Ligand Binding Properties and Structural Studies of Recombinant and Chemically Modified Hemoglobins Altered at β93 Cysteine[†]

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Received April 16, 2002; Revised Manuscript Received July 30, 2002

ABSTRACT: To investigate the roles of β93 cysteine in human normal adult hemoglobin (Hb A), we have constructed four recombinant mutant hemoglobins (rHbs), rHb (βC93G), rHb (βC93A), rHb (βC93M), and rHb (βC93L), and have prepared two chemically modified Hb As, Hb A−IAA and Hb A−NEM, in which the sulfhydryl group at β93Cys is modified by sulfhydryl reagents, iodoacetamide (IAA) and *N*-ethylmaleimide (NEM), respectively. These variants at the β93 position show higher oxygen affinity, lower cooperativity, and reduced Bohr effect relative to Hb A. The response of some of these Hb variants to allosteric effectors, 2,3-bisphosphoglycerate (2,3-BPG) and inositol hexaphosphate (IHP), is decreased relative to that of Hb A. The proton nuclear magnetic resonance (NMR) spectra of these Hb variants show that there is a marked influence on the proximal heme pocket of the β-chain, whereas the environment of the proximal heme pocket of the α-chain remains unchanged as compared to Hb A, suggesting that higher oxygen affinity is likely to be determined by the heme pocket of the β-chain rather than by that of the α-chain. This is further supported by NO titration of these Hbs in the deoxy form. For Hb A, NO binds preferentially to the heme of the α-chain relative to that of the β-chain. In contrast, the feature of preferential binding to the heme of the α-chain becomes weaker and even disappears for Hb variants with modifications at β93Cys. The effects of IHP on these Hbs in the NO form are different from those on HbNO A, as characterized by ^{1}H NMR spectra of the T-state markers, the exchangeable resonances at 14 and 11 ppm, reflecting that these Hb variants have more stability in the R-state relative to Hb A, especially rHb (βC93L) and Hb A−NEM in the NO form. The changes of the C2 proton resonances of the surface histidyl residues in these Hb variants in both the deoxy and CO forms, compared with those of Hb A, indicate that a mutation or chemical modification at β93Cys can result in conformational changes involving several surface histidyl residues, e.g., β146His and β2His. The results obtained here offer strong evidence to show that the salt bridge between β146His and β94Asp and the binding pocket of allosteric effectors can be affected as the result of modifications at β93Cys, which result in the destabilization of the T-state and a reduced response of these Hbs to allosteric effectors. We further propose that the impaired alkaline Bohr effect can be attributed to the effect on the contributions of several surface histidyl residues which are altered because of the environmental changes caused by mutations and chemical modifications at β93Cys.

Human normal adult hemoglobin (Hb A)[1] is composed of two α-chains and two β-chains, which are associated noncovalently along two distinct surfaces, called the $\alpha_1\beta_1$ (or $\alpha_2\beta_2$ by reason of symmetry) and the $\alpha_1\beta_2$ (or $\alpha_2\beta_1$) subunit interfaces (*1*−*3*). The allosteric interactions of Hb A are due to the subunit rearrangement in the tetramer that allows significant quaternary alterations to occur during ligand binding. Analyses of the crystallographic structures of Hb A in the unligated and ligated forms have revealed that the $\alpha_1\beta_2$ interface, compared to the $\alpha_1\beta_1$ interface, undergoes an extensive relative movement and rearrangement of contact amino acid residues upon ligand binding (*1*−*3*). A number of amino acid residues, particularly those in the $\alpha_1\beta_2$ subunit interface, play an important role in modulating function and structure of the Hb molecule (*1*−*3*).

The β93 cysteine residue, the only sulfhydryl-containing group located at the $\alpha_1\beta_2$ subunit interface, in the carboxyl-terminal region of the β-chain, and in close proximity to the proximal histidyl residue (*3*), has been thought to be involved in the transport and delivery of NO in vivo and to influence the ligand-binding affinity and the physicochemical properties of Hb (*4*−*8*). Moreover, β93Cys may also function as a scavenger of superoxide by forming the thioyl radical, a relatively stable free radical, which may inhibit the rate of

[†] This work is supported by a research grant from the National Institutes of Health (R01HL-24525).

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[1] Abbreviations: Hb A, human normal adult hemoglobin; rHb, recombinant hemoglobin; oxy-Hb (or HbO_2), oxyhemoglobin; deoxy-Hb, deoxyhemoglobin; carbonmonoxy-Hb (or HbCO), carbonmonoxy-hemoglobin; HbNO, nitrosylhemoglobin; SNO-Hb, *S*-nitrosohemoglobin; met-Hb, methemoglobin; P_{50}, partial O_2 pressure at 50% saturation; n_{50}, Hill coefficient; NMR, nuclear magnetic resonance; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; 2,3-BPG, 2,3-bisphosphoglycerate; IHP, inositol hexaphosphate; IAA, iodoacetamide; NEM, *N*-ethylmaleimide; PMB, *p*-mercuribenzoic acid; Bis-Tris, [bis(2-hydroxyethyl)-amino]tris(hydroxymethyl)methane; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid.

10.1021/bi0202880 CCC: $22.00
Published on Web 09/07/2002

autoxidation of Hb and reduce heme degradation (*9*). These functional roles of β93Cys are modulated by the overall Hb structure (*4*). Early investigations revealed that the reactivity of the β93Cys residue with sulfhydryl reagents varied significantly depending on the conformation of the Hb molecule. Therefore, this residue has served as a structural probe for detecting ligand-induced conformational changes in Hb (*10*−*12*). Recent studies report that, in vivo, the binding of oxygen to heme irons in hemoglobin promotes the binding of nitric oxide to β93Cys, forming *S*-nitrosohemoglobin (SNO-Hb), although in vivo NO-modified Hb is measured only to a very limited extent (*5*, *7*). Deoxygenation is accompanied by an allosteric transition in SNO-Hb from the R- (ligated) state to the T- (deoxygenated) state that releases NO to a free form or a form complexed with other small thiols. The structural origin for the reactivity of β93Cys has been postulated on the basis of the crystal structures of hemoglobin in the R- and the T-states. In deoxy-Hb, β93Cys points out toward the protein surface, and the γ sulfur of β93Cys is shielded by the salt bridge between β146His and β94Asp (*3*−*5*). Thus, in the T structure, the thiol (SH) reactivity of β93Cys toward sulfhydryl reagents is low. In contrast, in the R-state, the salt bridge between β146His and β94Asp is broken, and β93Cys points in and away from the solvent, which is expected to facilitate the deprotonation of the sulfur atom and promote its nucleophilicity. Thus, β93Cys is accessible to attack by sulfhydryl reagents in the R-state.

The role of the β93Cys residue in the oxygen-binding properties of hemoglobin has been extensively studied by mutations and chemical modifications (*13*−*18*). Compared to Hb A, all Hb variants with modifications at β93Cys that have been studied are reported to exhibit higher oxygen affinity, but there are significant differences in the extent. Some aspects of the oxygen-binding properties, however, remain controversial, and the detailed structural basis of these properties is not fully understood. Modification of Hb by various sulfhydryl reagents, such as *N*-ethylmaleimide (NEM) and iodoacetamide (IAA), results in higher oxygen affinity, impaired cooperativity, and reduced Bohr effect, but the extent of the effects produced differs with the blocking reagents (*10*, *17*). For SNO-Hb, Patel et al. (*19*) and McMahon et al. (*20*) reported a higher affinity for oxygen than Hb A and intact allosteric effects of organic phosphates. Decreasing the pH from 7.4 to 6.5 reduced the oxygen affinities of both Hb and SNO-Hb to a similar extent, suggesting that S-nitrosylation of Hb does not significantly affect the alkaline Bohr effect. More recently, it has been reported that Bis-Mal-PEG2000 Hb A, the cross-linked species for which β93Cys has been identified as the site of intramolecular cross-linking, shows only a limited effect on the oxygen affinity and cooperativity in Bis-Tris buffer at pH 7.4, compared to Hb A. However, the Bohr effect was reduced by ~60%. This cross-linked species of Hb A retains its sensitivity to allosteric effectors, 2,3-bisphosphoglycerate (2,3-BPG), inositol hexaphosphate (IHP), and chloride, but to a lesser degree compared with Hb A (*21*). To date, several recombinant mutants of Hb A modified at β93Cys, such as rHb (βC93A), rHb (βC93T), and rHb (βC93S), have been expressed to probe the structural and functional events occurring at the $\alpha_1\beta_2$ subunit interface upon ligand binding (*6*). These mutant rHbs have displayed increased oxygen affinities and impaired allosteric and Bohr effects. However, the nature of the structural changes responsible for these changes in the oxygen-binding properties is not clear. On the basis of the crystal structure of Hb A in the deoxy state, it has been proposed that the salt bridge between β146His and β94Asp might affect the oxygen-binding properties of Hb modified at the β93 site (*16*), but no direct evidence has been obtained from Hb in solution to support this hypothesis.

