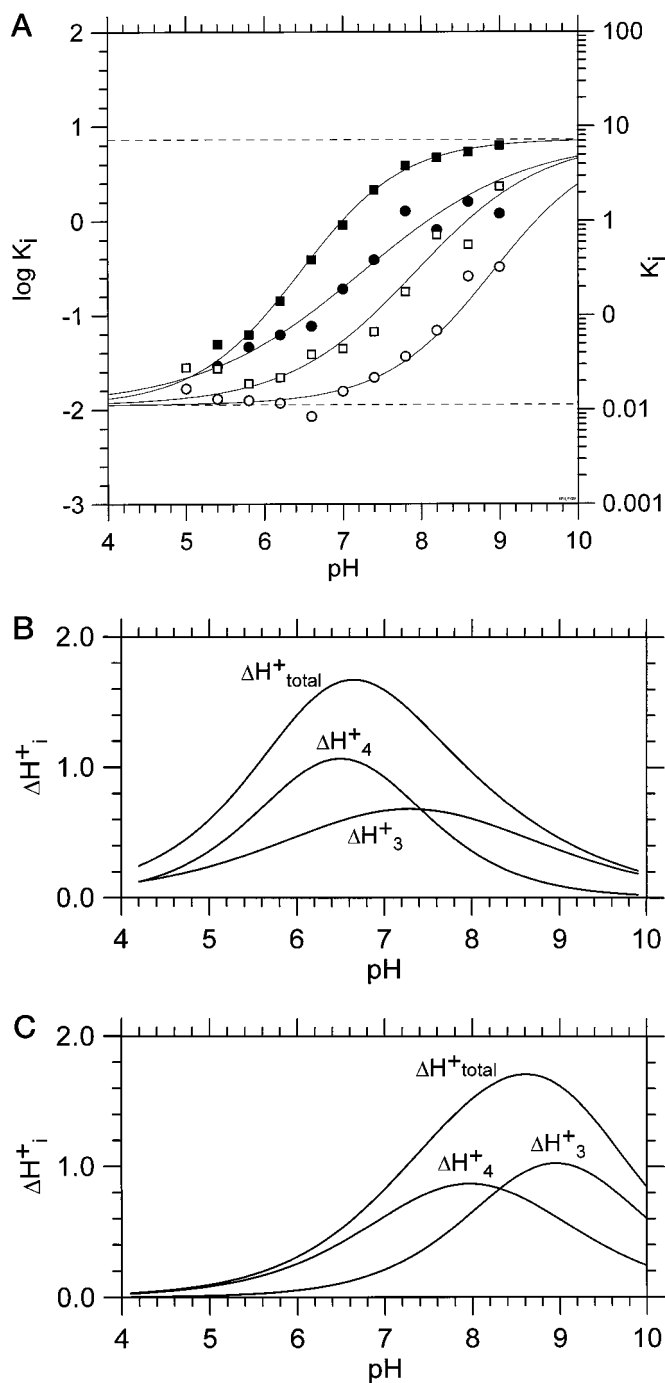


FIG. 5. pH dependence of the stepwise oxygen affinity for the third and fourth binding step, expressed as K_3 (circles) and K_4 (squares), respectively, in the absence (closed symbols) and presence (open symbols) of 2 mM IHP. A, data were calculated from oxygenation curves shown in Fig. 3. The slope of the respective smoothed curves from A, which correspond to conditions in the absence and presence of 2 mM IHP, are shown in B and C, respectively. The total Bohr effect ($\Delta H^+_{\text{total}}$) for each condition was obtained by adding curves for the third and fourth ligation.

IHP, cooperativity increased at more alkaline conditions (pH above 7), although n_{max} values were apparently higher ($n_{\text{max}} \sim 1.5$ around pH 8.2) than those obtained in the absence of IHP.

Reactivity of the Sulfhydryl Group of Cys⁶⁹³ as a Probe for the Quaternary Structure—In the presence of excess of 4-PDS, reactions were first-order with respect to concentrations of Hb derivatives. The apparent rate constants (k_{app}) for both oxy and deoxy Hb increased monotonically above pH 7. Moreover, rate