Proton nuclear magnetic resonance (NMR) spectroscopy has provided a considerable amount of detail regarding the tertiary and quaternary structures of Hb A in the R- and T-states. It has been used to obtain information involving surface histidyl residues in the Bohr effect (*22*, *23*). On the basis of the crystal structure, Perutz and co-workers (*1*, *24*) proposed β146His as an important contributor to the alkaline Bohr effect. Studies in our laboratory using ^{1}H NMR spectra have further elucidated the molecular mechanism of the alkaline Bohr effect (*23*, *25*−*28*). All C2 proton resonances of the surface histidyl residues of the α- and β-chains have been assigned, and their contributions to the Bohr effect have been estimated (*23*, *25*−*27*). These results show that several histidyl residues make a positive contribution to the Bohr effect, a few make a negative contribution, and a few do not make any contribution to the Bohr effect. The contribution of surface histidyl residues can account for 86% of the Bohr effect in 0.1 M HEPES containing 0.1 M chloride at pH 7.4 (*23*).

In the present work, to investigate the detailed structure−function relationship of β93Cys, we have extended the previous investigations by studying four recombinant hemoglobins (rHs) with substitutions of nonpolar amino acid side chains at β93Cys, rHb (βC93G), rHb (βC93A), rHb (βC93M), and rHb (βC93L), and two Hb As chemically modified by sulfhydryl reagents at β93Cys. The functional results show that all of these mutant rHbs and chemically modified Hb As exhibit common functional features with higher oxygen affinity and impaired cooperativity. Among them, rHb (βC93L) and Hb A chemically modified by NEM, which have bulky hydrophobic side chains, have a much higher oxygen affinity, lower cooperativity, and significantly reduced alkaline Bohr effect. ^{1}H NMR spectra have been used to investigate the structural basis of these altered functional features. We have found that modifications at β93Cys can lead to significant local environmental changes of the β-heme pockets that enhance ligand binding to the β-heme, but do not appear to alter the α-heme pockets. Moreover, the conformational changes triggered by the alteration of the β93 site can transfer to several surface histidyl residues and affect the salt bridge between β146His and β94Asp, causing an increase of ligand-binding ability due to the destabilization of the T-state and the reduction of the alkaline Bohr effect. Thus, this study has provided new insights into the molecular basis for the ligand-binding properties of Hb variants with modifications at β93.

MATERIALS AND METHODS

Construction of Plasmids. The *Escherichia coli* Hb expression vector pHE2, containing synthetic α- and β-globin genes and the *E. coli* methionine aminopeptidase gene, was previously constructed in this laboratory (*29*) and was used as the plasmid template for mutant rHbs. The four synthetic

oligonucleotides used as primers to provide the mutant plasmids are as follows: 5′-CTGTCTGAGCTCCACGCTGACAAACTGCACGTTGA-3′ for rHb (βC93A) (pHE2601), 5′-CTGTCTGAGCTCCACATGGACAAACTGCACGTTGA-3′ for rHb (βC93M) (pHE2602), 5′-CTGTCTGAGCTCCACCTGGACAAACTGCACGTTGA-3′ for rHb (βC93L) (pHE2603), and 5′-CTGTCTGAGCTCCACGGTGACAAACTGCACGTTGA-3′ for rHb (βC93G) (pHE2604). The mutant plasmids were confirmed by DNA sequencing.

Chemicals and restriction enzymes were purchased from the following suppliers, Fisher, Bio-Rad, Sigma, Pharmacia, U.S. Biochemical Corp., Inc., Promega, Boehringer Mannheim, and New England Biolabs, and were used without further purification.

Growth and Purification of Recombinant Hb. The plasmids for expression of rHbs were transformed into *E. coli* JM109 and grown in LB medium in a Microferm fermentor (New Brunswick Scientific, Model BioFlo 3000) according to the standard procedures described previously (*29*−*31*). The cells were harvested by centrifugation and stored frozen at −80 °C until needed for purification. The detailed procedures for the isolation and purification of mutant rHbs are described in our previous papers (*29*−*31*). Briefly, in the first step, the cells were lysed by high-pressure homogenization (Model EmulsiFlex-C5, Avestin), and then the supernatant from the lysate was incubated at 30 °C overnight. The recombinant Hb fractions were collected through a Q-Sepharose fast protein liquid chromatography column (Pharmacia) followed by an oxidation−reduction process. The resulting preparation was converted to the CO form. The rHb solution was then purified by eluting through a fast protein liquid chromatography Mono-S column (Pharmacia). Hb A was prepared from outdated human red blood cells obtained from the local blood bank according to the procedure described previously (*32*). The purified rHbs and Hb A, which were in the CO form, were added drop by drop to liquid nitrogen to make frozen Hb beads and then stored at −80 °C until use.

Characterization of Mutant rHbs. The electrospray ionization mass spectrometric analyses were performed on a VG Quattro-BQ (Fissons Instruments, VG Biotech, Altrincham, U.K.) as described in previous publications (*29*). Automated cycles of Edman degradation were performed on an Applied Biosystems gas/liquid-phase sequencer (Model 470/900A) equipped with an on-line phenylthiohydantoin amino acid analyzer (Model 120A). All mutant rHbs had the correct molecular weights and contained less than 3% methionine at the amino termini.

Chemically Modified Hb A. Two Hb As chemically modified at β93Cys were prepared and purified according to previously described procedures (*10*, *17*). Hb A in the CO form was reacted with the excess chemical reagents, IAA and NEM, in 0.1 M phosphate buffer, pH 7.0, at room temperature ([NEM]/[Hb A] = 5 for 4 h, [IAA]/[Hb A] = 10 for 2 h) and then was purified by eluting through a G-25 column to remove the excess reagents. The collecting solution was checked by titration with *p*-mercuribenzoic acid (PMB) in order to make sure that the −SH groups of β93 cysteine in Hb A had been reacted completely with the chemical reagents. Spectrophotometric titration of reactive SH groups of Hb at β93Cys was performed as described by Boyer (*33*). The reaction of PMB with the SH group of β93Cys was monitored at 255 nm on each addition of PMB.

Equilibrium Oxygen-Binding Properties of Recombinant and Chemically Modified Hbs. The oxygen dissociation curves of Hb A, rHbs, and chemically modified Hb As were measured by a Hemox-Analyzer (TCS Medical Products, Huntington Valley, PA) at 29 °C as a function of pH (from 6.0 to 8.5) in 0.1 M sodium phosphate buffer. Immediately before measurement, Hb in the CO form was converted to the oxy form by illuminating with a halogen lamp under a constant stream of pure oxygen in an ice bath. The concentration of Hb used for these measurements was about 0.1 mM per heme. The methemoglobin (met-Hb) reductase system was used if needed to reduce the amount of met-Hb in the sample (*34*, *35*). The met-Hb content of each sample was determined immediately after the oxygen equilibrium measurement by using the extinction coefficients for Hb reported by Antonini (*36*). The oxygen equilibrium data were analyzed by fitting the Adair equation to each oxygen dissociation curve by a nonlinear least-squares method. P_{50}, a measure of oxygen affinity, was determined at 50% oxygen saturation, and the Hill coefficient (n_{50}), a measure of cooperativity, was derived from the slope of the Hill plot by linear regression for oxygen saturation levels between 40% and 60%. The accuracy of P_{50} measurements (in mmHg) is ±8%, and that of n_{50} is ±10%.

Preparation of Deoxy-Hb, HbNO, and Met-Hb. Deoxy-Hbs (5%) were prepared by first flushing samples of HbCO with O_2 for 1 h on ice, under direct illumination from a halogen lamp, to exchange the tightly bound CO. The oxy-Hbs were flushed with nitrogen in the presence of ∼1 mM dithionite and 5% D_2O for 1 h to give deoxy-Hb samples which were then transferred anaerobically to sealed standard NMR tubes. Nitric oxide (NO) gas was washed through a deoxygenated solution of 1 M NaOH as well as a column of NaOH pellets at ∼1 atm for 25 min. The NO gas was then bubbled through deoxygenated 0.1 M phosphate buffer at pH 7.0 under argon gas (*37*, *38*) to prepare saturated solutions of NO (∼2 mM). HbNO solution was prepared by mixing deoxy-Hb with NO gas or NO solution. Met-Hbs were prepared by adding a 2-fold molar excess of potassium ferricyanide to Hbs in the CO form and then stirring for 1 h at room temperature. The met-Hbs were purified by eluting through a G-25 column against 0.1 M phosphate buffer (pH 7.0) to remove the excess potassium ferricyanide. All experiments using deoxy-Hbs titrated by NO and met-Hbs bound by NO were performed in an anaerobic plastic bag using gastight glass syringes.

NMR Measurements. ^{1}H NMR spectra of rHbs and chemically modified Hb As were obtained from Bruker AVANCE DRX-300 and/or AVANCE DRX-600 NMR spectrometers at 29 °C. HbCO and deoxy-Hb solutions were prepared as described (*25*−*27*) and were transferred anaerobically to sealed NMR tubes under positive pressure. Hbs in the NO form were prepared as described above. The Hb samples were in 0.1 M sodium phosphate buffer at pH 7.0 with 5% D_2O added for the signal lock or at pH 6.85 in D_2O or in 0.1 M HEPES buffer containing 0.1 M NaCl in D_2O at pH 6.85. The Hb concentration was about 5% (∼3 mM) unless otherwise noted. To avoid met-Hb formation, all deoxygenated samples were made ∼1−3 mM in deoxygenated sodium dithionite. The ^{1}H NMR spectra of Hbs in H_2O were obtained by using a jump-and-return pulse sequence with a delay time of 1.0 s (*39*). The ^{1}H NMR

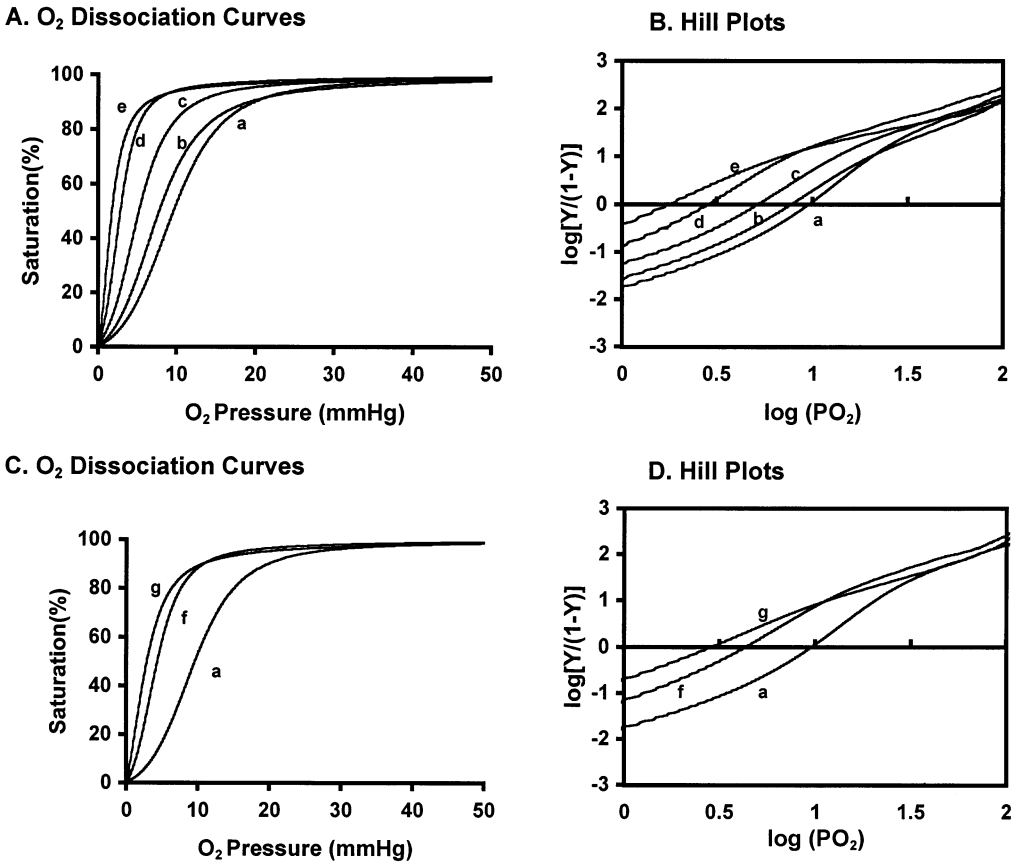

FIGURE 1: Oxygenation properties of mutant rHbs and Hb As chemically modified at β93Cys and Hb A: (A, C) oxygen dissociation curves; (B, D) Hill plots. (A) and (B) represent rHbs; the curves from right to left are Hb A (a), rHb (βC93A) (b), rHb (βC93G) (c), rHb (βC93M) (d), and rHb (βC93L) (e), respectively. (C) and (D) represent chemically modified Hb As; the curves from right to left are Hb A (a), Hb A−IAA (f), and Hb A−NEM (g), respectively. The experimental conditions were 0.1 mM Hb (heme) in 0.1 M sodium phosphate buffer at pH 7.4 and 29 °C and in the presence of a met-Hb reductase system.

spectra of Hbs in D_2O were obtained by using a single 90° pulse and a delay time of 1.0 s. Proton chemical shifts were referenced to the water resonance, which occurs at 4.76 ppm downfield from the methyl proton resonance of 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) at 29 °C.

RESULTS

Functional Studies. (A) Oxygen Affinity and Cooperativity of Mutant rHbs and Chemically Modified Hb As. The oxygen equilibrium curves of mutant rHbs and chemically modified Hb As with modifications at β93Cys were measured in 0.1 M sodium phosphate buffer as a function of pH at 29 °C. Figure 1 shows representative oxygen-binding curves of the Hb variants in 0.1 M sodium phosphate buffer at pH 7.4. These curves are shifted leftward relative to that of Hb A (Figure 1A,C). P_{50} values (half-saturation) obtained from these equilibrium curves, a measure of the oxygen affinity of Hb, are shown in Figure 2A. These results reveal that, relative to Hb A, these Hb variants have higher oxygen affinity over all the pH values examined, especially rHb (βC93L), rHb (βC93M), and Hb A−NEM, which have very high oxygen affinity. The order of oxygen affinity is as follows: rHb (βC93L) > Hb A−NEM ≈ rHb (βC93M) > Hb A−IAA > rHb (βC93G) > rHb (βC93A) > Hb A (Figure 2). When these values are expressed in the form of the plot log P_{O_2} versus log[Y/(1 − Y)], where Y is the fractional oxygen saturation, it becomes clear that the leftward shift of the oxygen equilibrium curves of these mutant rHbs and chemically modified Hb As is asymmetric in that the differences in oxygen affinity are most pronounced at low P_{O_2} values (Figure 1B,D), similar to the case of *S*-nitrosohemoglobin reported by McMahon et al. (*20*). The asymmetric shifts in the oxygen affinity of these Hb variants at the β93 site result in the decrease to different extents of the cooperativity of oxygen binding as measured by the slope of the Hill plots at 40−60% of the oxygen-binding curves (n_{50}), as illustrated in Figure 2B. Among them, Hb A−NEM exhibits a significantly lower cooperativity with $n_{50} = 1.5$ at pH 7.4 as compared to the value for Hb A of 3.0.

(B) Bohr Effect of Recombinant Hbs and Chemically Modified Hb As. The Bohr effect can be expressed as the number of Bohr protons released upon oxygenation and can be estimated from $-\Delta \log P_{50}/\Delta$pH (*40*). Table 1 shows the number of H^+ ions released per heme over the pH range from 6.5 to 8.0 for Hb variants and Hb A under the same experimental conditions. Hb A releases 0.51 H^+ ion per heme, in good agreement with the result of Imai et al. (*41*). Under the same conditions, Hb variants at β93Cys have a decrease of H^+ ions released to different extents. Interestingly, rHb (βC93L) has approximately half the release of H^+ ions (0.23 proton/heme), and Hb A−NEM has a 76% reduction (0.12 proton/heme) relative to Hb A, suggesting that they possess a very much lower alkaline Bohr effect than Hb A.

(C) Comparative Allosteric Effects of Organic Phosphates on Mutant rHbs and Chemically Modified Hb As. The

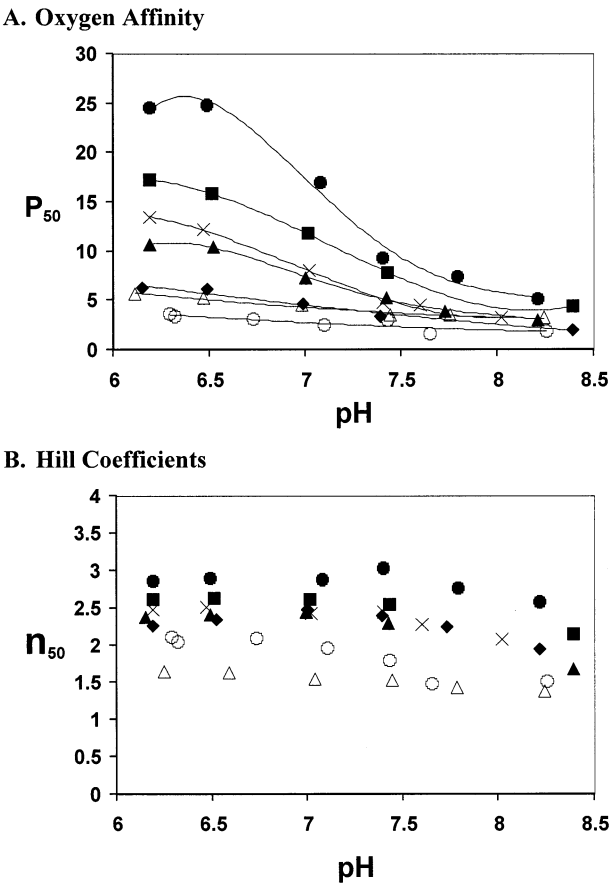

FIGURE 2: pH dependence of (A) oxygen affinity (P_{50}) and (B) Hill coefficient (n_{50}) of mutant rHbs and Hb As chemically modified at β93Cys in 0.1 M sodium phosphate buffer at 29 °C: (●) Hb A; (■) rHb (βC93A); (×) rHb (βC93G); (▲) Hb A−IAA; (◆) rHb (βC93M); (△) Hb A−NEM; (○) rHb (βC93L). Oxygen dissociation data were obtained with 0.1 mM Hb (heme). The oxygen-binding properties of these Hb variants were measured, if needed, in the presence of a met-Hb reductase system to reduce the amount of met-rHb formed to less than 3% during the oxygenation process. n_{50} values were obtained from the results of linear regression in the range of 40−60% oxygen saturation.