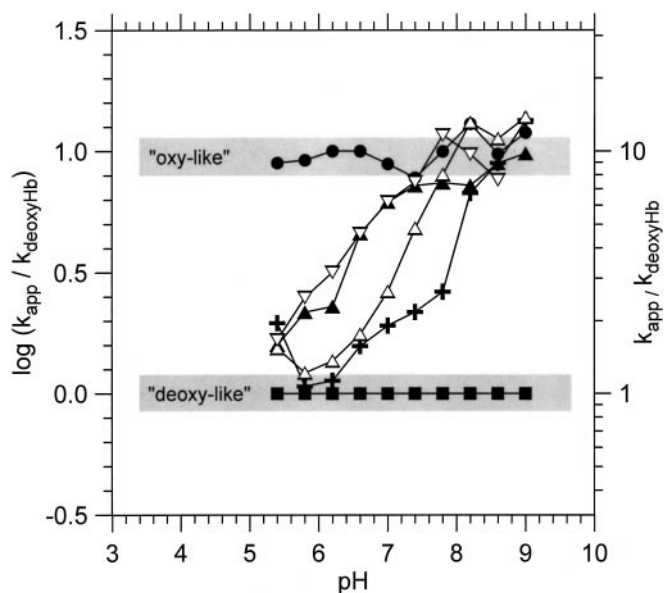


FIG. 6. pH dependence of the quaternary structural change of native HbA and several derivatives of α -nitrosyl Hb, as probed by the sulfhydryl reactivity of Cys⁶⁹³ toward 4-PDS in the absence (closed symbols) and presence (open symbols) of 2 mM IHP. Reaction rates were normalized against those obtained for deoxy Hb. Squares, native deoxy Hb; circles, native oxy Hb; triangles, $\alpha(\text{Fe-NO})_2\beta(\text{Fe-O}_2)_2$; inverted triangles, $\alpha(\text{Fe-NO})_2\beta(\text{Fe-CO})_2$; crosses, $\alpha(\text{Fe-NO})_2\beta(\text{Fe})_2$. Sample concentration was 40 μM heme in 50 mM bis-Tris-propane buffer, containing 0.1 M Cl^- , at 15 $^\circ\text{C}$. 4-PDS was added to a final concentration of 160 μM . The reaction was followed by monitoring absorbance change at 324 nm due to the release of 4-thiopyridine.

constants for oxy Hb were about 10 times those corresponding to deoxy Hb at any pH studied (not shown). To ease comparison, the relative reactivity of a derivative at any pH was normalized with respect to rate constants for deoxy Hb corresponding to the same pH, as shown in Fig. 6. Thus, rate constants for deoxy Hb were equal to 1, and those for oxy Hb were approximately 10. In the same figure, reactivity profiles for various derivatives of α -nitrosyl Hb were included.

Sulfhydryl reactivities of all α -nitrosyl Hb species, including β -ligated derivatives, were strongly pH-dependent and varied from that characteristic of deoxy Hb to that of oxy Hb when solution conditions changed from acidic to alkaline, respectively. Moreover, the transition from one state to the other seemed to be related to the degree of the affinity of the ligand toward the β -subunits and the presence of organic phosphate, such as IHP. $\alpha(\text{Fe-NO})_2\beta(\text{Fe-O}_2)_2$ underwent a transition between pH 6 and 7 in the absence of IHP, whereas this transition occurred under more alkaline conditions (between pH 7 and 8) in the presence of IHP. In the same manner, $\alpha(\text{Fe-NO})_2\beta(\text{Fe-CO})_2$ showed rate constants that were shifted toward those of an “oxy-like” derivative because even under acidic conditions, rate constants were intermediate to those of the “oxy-like” and “deoxy-like” conformations. However in the presence of IHP and under acidic conditions, the rate constants resembled those of $\alpha(\text{Fe-NO})_2\beta(\text{Fe-O}_2)_2$ in the absence of IHP, indicating that the transition to a “deoxy-like” conformation had taken place. Sulfhydryl reactivities above pH 7.4 in the presence of IHP for all α -nitrosyl Hb derivatives were indistinguishable from those obtained in the absence of IHP. This is consistent with the fact that IHP interacts more strongly with Hb under acidic than alkaline conditions by shifting the allosteric equilibrium toward the low affinity state.

DISCUSSION

Stability of α -Nitrosyl Hb—Nitrosyl derivatives of Hb are less stable than native Hb under aerobic conditions. Their

oxidative decomposition leads to the formation of fully and partially met Hbs and NO_3^- . α -Nitrosyl Hb was converted to met Hb more rapidly than native Hb but more slowly than tetranitrosyl Hb. Moreover, the rate for all derivatives diminished as the temperature was reduced. At 15 °C, the half-life time for α -nitrosyl Hb was 16 h; this time span was reasonably long enough for all experiments to be conducted on this derivative without the concern of formation of significant amounts of met Hb. This rather moderate stability of α -nitrosyl Hb derivative upon exposure to air might conflict with common expectation. We have observed, however, that α -nitrosyl Hb formed within intact and fully functional erythrocytes was much more rapidly decomposed at 37 °C ($t_{1/2} \approx 21$ min) than in solution conditions.² This could explain why this derivative has been seldom found *in vivo* under normal conditions, but only observed when large amounts of NO were produced in the blood, such as in shock, in inflammation, or upon administration of cytokines, nitrite, and organic nitrates (7–12). Under such conditions, steady-state concentrations of α -nitrosyl hemes so formed could reach several percent of the total hemes of Hb, which corresponds to steady-state intra-erythrocyte concentrations of over 400 μM α -nitrosyl heme. This indicates an active role of Hb in removing NO from the plasma and sequestering/concentrating NO as α -nitrosyl hemes, as plasma concentrations of NO range only from $\sim 10^{-7}$ to $\sim 10^{-5}$ M.

Another matter of concern in the present work was the stability of the ligation within samples because it has been reported the ability of NO bound to heme under levels below saturation to redistribute among Hb subunits (1–3). This implies migration of NO molecules already bound to hemes, from α -subunits to β -subunits, and *vice versa*. It has to be pointed out that the α -nitrosyl Hb was always kept, unless indicated, in its oxygenated form, $\alpha(\text{Fe-NO})_2\beta(\text{Fe-O}_2)_2$. In fact, no variation in the percentage of the NO-bound α subunits in α -nitrosyl Hb for the time span of the measurements was detected by EPR under any of our experimental conditions.

We have found that the affinity of NO for the α -hemes of Hb is more than 100-fold stronger than that for the β -hemes under physiological conditions. This was reflected principally in their dissociation rate constants: $k_{\text{off}}(\alpha) < 10^{-5} \text{ s}^{-1}$ versus $k_{\text{off}}(\beta) \approx 10^{-3} \text{ s}^{-1}$ at acidic pH and 15 °C, particularly in the presence of organic phosphate effectors (29).² Of course, it is likely that these values increase substantially at physiological temperature of 37 °C.² Therefore, the primary product of the reaction of deoxy Hb with NO at equilibrium would be α -nitrosyl, β -deoxy Hb, $\alpha(\text{Fe-NO})\alpha(\text{Fe})\beta(\text{Fe})_2$ and/or $\alpha(\text{Fe-NO})_2\beta(\text{Fe})_2$, when $[\text{NO}]/[\text{heme}] \ll 0.5$, as evidenced by *in vitro* experiments in solution (1–3) and in erythrocytes (4–6) as well as *in vivo* results in the blood (7–12). These two types of α -nitrosyl Hbs are indistinguishable from one to the other by EPR or spectrophotometry. Furthermore, the former can exist only in dynamic equilibrium and cannot be isolated without chemical manipulation such as intersubunit cross-linking. Therefore, we have prepared in the present work the latter in pure state and examined its characteristics in detail.

The Action of NO on the α -Hemes of Hb—The tenet of the cooperative mechanism of Hb is that the quaternary structural equilibrium of deoxy Hb in the T (low affinity) state ($1 < L < \infty$) reversibly shifts toward the R- (high affinity) state upon successive binding of four ligands to its heme groups (30, 31), as schematically illustrated in Fig. 7. (L is an allosteric parameter defined as $[\text{T}_0]/[\text{R}_0]$ or a ratio of molar concentrations of deoxy Hb in the T- and R-states, respectively (31).) The stronger the

affinity of the ligand, the more effective is the shift of the equilibrium toward the R state. Such a homotropic (or positively allosteric) property of ligand has been unequivocally proven for diatomic ligands like O_2 and CO. Low concentrations of CO, which has more than 10-fold higher affinity than O_2 , effectively compete with O_2 for the hemes of deoxy Hb under physiological conditions. In addition, its binding shifts the quaternary structure of Hb toward the R- (high affinity) state, so that partial binding of CO renders Hb ineffective in its delivery of O_2 to tissues, which is the primary cause of CO poisoning. The United States Environmental Protection Agency sets the Federal occupational limits of CO to be 8 and 35 ppm for 9-h and 1-h inhalation, respectively (32). Such a homotropic behavior has been generally assumed for NO, another diatomic ligand, which has an extremely high affinity of $\sim 10^{-12}$ M for deoxy Hb (33). Thus NO has more than 10^3 -fold stronger affinity for deoxy Hb than CO. However, several doses of 1-h inhalation of ~ 80 ppm NO have been successfully prescribed for clinical treatments of persistent pulmonary hypertension for the newborn and other pulmonary distress syndrome for the adult with no apparent adverse effect (34). Therefore, one must wonder why there have been neither observation nor report of NO poisoning.

The present work has demonstrated that NO behaves quite differently from other diatomic ligands (O_2 and CO) toward Hb. The principal difference between them lies in the difference in their coordination chemistry with heme (35–40), as summarized in Table I. The binding of O_2 and CO favors the 6-coordinate heme over the 5-coordinate heme. Thus, the affinity of O_2 or CO toward heme increases synergistically with the affinity of the trans-axial imidazole ligand. In contrast, the affinity of NO toward heme is stronger in a 5-coordinate state than in a 6-coordinate state. Thus, binding of NO to heme tends to weaken the Fe-axial imidazole bond as much as $\sim 10^3$ -fold, or sometimes to cleave the bond. Such a trans-axial bond cleavage upon ligation of NO has been observed in the cases of α -hemes of Hb in the presence of IHP (13–15, 26, 27), $\alpha(\text{Fe-NO})_2\beta(\text{Fe})_2$ even in the absence of IHP (Refs. 1, 3, 14, and 15, and this paper), and the heme group in the regulatory subunit of soluble guanylyl cyclase (41). These heme Fe-His bonds were known to be constrained or distorted and thus susceptible to cleavage (30, 42). The ligation of NO could cause the trans-axial bond cleavage under appropriate conditions as demonstrated here. This is the reason why only NO, a simple diatomic molecule, but not O_2 and CO, can act as a paracrine inducer of conformational changes in certain hemoproteins.