Table 1: Bohr Effect of Mutant rHbs and Hb As Chemically Modified by Sulfhydryl Reagents at β93Cys in 0.1 M Sodium Phosphate Buffer (pH 6.5−8.0) at 29 °C[a]

	$-\Delta P_{50}/\Delta$pH	% reduction
Hb A	0.51	
rHb (βC93G)	0.40	22
Hb A−IAA	0.35	31
rHb (βC93A)	0.33	35
rHb (βC93M)	0.28	45
rHb (βC93L)	0.23	55
Hb A−NEM	0.12	76

[a] The Bohr effect is measured by $-\Delta \log P_{50}/\Delta$pH, which gives the number of H^+ ions released upon ligand binding.

propensity of the allosteric effectors, 2,3-BPG and IHP, to reduce the oxygen affinity of mutant rHbs and chemically modified Hb As was determined in 0.1 M sodium phosphate buffer at pH 7.4, as shown in Table 2. The oxygen equilibrium studies indicate a decreased response of rHb (βC93L), Hb A−NEM, and Hb A−IAA to organic phosphates relative to Hb A. For example, the addition of 2 mM IHP to Hb A−NEM or rHb (βC93L) causes ≈1.9−2.7-fold reduction in oxygen binding (see P_{50} values), while no significant effect of 2 mM 2,3-BPG on the oxygen affinity is observed.

Table 2: Oxygen-Binding Properties of Mutant rHbs and Hb As Chemically Modified by Sulfhydryl Reagents in 0.1 M Sodium Phosphate Buffer in the Presence of 2 mM 2,3-BPG or 2 mM IHP at 29 °C

	pH	P_{50}	n_{50}
Hb A	7.40	9.3	3.0
+2,3-BPG	7.39	11.6	2.8
+IHP	7.40	35.1	2.6
rHb (βC93A)	7.43	7.9	2.6
+2,3-BPG	7.41	10.2	2.6
+IHP	7.41	33.1	2.8
rHb (βC93G)	7.40	4.8	2.5
+2,3-BPG	7.35	7.8	2.4
+IHP	7.35	22.9	2.6
rHb (βC93M)	7.39	3.4	2.4
+2,3-BPG	7.37	4.9	2.3
+IHP	7.37	12.7	2.8
rHb (βC93L)	7.43	3.0	1.8
+2,3-BPG	7.36	3.2	2.0
+IHP	7.36	8.1	2.7
Hb A−NEM	7.44	3.5	1.5
+2,3-BPG	7.45	3.5	1.6
+IHP	7.44	6.8	1.6
Hb A−IAA	7.42	5.3	2.3
+2,3-BPG	7.40	6.1	2.4
+IHP	7.39	16.7	2.4

Structural Studies. ^{1}H NMR Studies of the Structures of Hb Variants with Modifications at β93Cys. ^{1}H NMR spectroscopy has proven to be a valuable technique in the study of the tertiary and quaternary structures of Hbs (*22*). The exchangeable proton resonances and the ring-current-shifted proton resonances of the four Hb mutants at the β93 site, rHb (βC93A), rHb (βC93G), rHb (βC93M), and rHb (βC93L), in the CO form compared to those of HbCO A are illustrated in Figure 3. The exchangeable proton resonances arise from the exchangeable protons in the $\alpha_1\beta_1$ subunit interface. The ^{1}H resonances at 12.9 and 12.1 ppm have been reassigned to the intersubunit H-bonds between α122His and β35Tyr and between α103His and β131Gln, respectively (*28*). Compared to Hb A, there is no observable difference in these exchangeable resonances in the four mutant rHbs, suggesting that the $\alpha_1\beta_1$ subunit interface is unaffected by the substitution at the β93 site in these Hb variants. The ring-current-shifted resonances are sensitive to the tertiary structure in the heme pocket and the heme conformation (*22*). In Hb A, the resonances at −1.75 and −1.85 ppm can be assigned to the γ_2-CH_3 protons of E11Val in the α- and β-chains, respectively, while the resonance at −1.1 ppm is from the γ-CH_3 protons of E11Val in the β-chain (*22*). It is clear that these Hb variants display some differences in the ring-current-shifted resonances relative to those of Hb A, especially in the γ-CH_3 proton resonances of E11Val in the β-chain. The −1.1 ppm resonance is missing in three mutant rHbs [rHb (βC93L), rHb (βC93M), and rHb (βC93G)] and becomes significantly weaker in rHb (βC93A). Moreover, the resonances in rHb (βC93L) assigned to the γ_2-CH_3 protons of E11Val in the α- and β-chains are shifted downfield to −1.7 and −1.8 ppm, respectively. These differences in the ring-current-shifted resonances indicate conformational changes of the heme and heme pockets, especially for the β-chain, due to the mutations at the β93 site. No obvious changes were observed for the spectra of these rHbs in the presence of 2,3-BPG (results not shown).

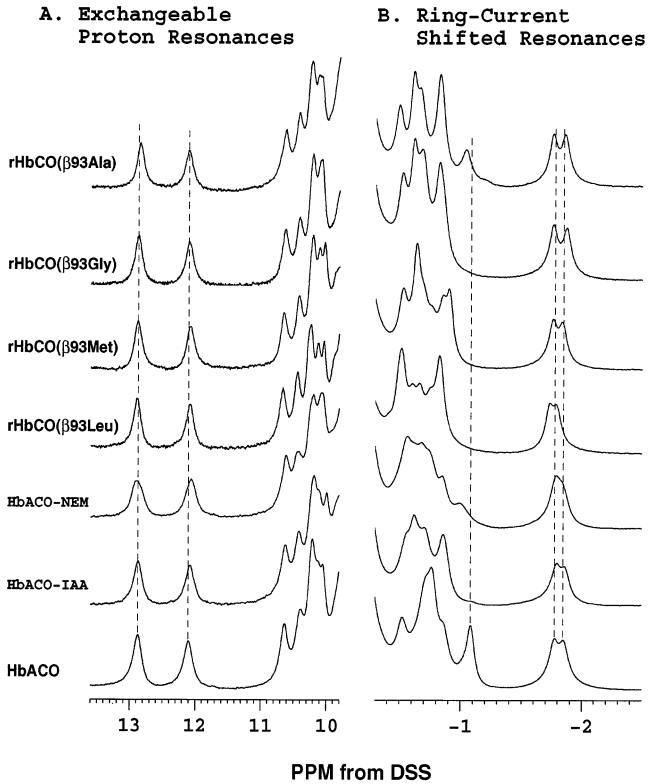

FIGURE 3: 300 MHz ^{1}H NMR spectra of 4−6% solutions of HbCO A, Hb ACO−IAA, Hb ACO−NEM, rHbCO (βC93A), rHbCO (βC93G), rHbCO (βC93M), and rHbCO (βC93L) in 0.1 M sodium phosphate buffer at pH 7.0 and 29 °C: (A) exchangeable proton resonances; (B) ring-current-shifted proton resonances.

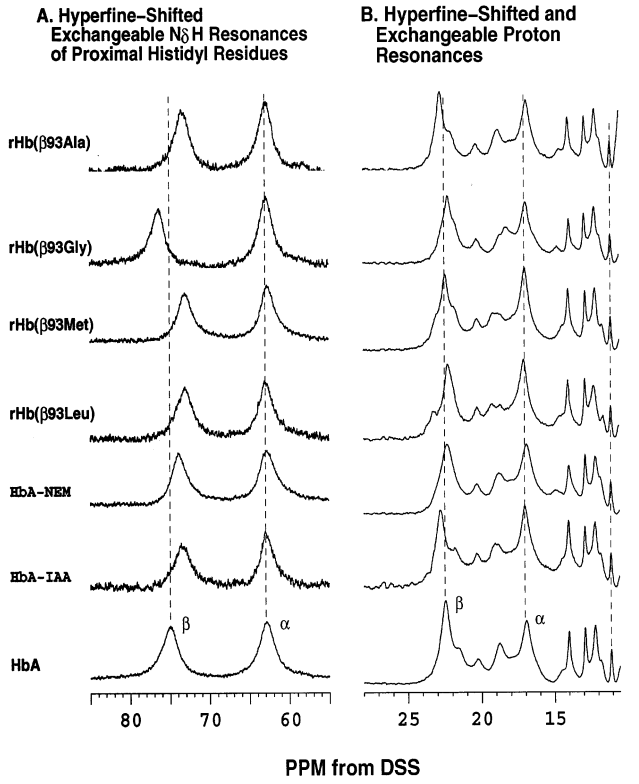

FIGURE 4: 300 MHz ^{1}H NMR spectra of hyperfine-shifted and exchangeable proton resonances of 4−6% solutions of Hb A, Hb A−IAA, Hb A−NEM, rHb (βC93A), rHb (βC93G), rHb (βC93M), and rHb (βC93L) in the deoxy form in 0.1 M sodium phosphate buffer at pH 7.0 in H_2O and 29 °C, containing 1 mM dithionite: (A) hyperfine-shifted exchangeable $N_\delta H$ resonances of proximal histidyl residues; (B) hyperfine-shifted and exchangeable resonances.