Effect of Cleavage/Elongation of the α -Heme-F Helix Linkage on the Quaternary Structure of Hb—Several years ago, we demonstrated that the weakening/cleavage of the α -heme-His (F8) bonds causes the permanent shift of the quaternary structure of Hb to a T- (low affinity extreme) state (43), where $L = \infty$ (31), as exemplified by $\alpha(\text{porphyrin})_2\beta(\text{Fe})_2$ (43), $\text{HbM}_{\text{Iwate}}(\text{His}^{\alpha 87} \rightarrow \text{Tyr})$ (44), and $\text{HbM}_{\text{Boston}}(\text{His}^{\alpha 58} \rightarrow \text{Tyr})$ (45). The recently reported recombinant Hb ($\text{His}^{\alpha 87} \rightarrow \text{Gly}$) (46) probably belongs to this category. The $\alpha(\text{Ni})_2\beta(\text{Fe})_2$ hybrid, the $\alpha(\text{Ni})$ -subunits of which are predominantly in a 4-coordinate state, having Ni bonding with neither a distal ligand nor the $\text{His}^{\alpha 87}$ (F8) residue (47), shows similar structural and functional characteristics (47).³ Therefore, it can also be considered to belong to this category as well.³ These artificial and natural hybrid Hbs exhibit the lowest O_2 affinity attainable for the β -subunits of Hb with no or substantially diminished cooperativity and allosteric responses toward proton and organic phosphates, characteristics associated with the T- (low affinity extreme)

² T. Yonetani, A. Tsuneshige, Y. Zhou, and X. Chen, unpublished results.

³ A. Tsuneshige and T. Yonetani, unpublished results.