Figure 4 shows the hyperfine-shifted and exchangeable proton resonances of Hb A and the four mutant rHbs in the deoxy form in 0.1 M sodium phosphate buffer at pH 7.0 at 29 °C. The two hyperfine-shifted resonances, 75.5 and 63.0 ppm, have been assigned to the $N_\delta H$ protons of the β- and α-chain proximal histidyl residues, β92His (F8) and α87His (F8), respectively (*42*, *43*). These resonances are sensitive to the local structure and conformation of the heme groups because they result from hyperfine interactions (either contact or dipolar or both) with the unpaired electrons of the high-spin deoxy-heme iron in the heme pocket of each subunit and the nearby amino acid residues (*22*). No change of the resonance at 63.0 ppm is observed for the four mutant rHbs as compared to Hb A, suggesting that there is no influence on the environment of the proximal heme pocket of the α-chain due to the mutations at β93Cys. However, there are significant shifts in the resonance assigned to the $N_\delta H$ protons of the β-chain proximal histidyl residue in the four mutant rHbs. The resonance of three mutant rHbs, rHb (βC93L), rHb (βC93M), and rHb (βC93A), shifts about 2.5 ppm upfield to 73 ppm. Unlike these mutant rHbs, the resonance of rHb (βC93G) is shifted 1.5 ppm downfield to 77 ppm. The several resonances in the range of 10−24 ppm (Figure 4B) originate from the hyperfine-shifted resonances of the porphyrin ring and the amino acid residues located in the proximity of the heme pockets and the exchangeable proton resonances (*22*). There are slight changes over the region from 18 to 24 ppm, further indicating conformational changes in the environment of the heme pockets of the β-chain. Our results also show that the addition of 2,3-BPG or IHP does not significantly affect these changes occurring at the nearby heme pocket of the β-chain as a result of mutation at β93Cys (results not shown).

Figure 3 shows the exchangeable proton resonances and the ring-current-shifted proton resonances of the two Hb As chemically modified at β93Cys, Hb A−IAA and Hb A−NEM, in the CO form in 0.1 M phosphate buffer, pH 7.0, at 29 °C. Interestingly, unlike the case in the mutations of the β93 site, the exchangeable proton resonance assigned to the H-bond between α103His and β131Gln in the $\alpha_1\beta_1$ subunit interface is shifted slightly upfield in Hb A−NEM relative to the resonance at 12.1 ppm of Hb A. This change reflects a slight influence on the H-bond between α103His and β131Gln and adjustments of the conformation involving $\alpha_1\beta_1$ interfacial histidines. The significant spectral change of the ring-current-shifted resonances is that the resonance assigned to the γ-CH_3 protons of E11Val in the β-chain almost disappears in the spectra of Hb A−NEM and Hb A−IAA, which is similar to the observation for the mutations at β93Cys. For Hb A−NEM, the resonance assigned to the γ_2-CH_3 protons of E11Val in the β-chain is shifted slightly downfield so that two slightly separated peaks become a single broader peak. However, no significant alteration in this spectral region (−1.5 to −2 ppm) is observed in the spectrum of Hb A−IAA. Moreover, the addition of IHP does not markedly affect these exchangeable proton resonances and ring-current-shifted proton resonances. In the ^{1}H NMR spectra of Hb A−NEM and Hb A−IAA in the deoxy form (Figure 4), the resonance assigned to the $N_\delta H$ protons of the β-chain proximal histidyl residues has an upfield shift

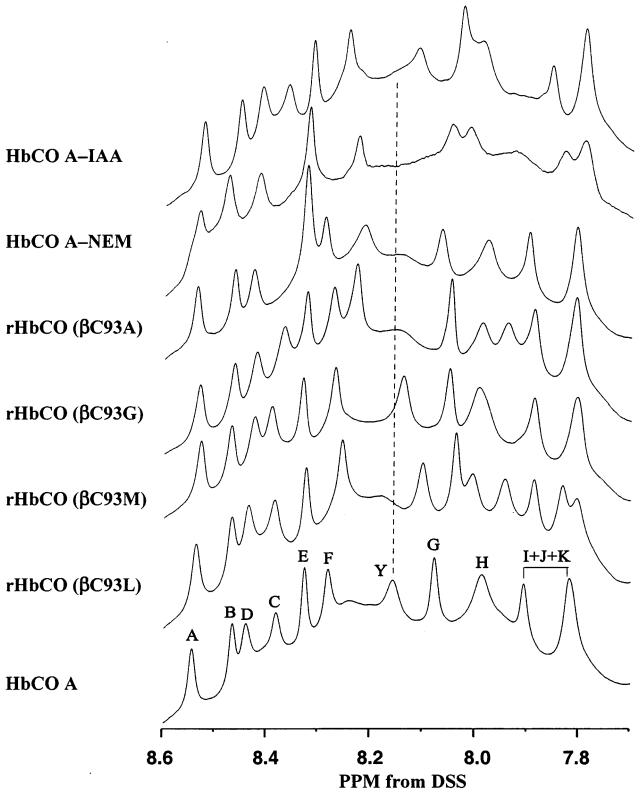

FIGURE 5: 300 MHz ^{1}H NMR spectra of 4−6% solutions of rHb (βC93A), rHb (βC93G), rHb (βC93M), rHb (βC93L), Hb A−IAA, Hb A−NEM, and Hb A in the CO form in D_2O in 0.1 M sodium phosphate buffer at pH 6.85 and 29 °C. The labeling of the C2 proton resonances of the surface histidyl residues in the CO form alphabetically follows the system used our laboratory (*23*).

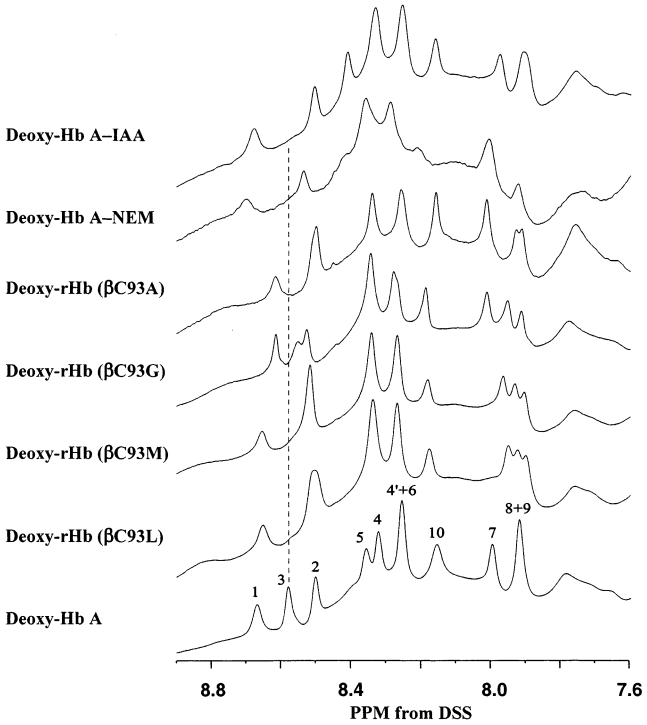

FIGURE 6: 300 MHz ^{1}H NMR spectra of 4−6% solutions of rHb (βC93A), rHb (βC93G), rHb (βC93M), rHb (βC93L), Hb A−IAA, Hb A−NEM, and Hb A in the deoxy form in D_2O in 0.1 M sodium phosphate buffer at pH 6.85 and 29 °C, containing 1 mM dithionite. The labeling of the C2 proton resonances of the surface histidyl residues in the deoxy form numerically follows the system used our laboratory (*23*).

of 2 ppm in both spectra, while the resonance corresponding to the α-chain is identical to that of Hb A. There are minor differences in the position of the hyperfine-shifted resonances of the porphyrin ring in the β-chain from 18 to 24 ppm, indicating that the heme pocket of the β-chain is perturbed by the chemical modifications at β93Cys. In addition, the resonances of this region are also affected slightly by the addition of an allosteric effector, IHP (results not shown).

Surface Histidyl Residues. To gain an insight into the structural basis of the effect on the alkaline Bohr effect caused by modifications at β93Cys, we have investigated the proton resonances of the surface histidyl residues of the Hb variants derived from both mutations and chemical modifications at β93Cys. The C2 proton resonances of the histidyl residues of Hb A and Hb variants in the CO and deoxy forms in D_2O in 0.1 M phosphate buffer at pH 6.85 and 29 °C are shown in Figures 5 and 6, respectively. The assignments of these resonances (six α-chain and seven β-chain surface histidyls) were obtained from our previous ^{1}H NMR studies on Hbs (*23*, *25*−*28*). Although we cannot identify all of the resonances of these mutant rHbs and chemically modified Hb As, it is clear that, compared with Hb A, there are substantial changes in the intensity and chemical shift of the C2 proton resonances of the surface histidyl residues of these Hb variants. The resonance arising from β146His (resonance Y in HbCO A and resonance 3 in deoxy-Hb A), a main contributor to the alkaline Bohr effect, has noticeable changes in both the CO (Figure 5) and deoxy (Figure 6) forms. In 0.1 M HEPES buffer containing 0.1 M NaCl at pH 6.85, we have also observed significant resonance changes in several of the surface histidyl residues in the CO and deoxy forms of mutant Hbs, such as the C2 proton resonances of β2His (resonance G in the CO form and resonance 10 in the deoxy form) and β143His. These two His residues are located at the central cavity of the Hb molecule and the residues involved in the binding of 2,3-BPG (*3*). These findings indicate that the local environments of several surface histidyl residues are affected by the mutations at the β93 site.