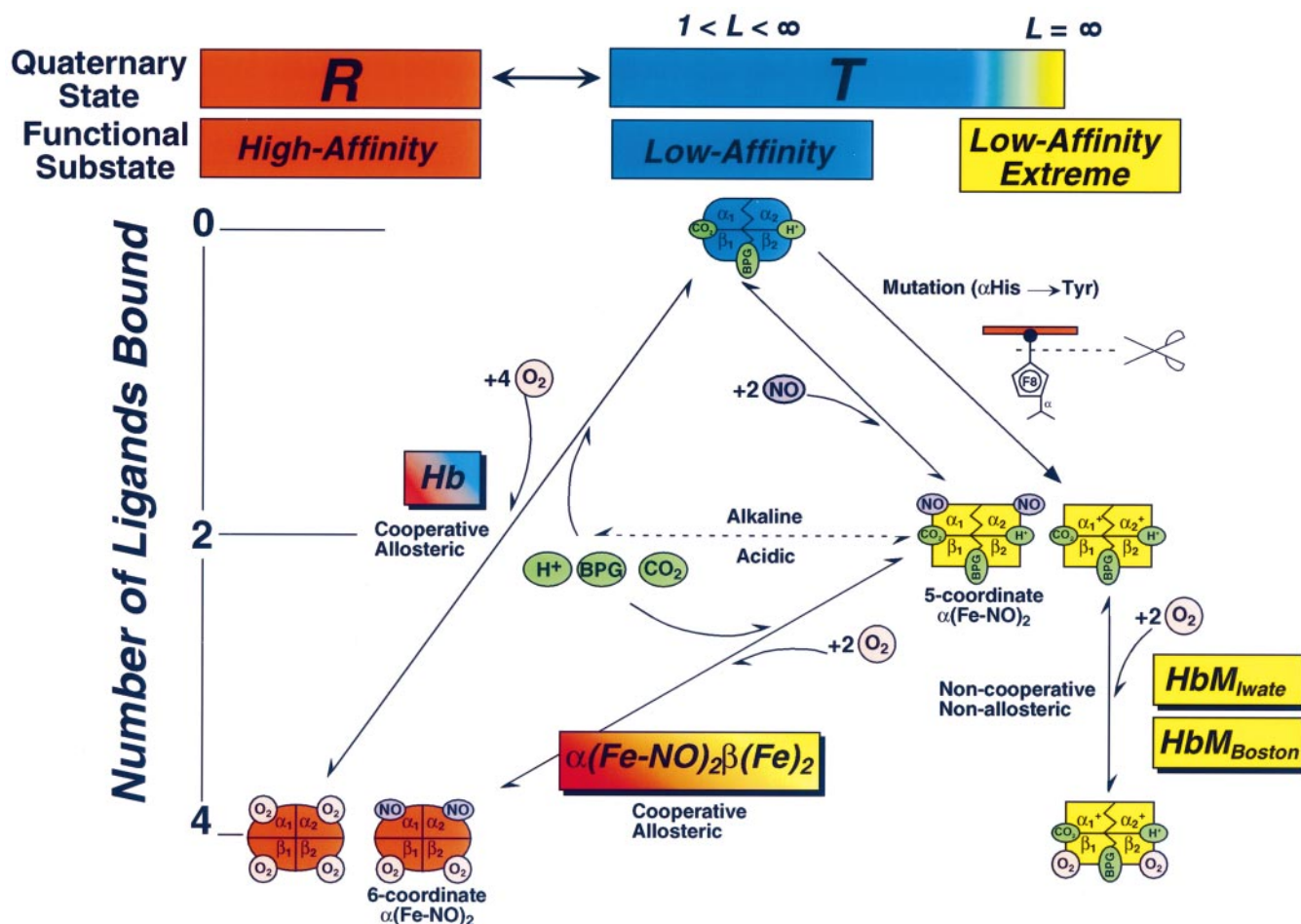


FIG. 7. Schematic presentation of structure-function relationship among Hb, HbM_{Iwate} , HbM_{Boston} , and α -nitrosyl Hb in O_2 binding equilibria. α^+ represents a met heme-containing α -subunit. The reversible four-step O_2 binding to native Hb takes place between the T- (low affinity) and the R- (high affinity) states. The His $^{\alpha}$ \rightarrow Tyr mutations in HbMs shift the quaternary/functional state of HbMs to the T- (low affinity extreme) state irreversibly. The O_2 binding to their β -subunits occurs within the T- (low affinity extreme) state and, thus, is noncooperative and nonallosteric. The ligation of two molecules of NO to the α -hemes of Hb transforms the quaternary/functional state of Hb toward the T- (low affinity extreme) state. However, this shift is reversibly modulated by ligation in the β -hemes, pH, and organic phosphates. Therefore, the O_2 binding of $\alpha(Fe-NO)_2\beta(Fe)_2$ occurs between T- (low affinity extreme) and R- (high affinity) states and, thus, is cooperative and allosterically sensitive.

state with $L = \infty$ (Fig. 7). The common structural denominator among these hybrid Hbs is the cleaved α -heme Fe-proximal bonds or an extended linkage between the α -heme Fe and the α -carbon of the F8 residue (43). This permanent quaternary structural shift is surprisingly independent of the nature/oxidation state of the porphyrin metal ion, the presence and absence of the porphyrin metal ion, and/or the presence and absence of distal ligation. It solely depends upon the geometry (distance/orientation) of the proximal linkage between the α -porphyrin metal and F helix. This might imply that the F helices in the α -subunits of Hb have an inherent tendency to move away from the α -heme planes. Thus, if the α -heme-F helix linkage is removed, Hb would shift its quaternary structure to the energetically more stable T- (low affinity extreme) state with $L = \infty$. The presence of this linkage might be actually constraining and preventing Hb to settle into such a stable state with $L = \infty$ (in nonequilibrium) and keeping deoxy Hb at the energetically more dynamic T- (low affinity) state with $1 < L < \infty$ (in equilibrium), which can readily and reversibly shift to the R- (high affinity) state in response to ligand binding and/or to interaction with allosteric effectors.












Whether the α -heme-F helix linkage plays such an important role in the actual mechanisms of trigger and cooperativity in native Hb may be debatable. There is no clear evidence for the reversible cleavage of the α -heme Fe-F-helix linkage during the

reversible ligation of O_2 and CO to Hb. However, the excessively out-of-the plane position of the α -heme Fe observed in deoxy Hb in the T-state may be a subtle indication of such an inherent pull by the F helix of deoxy Hb in the energetically more dynamic T- (low affinity) state with $1 < L < \infty$ (Fig. 7). One wonders whether it is technically feasible to test such a hypothesis by measuring the effect of artificial cleavage of the α -heme Fe-His (F8) bonds on the atomic (quaternary) structure of a stable R-state, ligated Hb such as tetracarboxymonoxyl Hb by an energy-minimization technique. The widely held hypothesis that the out-of-plane movement of the α -heme Fe (high spin) in deoxy Hb pushes the F helix away from the α -heme plane (30) does not adequately explain why $\alpha(\text{porphyrin})_2\beta(Fe)_2$ settles into the T- (low affinity extreme) state in the absence of the α -porphyrin-F helix linkage. The distal ligation-induced movement of the α -heme Fe toward the in-plane position would pull the F helix back toward the α -heme plane and thus might contribute in part to the triggering and the shift in the quaternary structure from the T- (low affinity) state toward the R- (high affinity) state. It may be possible that such a general mechanism of the cooperativity of Hb is more exaggeratedly revealed in α -nitrosyl Hb by the unique coordination characteristics of NO, namely, its ability to trans-axially cleave the α -heme Fe-His (F8) bonds. Stereochemically speaking, this ability is due to a shorter interatomic

TABLE I

Affinities (equilibrium constant, K_E , in M^{-1}) of heme for diatomic ligands (CO and NO) and an imidazole base

Data have been compiled from Refs. 35–40.

	5-Coordinate	ΔK_E	6-Coordinate
Ligand Affinity	2×10^4  $\sim 10^{15}$ 	 2×10^4  10^{-3}	4×10^8  10^{12} 
Base Affinity	3×10^4 	 2×10^4  3×10^{-3}	6×10^8  ~ 10 

distance between the α -heme Fe and NO and consequently the out-of-plane position of the Fe in the opposite (distal) direction in α -nitrosyl hemes (48).

The β -heme Fe-F helix linkage, on the other hand, does not seem to have such a critical role in defining the quaternary/functional structure of Hb (43).³ For example, it has been shown that $\alpha(\text{Fe})_2\beta(\text{porphyrin})_2$, which has no direct linkage between β -porphyrin and F helix, exhibits a cooperative O_2 binding at the α -subunits (43). Therefore, an alternate mechanism must be considered for the quaternary transition induced by the β -ligation.

Effect of Ligation of NO at the α -Hemes on the Tertiary and Quaternary Structural Equilibria and O_2 Binding Characteristics of Hb—It is, therefore, obvious from the preceding discussion that the NO-induced cleavage of the α -heme Fe-His (F8) bonds would transform $\alpha(\text{Fe-NO})_2\beta(\text{Fe})_2$ into a T- (low affinity extreme) state, despite ligation of two molecules of a high affinity ligand (NO) per tetramer. In other words, two NO molecules bound to the α -hemes act as a negative allosteric ligand rather than as a homotropic (or positive allosteric) ligand by breaking the α -heme Fe-His (F8) bonds and thus, shifting the quaternary structural equilibrium from the T- (low affinity) state with $1 < L < \infty$ (in equilibrium) toward the far end of the T-state, *i.e.* the T- (low affinity extreme) state with $L \rightarrow \infty$ (in nonequilibrium), so that the O_2 affinity in the β -subunits of $\alpha(\text{Fe-NO})_2\beta(\text{Fe})_2$ is substantially reduced. In the above-mentioned hybrids, $\alpha(\text{porphyrin})_2\beta(\text{Fe})_2$, HbM_{Iwate}(His ^{α 87} \rightarrow Tyr), HbM_{Boston}(His ^{α 58} \rightarrow Tyr), and recombinant Hb (His ^{α 87} \rightarrow Gly), the α -heme Fe-F helix linkages are irreversibly either cleaved or elongated by chemical modification or mutation. Therefore, their quaternary structure is permanently locked into the T- (low affinity extreme) state with $L = \infty$ (in nonequilibrium), and thus their O_2 binding process is not only of low affinity but also noncooperative and allosterically insensitive (Fig. 7). On the other hand, the cleavage of the α -heme Fe-His (F8) bonds in $\alpha(\text{Fe-NO})_2\beta(\text{Fe})_2$ is reversible. Varying degrees of re-formation of the α -heme Fe-His (F8) bonds upon ligation in the β -subunits, in the absence of IHP, and/or at higher pH (Fig. 2) represent the transition of the quaternary structural equilibrium toward the R- (high affinity) state (Fig. 7). Thus, it is evident that the reversible, two-step O_2 binding to the β -subunits of $\alpha(\text{Fe-NO})_2\beta(\text{Fe})_2$ is accompanied by the T- (low affinity

extreme) \leftrightarrow R (high affinity) quaternary transition and is a cooperative and allosterically sensitive process (Fig. 7).

The above-mentioned general framework of the correlation between the tertiary and quaternary structures and functional properties of $\alpha(\text{Fe-NO})_2\beta(\text{Fe})_2$ in O_2 binding (Fig. 7) has been more quantitatively confirmed by the present EPR measurements of the 5- \leftrightarrow 6-coordination equilibrium of the α -hemes (Figs. 1 and 2), the O_2 equilibrium measurements (Figs. 3 and 4), and the probing of the quaternary structure by the Cys⁶⁹³ reactivity (Fig. 6). From the present oxygenation experiments (Fig. 3), we can draw the following conclusions: (a) the overall O_2 affinity (P_{50}) of α -nitrosyl Hb varied with pH from a low affinity at low pH to a high affinity at high pH, (b) cooperativity was virtually absent at acidic extreme and was substantially reduced at alkaline extreme, (c) the maximal cooperativity and maximal Bohr effect occurred around pH 7, and (d) IHP, a negative allosteric effector, shifts the O_2 affinity toward the low affinity side over an entire pH range (less so at the acidic extreme). The EPR measurements of the α -heme coordination equilibria (Fig. 2), on the other hand, showed that (e) the coordination equilibrium of the α -nitrosyl hemes shifted in favor of the 5-coordinate at lower pH, (f) the β -ligation-induced shift in the α -nitrosyl heme coordination was considerably reduced under acidic conditions and was somewhat diminished under alkaline conditions, (g) the largest change in the β -ligation induced change in the α -nitrosyl heme coordination occurred around pH 7.4, and (h) IHP shifts the coordination equilibrium of the α -hemes in favor of the 5-coordinate over a wide pH range (less effectively at acidic extreme). Thus, the increase in the 5-coordinate α -heme tertiary structure is correlated to the shift of its functional characteristics toward the more low affinity state. The degree of the β -ligation-induced changes in the coordination equilibrium of the α -heme tertiary structure is related to the degree of the functional cooperativity exhibited by α -nitrosyl Hb. Both functional and tertiary structural changes of α -nitrosyl Hb induced by allosteric effectors such as proton and organic phosphates are most significant around physiological pH of 7.4 and substantially diminished at acidic extreme. The reactivity of Cys⁶⁹³ toward 4-PDS of various derivatives of α -nitrosyl Hb (Fig. 6) is entirely consistent with their predicted quaternary structures associated with respective functional characteristics.