^{1}H NMR Studies of Deoxy-Hbs and Met-Hbs Bound by NO. Figure 7 shows the hyperfine-shifted resonances and exchangeable proton resonances of deoxy-Hb A bound by various amounts of NO in 0.1 M phosphate buffer at pH 7.0 and 29 °C. On the basis of the assignments of the hyperfine-shifted resonances, 75.5 and 63.0 ppm, to the $N_\delta H$ protons of the β- and α-chain proximal histidyl residues [β92His (F8) and α87His (F8), respectively] and of 22.8 and 17 ppm to the porphyrin rings (the β- and α-chains, respectively), these resonances can be used to measure NO binding to β- and α-hemes individually. As shown in Figure 7, the resonances (63 and 17 ppm) arising from the α-heme decrease in intensity much faster than the resonances (75.5 and 22.8 ppm) arising from the β-heme, during the nitrosylation of Hb A, in agreement with previous results (*44*). This suggests that NO binds preferentially to the α-chain. For Hb A−NEM, the difference in the decrease in intensity is less marked, while the intensities of resonances at 63 and 75.5 ppm, or at 22.8 and 17 ppm, decrease at close to the same rate for rHb (βC93L) (Figures 8 and 9). This result indicates that NO might bind to the α- and β-hemes with the same binding affinity, i.e., no preferential binding. Moreover,

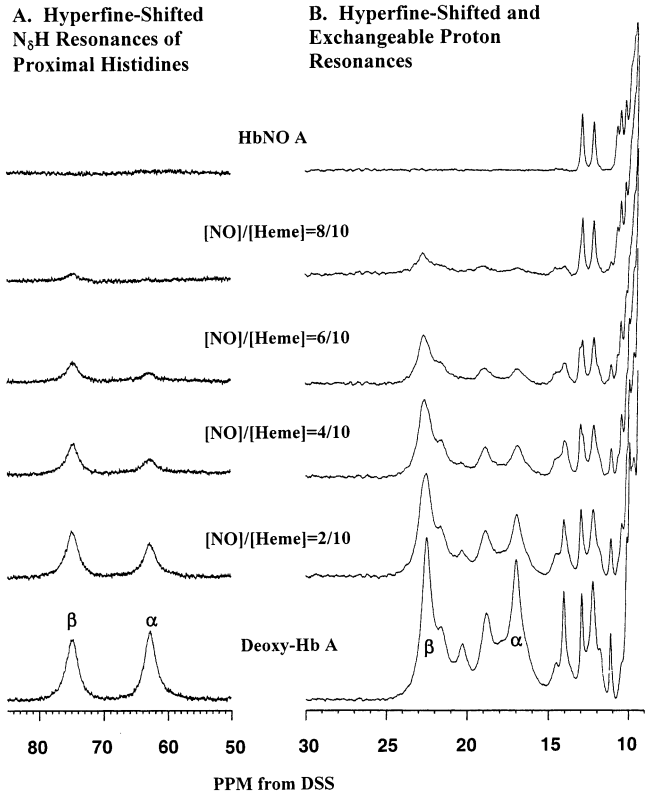

FIGURE 7: 300 MHz ^{1}H NMR spectra of hyperfine-shifted and exchangeable proton resonances of a 5% solution of deoxy-Hb A bound by increasing concentrations of NO in 0.1 M sodium phosphate buffer at pH 7.0 in H_2O and 29 °C, containing 1 mM dithionite: (A) hyperfine-shifted exchangeable $N_\delta H$ resonances of proximal histidyl residues; (B) hyperfine-shifted and exchangeable resonances.

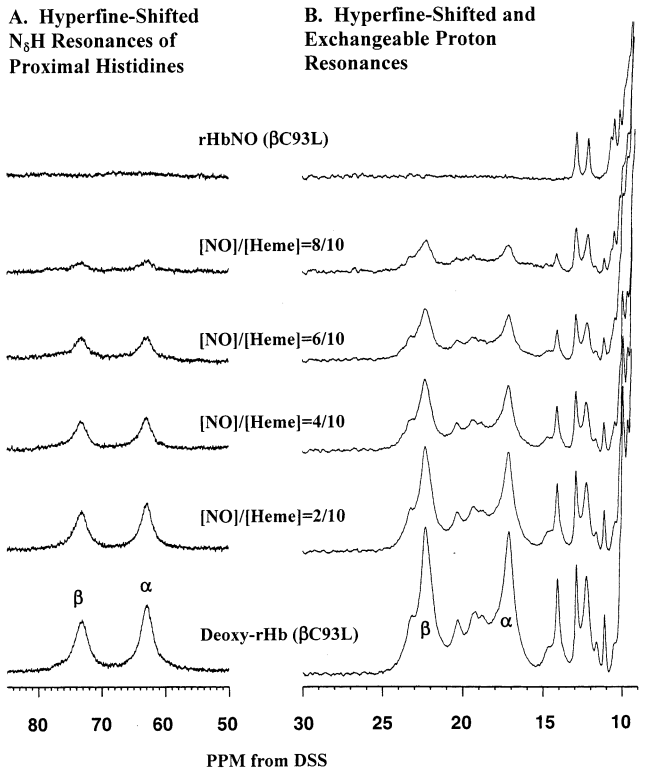

FIGURE 8: 300 MHz ^{1}H NMR spectra of hyperfine-shifted and exchangeable proton resonances of a 5% solution of deoxy-rHb (βC93L) bound by increasing concentrations of NO in 0.1 M phosphate buffer in H_2O at pH 7.0 and 29 °C, containing 1 mM dithionite: (A) hyperfine-shifted exchangeable $N_\delta H$ resonances of proximal histidyl residues; (B) hyperfine-shifted and exchangeable resonances.

further studies show that the preferential decrease in the intensity of the hyperfine-shifted resonances arising from the α-chain is much less pronounced for other Hb variants at the β93 site as compared to Hb A as the nitrosylation proceeds (results not shown). The order of change compared to Hb A is as follows: rHb (βC93L) > Hb A−NEM > rHb (βC93M) > Hb A−IAA > rHb (βC93G) ≈ rHb (βC93A), consistent with their oxygen-binding affinity. Thus, we believe that having β93Cys substituted or blocked can promote the affinity of the β-chain for NO relative to the α-chain to different extents. In addition, it should be noted that although NO is paramagnetic, no significantly shifted or line-broadened resonances have been observed upon NO binding to these Hbs, presumably due to the long electronic relaxation time of the NO radical (*44*).

The resonances at 14.2 and 11.3 ppm in the ^{1}H NMR spectrum of deoxy-Hb A have been assigned to the intersubunit hydrogen bonds between α42Tyr and β99Asp and between α94Asp and β37Trp in the $\alpha_1\beta_2$ interfaces, respectively (*22*). Both of these H-bonds are formed in the T quaternary structure and thus serve as excellent markers for T-state structure (*22*, *45*). Hence, nitrosylhemoglobin (HbNO A) is in the R-state as shown by the disappearance of the T markers (Figure 10A), just like Hb A in the CO or O_2 form. Our ^{1}H NMR studies also show that the addition of 5 mM IHP can result in the appearance of the two T-state markers (14.2 and 11.3 ppm) in nitrosylhemoglobin, suggesting that the R quaternary structure of Hb A can switch to the T quaternary structure with the NO ligands intact by the addition of an allosteric effector, IHP (Figure 10B). As with HbNO A, it is clear that IHP also can stimulate the transition of rHb (βC93A) and rHb(βC93G) in the NO form from the R- to the T-state, but the effect of IHP on this kind of transition becomes weaker gradually for the nitrosylation of Hb A−IAA, rHb (βC93M), and Hb A−NEM, because much weaker T markers occur. For rHb (βC93L) with NO ligation, evidently, the addition of IHP does not result in the transition from R-state to T-state (Figure 10B).