Shifts in the 5- \leftrightarrow 6-coordination equilibrium of the α -nitrosyl hemes in $\alpha(\text{Fe-NO})_2\beta(\text{Fe})_2$ upon ligation at the β -hemes have been observed previously (12, 14, 15). However, the interaction of $\alpha(\text{Fe-NO})_2\beta(\text{Fe})_2$ with O_2 , the physiologically most important ligand of Hb, has not been investigated in detail, probably on the assumption that NO and O_2 could not be mixed and that $\alpha(\text{Fe-NO})_2\beta(\text{Fe})_2$ might be readily oxidized to met Hb in the presence of O_2 . The present work demonstrates that the intersubunit transfer of structural information in Hb occurs in both directions (α -subunits \leftrightarrow β -subunits): ligation of NO to the α -hemes induces the trans-axial breakage of the α -heme Fe-His (F8) bonds, followed by the quaternary structural shift toward the T- (low affinity extreme) state, which in turn lowers the ligand affinity of the β -subunits in $\alpha(\text{Fe-NO})_2\beta(\text{Fe})_2$. Conversely, binding of ligand (CO , O_2 , or NO) to the β -hemes shifts the quaternary structure of $\alpha(\text{Fe-NO})_2\beta(\text{Fe})_2$ toward the R- (high affinity) state, which in turn causes the re-formation of the α -heme Fe-His (F8) bonds, which resulted in the significant decrease in the affinity of α -hemes for NO. In the 6-coordinate state, the affinity of heme for NO is known to decrease as much as 10^3 -fold from that in the 5-coordinate heme in the case of model heme systems (Table I). Therefore, the coordination equilibrium of the α -nitrosyl hemes in $\alpha(\text{Fe-NO})_2\beta(\text{Fe})_2$ as measured by EPR (Fig. 2) could serve a convenient EPR probe

to assess the quaternary structural equilibrium as well as the O₂ binding equilibrium of $\alpha(\text{Fe-NO})_2\beta(\text{Fe})_2$. The α -nitrosyl heme-His (F8) coordination complex not only acts as a signal transducer of the quaternary structural and functional changes but also may behave as a Bohr group in α -nitrosyl Hb. Therefore α -nitrosyl Hb can mimic allosteric functions of native Hb remarkably well. Although its O₂ binding function is a two-step process, α -nitrosyl Hb can modulate the O₂ affinity of its $\beta(\text{Fe})$ subunits as much as 700-fold (Fig. 5A), the magnitude comparable with that observed in the (Fe)-subunits of native Hb.

The Enhanced Bohr Effect in α -Nitrosyl Hb—The enhanced Bohr effect, namely, an increased pH effect on the O₂ affinity of the β -subunits observed in α -nitrosyl Hb (Fig. 4) is striking, where only the two-step ligation was involved. Its Bohr coefficient, ΔH^+ , around physiological pH of 7.4 in the absence of organic phosphates (Fig. 4) is almost double that for native Hb, which involves the four-step ligation. This indicates that the ligation-linked Bohr groups of α -nitrosyl Hb ionize more easily than in native Hb, or that additional groups such as His⁶⁸⁷ are involved in the process. In the presence of IHP, the alkaline Bohr effect was similar to that in the absence of IHP, except that the curve was shifted toward the alkaline side about 1.5 pH units. Comparable features were also detected in EPR experiments. Because the top and bottom on each bar (Fig. 2, *open* and *closed rectangles*, respectively) are roughly related to K_3 and K_4 , respectively, estimated from oxygenation experiments, a midpoint between these two extremes (Fig. 2, *open* and *closed circles*) could be a convenient way to indicate qualitatively the O₂ affinity. It should be remarked here that there is no attempt to imply any strict correlation between these parameters. Note that the curve connecting the midpoints does not follow a linear dependence over pH. The shape of this curve reflects how the ligation-linked allosteric transition takes place in the α -nitrosyl Hb by varying pH and indirectly reflects its Bohr effect. Under acidic conditions, the enhanced effect of protons on the trans-axial bond cleavage in the α -subunits is such that the molecule shifts to the extreme side of the T- (low affinity) conformation, *i.e.* the T- (low affinity extreme) state with $L \rightarrow \infty$ (Fig. 7). This makes its quaternary structure and functions virtually insensitive to any allosteric effectors (such as homotropic ligation in the β -subunits and/or negatively allosteric organic phosphates). Under alkaline conditions, where the α -heme Fe–His (F8) bond is partially restored, the cooperativity between two β -subunits seems to be re-established. It becomes then evident that the overall O₂ affinity in Hb is closely related to the coordination equilibrium of the α -heme Fe–His (F8) bonds in α -nitrosyl Hb and consequently to its quaternary state. For α -nitrosyl Hb, all allosteric effectors studied in the present work have proved to trigger this transition. The breakage of the α -heme Fe–His (F8) bonds implies the ionization of the His⁶⁸⁷ side chains; this could cause a change in pK values of other Bohr groups, or rather be the consequence of these alterations. More stretched/tilted or even absent α -heme Fe–His (F8) bonds promote the transition of the molecule to T-states, either (low affinity) or (low affinity extreme) (43). Earlier resonance Raman studies (42, 49) suggested that the protein control of O₂ binding in Hb is regulated by the state of the heme Fe-proximal His bonds without specifying the identity of the subunits involved.