We have also examined the effect of NO on the reduction of met-Hb by means of ^{1}H NMR spectroscopy. Structural features of met-rHb (βC93L), met-Hb A−NEM, and met-Hb A are shown in Figure 11. The high-spin ferric hyperfine-shifted and exchangeable proton resonances of these Hbs in the ferric form are displayed over the spectral regions from 15 to 85 ppm and from 9 to 15 ppm (22), respectively. There are no differences in the spectra of the met-Hbs in the spectral region from 9 to 15 ppm, arising from the exchangeable proton resonances of these Hbs in the ferric form. However, there are some spectral changes for the high-spin ferric hyperfine-shifted proton resonances (15−85 ppm) originating from the protons on the porphyrin rings and the amino acid residues situated in the vicinity of the heme groups, reflecting changes in the environment of the heme as a result of the β93Cys substitution and modification. On the addition of excess NO to the met-Hbs, no spectral changes are observed in the spectral region from 9 to 15 ppm. Of special interest is the complete disappearance of spectral features representing the met-Hb structure (15−85 ppm) in the presence of excess NO, suggesting that excess NO can reduce these met-

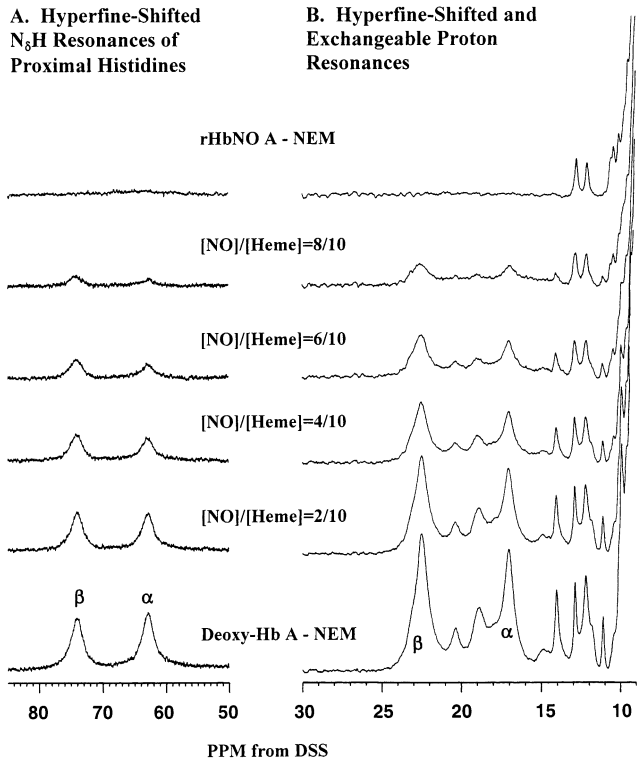

FIGURE 9: 300 MHz ^{1}H NMR spectra of hyperfine-shifted and exchangeable proton resonances of a 5% solution of deoxy-Hb A−NEM bound by increasing concentrations of NO in 0.1 M phosphate buffer in H_2O at pH 7.0 and 29 °C, containing 1 mM dithionite: (A) hyperfine-shifted exchangeable $N_\delta H$ resonances of proximal histidyl residues; (B) hyperfine-shifted and exchangeable resonances.

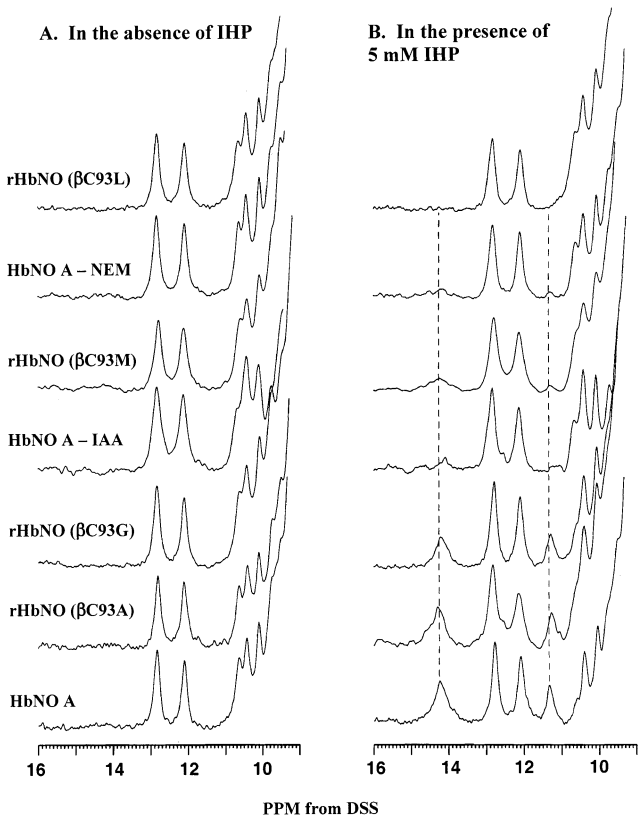

FIGURE 10: 300 MHz ^{1}H NMR spectra of exchangeable proton resonances of a 5% solution of Hb A, four rHbs, and two chemically modified Hb As in the NO form in 0.1 M phosphate buffer in H_2O at pH 7.0 and 29 °C in the absence (A) and presence (B) of 5 mM IHP, containing 1 mM dithionite.

Hbs to nitrosylhemoglobins, because the spectra are the same as those of their direct products of nitrosylation. But in the presence of [NO]/[metHbs] ≤ 1, no reduction effect of NO on met-Hbs has been observed in 1 h (results not shown).

DISCUSSION

Ligand-Binding Properties of Hb Variants with Modifications at β93Cys. Our results presented here show that the common features of the oxygen-binding properties of the mutant rHbs and Hb As chemically modified at the β93 position are higher oxygen affinity, lower cooperativity, and reduced alkaline Bohr effect relative to those of Hb A. Of interest is the finding that rHb (βC93L) possesses very high oxygen affinity and greatly reduced cooperativity and alkaline Bohr effect. Hb A−NEM exhibits a similar but even more impaired heme−heme interaction and an almost completely diminished alkaline Bohr effect. The oxygen-binding properties of rHb (βC93A) are similar to the previous results reported by Mawjood et al. (*6*), who found a 1.3-fold higher oxygen affinity, lower cooperativity ($n_{max} = 2.0$), and reduced Bohr effect ($\delta H^+ = 0.27$) as compared to those of Hb A in 0.05 M Bis-Tris buffer at pH 7.4 containing 0.1 M Cl^- at 25 °C. However, for the Hb As chemically modified at β93Cys, Hb A−IAA and Hb A−NEM, the functional characteristics that we have obtained exhibit some differences from earlier published results in the literature (*18*, *46*). The values of n_{50} for Hb A treated with iodoacetamide and *N*-ethylmaleimide as reported in the past are higher than these presented here. Our experimental results on the titration of the sulfhydryl groups with PMB in these chemically modified Hb A show that the SH groups at β93Cys have been completely blocked by IAA or NEM. A possible explanation for these discrepancies is that, in the previous experiments, the SH groups at β93 were not completely reacted.

Origin of the High Oxygen Affinity of Hb Variants with Modifications at β93Cys. The structural feature of β93Cys is that it is adjacent to the proximal histidine, located at the $\alpha_1\beta_2$ interface and the C-terminal region of the β-chain. In the T-state of Hb A, the side chain of β93Cys is stretched out and is shielded by the imidazole group of β146His, which forms salt bridges with β94Asp and α40Lys. In the R-state of Hb A, β93Cys becomes internal but is partially exposed to the solvent due to the breaking of the salt bridge between β146His and β94Asp (*1*). According to Fronticelli et al. (*47*), the results of differential gel filtration indicate that rHb (βC93A) does not have altered dimer−tetramer association constants relative to Hb A. This means that this rHb has a tetrameric assembly identical to that of Hb A, as has been further supported by the study of Mawjood et al. (*6*). They reported that no significant amount of dimers was present in rHbs mutated at the β93 site, such as rHb (βC93A) and rHb (βC93T). In addition, the tetrameric nature of the chemically modified Hb As was also documented in earlier studies (*48*). Based on these facts, it is reasonable to assume that the changes in the oxygen-binding properties of Hb variants with modifications at the β93 site cannot be interpreted as resulting from the dissociation of the tetramer into $\alpha\beta$ dimers.

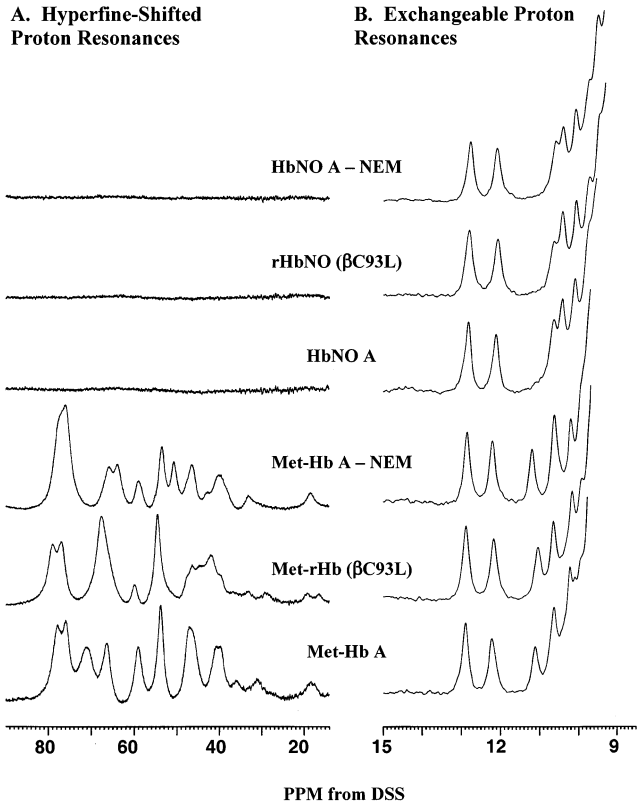

FIGURE 11: 300 MHz ^{1}H NMR spectra of hyperfine-shifted and exchangeable proton resonances of a 5% solution of met-Hb A, met-rHb (βC93L), and met-Hb A−NEM (lower spectra). These Hbs were reduced completely by NO in 0.1 M sodium phosphate buffer in H_2O at pH 7.0 and 29 °C (upper spectra): (A) hyperfine-shifted proton resonances; (B) exchangeable resonances.