The Mode of Reaction of Hb with NO—The fact that NO reacts with the α -hemes of Hb as a negative allosteric ligand rather than as a high affinity, homotropic ligand explains the previously unexplained puzzle that although $\alpha(\text{Fe-NO})_2\beta(\text{Fe})_2$ is in the T- (low affinity extreme) state, the NO in the $\alpha(\text{Fe-NO})$ subunits is very tightly bound, because it is in a 5-coordinate state (Table I). Nitric oxide binds to the β -hemes of Hb as a

conventional homotropic ligand in a 6-coordinate state, so that it is released readily from the $\beta(\text{Fe-NO})$ subunits of Hb in the T- (low affinity) state, as the two-state model predicts under acidic conditions. In other words, the interaction of two molecules of NO with the β -hemes of $\alpha(\text{Fe-NO})_2\beta(\text{Fe})_2$ involves a quaternary structural transition between T- (low affinity extreme) and R- (high affinity) states and thus conforms quite well to a two-state model under acidic conditions. However, a simple two-state model cannot adequately explain the four-step reaction of deoxy Hb with NO at acidic and/or neutral pH, because the first two molecules of NO act as a negative allosteric ligand and the subsequent two molecules of NO behave as a positive allosteric ligand. Therefore, NO present in low concentrations in the blood would react with deoxy Hb only as a negative allosteric effector. Thus, NO is not detrimental to the physiological functions of Hb as an O₂/CO₂ transporter in the blood, although the O₂-carrying capacity of Hb would decline as much as 50% by ligation of NO at the α -heme sites.

Physiological Scavenging of NO by Hb—The NO present in the blood is synthesized primarily by NO synthases, particularly those in the endothelial cells of blood vessels throughout the circulatory system upon local chemical and physical stimulation. It activates soluble guanylyl cyclases in adjacent cells as a paracrine signal transducer. Excess NO so produced in the rapidly moving blood must be scavenged as quickly as possible in order to prevent its action at unintended locations elsewhere downstream. Low concentrations of NO in the blood ($\sim 10^{-7}$ M $< [\text{NO}] < \sim 10^{-5}$ M) can be effectively ($K_D < 10^{-12}$ M) and rapidly ($k_{\text{on}} = 10^7 \text{ M}^{-1} \text{ s}^{-1}$) sequestered by Hb through eventual coordination at its α -hemes. The $\alpha(\text{Fe-NO})_2\beta(\text{Fe})_2$ thus formed becomes a cooperative, low affinity O₂ carrier that can deliver O₂ to tissues as efficiently as native Hb under physiological conditions, although it can carry only two molecules of O₂ per tetramer. In contrast, the partially CO-bound Hb, which enters a higher-affinity state, is less effective in delivery of O₂ to tissues. The α -nitrosyl hemes in $\alpha(\text{Fe-NO})_2\beta(\text{Fe})_2$ are eventually oxidized by O₂ to met hemes and NO₃⁻ ions at reasonable rates ($t_{1/2} \approx 21$ min in erythrocytes at 37 °C).² The partially met Hb thus formed will be effectively reduced to deoxy Hb by Hb reductase in the erythrocytes to complete the process of NO scavenging. We have shown that during the scavenging of NO through binding at the α -subunits, Hb transforms itself into α -nitrosyl Hb, a cooperative, low affinity O₂ carrier. Thus, the ability of effective O₂ delivery to tissues of Hb would not be impaired in the presence of low concentrations of NO. To put simply, Hb will not be poisoned in the presence of low concentrations of NO. This may explain in part why NO causes no acute adverse effect on newborn infants during clinical treatments with inhaled NO (34), even though NO has a substantially higher ($>10^3$ -fold) affinity for Hb than CO. Thus, we find Hb to be much more agile than we have previously assumed. Hemoglobin can function simultaneously as a NO sequestering agent as well as an efficient O₂ carrier in the hostile environment of the blood, where NO, a high affinity ligand, is always present in low concentrations.

Amounts of α -nitrosyl Hb formed *in vivo* are rather small, less than several percent of the intra-erythrocyte concentrations of Hb (12). Therefore, its impact on and contribution toward the overall O₂ affinity (P₅₀) of the erythrocytes are negligible. It has been recently reported that the O₂ binding curves of the blood of nitroglycerin-treated rats were shifted to the right by ~ 10 mm Hg at 37 °C (50). Although the observed, apparent right shift was attributed solely to low affinity α -nitrosyl Hb (50), it is theoretically impossible that such a degree of the right shift could be effected by such low concentrations of α -nitrosyl Hb present (only several percent of the total Hb in

the blood at most). The observed change, therefore, must be attributed to some unknown causes other than the low O_2 affinity of α -nitrosyl Hb.

The most outstanding feature of NO among diatomic gases as a biological signal transducer is its extremely high affinity for ferrous hemes and its ability to trans-axially break the heme-proximal His bond in its receptors, such as soluble guanylyl cyclase and Hb, causing conformation changes in the receptors. The latter feature is absent in other diatomic ligands, such as O_2 and CO (Table I). The free radical nature and chemical reactivity of NO at submicromolar concentrations do not seem to play major roles in a wide range of NO-induced biochemical, cellular, and physiological responses in which soluble guanylyl cyclase is involved.

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