According to the X-ray crystallographic analysis by Moffat (*49*), chemical modifications at β93Cys caused a structural change at the $\alpha_1\beta_2$ subunit interface. Imai and co-workers (*6*, *50*) obtained a similar result from observation of the ultraviolet difference and differential spectra of Hb A modified by NEM and IAA, respectively, as well as of rHb (βC93A) and rHb (βC93T). They found that the conformation of β37Trp at the $\alpha_1\beta_2$ interface is altered by modification or substitution at β93Cys. This finding also indicated a structural change at the subunit interface.

In a comparison of Hb variants modified at the β93 site with Hb A, the ^{1}H NMR spectra presented in this paper offer a clue to understanding the nature of the structural origin of the functional alterations of these Hb variants. Our results from observing the exchangeable proton resonances have revealed that the $\alpha_1\beta_1$ subunit interface is unaffected by mutations at β93 (Figure 3A). However, the conformational changes of Hb A modified by NEM at β93Cys might involve this interface as indicated by the small influence on the resonance at 12.1 ppm arising from the intersubunit H-bonds between α103His and β131Gln, as shown in Figure 3A. The NMR spectral results show that the tertiary structure around the β-heme pocket is significantly altered by mutations at the β93 site, while that of the α-heme pocket is unaffected in the tetramer, as characterized by the resonances of the proximal histidine and E11Val in the α- and β-heme pockets (Figure 3B). Perutz (*1*) reported that the distal valine residue of the β-chain overlaps the ligand-binding site in the unligated T-state Hb but moves about 1 Å away from it in the ligated R-state Hb. He suggested that this residue sterically hinders ligand binding, unlike the α-chain distal valine, which lies further from the heme. Thus, the local environmental change of E11Val in the β-heme pockets due to substitution or chemical modification at β93 might reduce its steric hindrance to ligand binding. Moreover, it should be noted that the influence of rHb (βC93G) on the conformation of the β-heme pocket might be different from that of the other three mutant rHbs as evaluated by the reverse shift of the β-chain proximal histidine resonance (Figure 4A). Spectral features involved in the structure that are apparently dependent upon the size of the hydrophobic side chain may be associated with a steric effect of these amino acid residues, because glycine is the smallest replacement studied. The β93Cys, with the only chemically reactive sulfhydryl group in Hb, is located ~12 Å from the iron in proximity to the β-heme pocket (*51*). It is, therefore, likely that even if the quaternary structure of these mutant rHbs does change, the local environment of the α-heme does not change as a consequence and that NMR spectral changes primarily reflect the localized tertiary changes around the β-heme. The present NMR data give the first direct indication of the preferential tertiary structural alteration in the β-chain. As demonstrated by the results of the NO titration (Figures 8 and 9), the environmental changes of the β-heme promote ligand binding to the β-heme, while the ligand affinity of the α-heme is unaffected. This might also be the cause for the high oxygen affinity of these Hb variants. The effect of modification by NEM and IAA is similar to the effect of mutation at β93Cys on the Hb structure, as shown in the hyperfine-shifted and exchangeable proton resonances in the deoxy form (Figure 4). The binding of the sulfhydryl reagents to the SH group at β93Cys might also perturb only the local environment of the β-heme but not that of the α-heme. On the basis of these results, we conclude that the functional alterations of both the mutant rHbs and Hb As chemically modified at the β93 site are closely related to conformational changes around the β-heme pockets rather than the α-heme pocket.

A number of studies have indicated that several surface histidyl residues play crucial roles in the Bohr effect of Hb A (*1*, *23*, *25*−*27*). ^{1}H NMR spectra of surface histidyl residues show that both mutation at β93Cys and modification of Hb A by sulfhydryl reagents can cause local environmental changes of these amino acid residues relative to those of Hb A, especially for β146 and β2 (Figures 5 and 6). This means that amino acid substitution or chemical modification at β93Cys can induce long-range conformational effects, which may affect a number of surface histidyl residues. Thus, the salt bridge between β146His and β94Asp in the T-state can be affected as the result of mutation or modification at the β93 sites, which further results in the destabilization of the T-state. This might be the origin of the higher oxygen affinity and lower cooperativity of these Hb variants. Also, according to the crystal structure of deoxy-Hb A complexed with 2,3-BPG, β2His and β143His are believed to be two binding sites of the allosteric effector, 2,3-BPG, forming salt bridges in Hb (*52*). Conformational changes of the binding pocket for allosteric effectors might reduce their binding abilities and impair the two salt bridges between β2His and 2,3-BPG and between β143His and 2,3-BPG, which could lead to the reduced response of the oxygen-binding properties to allosteric effectors for rHb (βC93L), Hb A−NEM, and

Hb A−IAA as shown in Table 2. Moreover, this is also supported by the effect of IHP on the nitrosyl-Hb variants (Figure 10). NO or a weaker T marker occurring in the presence of IHP indicates that these Hb variants are more stable in the R-state than in the T-state as compared to Hb A.

Effects of Modifications at β93Cys on the Conformations of Surface Histidyl Residues. Table 1 summarizes the results on the effects of modifications at β93Cys on the Bohr effect of Hb A. The magnitude of the reduction of the Bohr effect varies with the nature of the mutation and chemical modification at β93Cys. The substitution of Gly for Cys produces the least effect (22% reduction) on the Bohr effect, whereas the alkylation of the SH group by NEM produces the largest reduction (76%) of the Bohr effect. Our results are consistent with the finding of Benesch and Benesch (*46*) that the SH group of β93Cys does not directly participate in the Bohr effect.

The impairment of the Bohr effect of the Hb variants with modifications at β93Cys can be attributed to the changes in the local environments of those amino acid residues which are involved in the Bohr effect. In other words, the conformational changes in the vicinity of β93Cys caused by amino acid substitution and/or chemical modification can be propagated to other regions of the Hb molelcule, including those surface histidyl residues that are involved in the Bohr effect. Our previous results (*23*) have revealed that the sum of the contributions from 26 surface histidyl residues can account for 86% of the alkaline Bohr effect in 0.1 M HEPES buffer containing 0.1 M NaCl at pH 7.4 and 29 °C. Among them, α50His, α72His, α89His, β97His, and β116His have moderate positive contributions; α20His, α112His, and β117His have essentially no contribution; and β2His and β77His have a moderate negative contribution to the Bohr effect. β146His has the predominant contribution to the alkaline Bohr effect, which reaches 63% at pH 7.4. On the basis of an analysis of the crystal structures of hemoglobin, Perutz and co-workers (*1*, *53*) suggested that β146His binds and releases H^+ though the formation and breakage of a salt bridge between β146His and β94Asp and therefore contributes to the Bohr effect and that β146His is a major contributor to the alkaline Bohr effect. Recently, Mawjood et al. (*6*) suggested that the reduced Bohr effect of the mutant rHbs, rHb (βC93A) and rHb (βC93T), might be closely related to the impairment of the salt bridge between β146His and β94Asp due to the mutation at the β93 site, but without providing direct experimental evidence. According to the crystal analysis reported by Vàsquez et al. (*16*), alanine substitution at β93Cys increases the conformational freedom of β146His and then weakens the interaction of this residue with β94Asp, but no detectable changes of quaternary structure are observed as compared to Hb A. From a comparison of ^{1}H NMR spectra of the mutant rHbs and Hb As chemically modified at β93Cys with that of Hb A in 0.1 M phosphate buffer at pH 7.0 (Figures 5 and 6), it is clear that there are significant changes in the intensity and/or chemcal shift for the C2 proton resonances of several surface histidyl residues of Hb variants relative to those of Hb A, especially for the resonance arising from β146His. These findings suggest that the local environments of several surface histidine residues are perturbed as the result of mutation or chemical modification at the β93 site. The conformational change induced at the side chain of β146His could destabilize the salt bridge that normally exists between β146His and β94Asp in the T-state, resulting in an increase in oxygen affinity and a decrease in the Bohr effect. In addition, local conformational alterations of other surface histidyl residues also can affect their contributions to the Bohr effect. Thus, the reduced Bohr effect of the four mutant Hbs and two chemically modified Hb As studied in this work may arise from a combined effect of several surface histidyl residues due to the local conformational changes triggered by mutation or chemical modification at β93Cys.

Interaction of NO with Hb. To date, four well-characterized interactions of NO with Hb are known (*7*, *8*, *54*−*57*): (i) forming *S*-nitrosohemoglobin (SNO-Hb) with β93Cys, (ii) forming HbNO with deoxy-Hb, (iii) forming met-Hb and nitrate with oxy-Hb, and (iv) reducing met-Hb to HbNO. Actually, in vivo, the interaction of NO with hemoglobin is more complicated, and several of the reactions mentioned above might be involved under certain conditions. All of these reactions are closely related to the concentration of NO and the structural state of Hb. Stamler et al. (*5*) speculated that SNO-Hb is formed from HbNO via intramolecular NO transfer from the heme to the β93Cys accompanying the switch from the R-state to the T-state. However, recent results obtained by Hrinczenko et al. (*58*) have cast doubt on this unique explanation. They found that β93Cys is still able to be effectively S-nitrosylated even when the heme iron is blocked with either CO or CN, excluding any possibility of an intramolecular NO transfer from HbNO to β93Cys. Evidently, if β93Cys is mutated or modified by sulfhydryl reagents, the S-nitrosylation of Hb does not occur. Our titration results presented here show that the ability of NO to bind to the α-heme is essentially identical with the ability to bind to the β-heme in rHb (βC93L) and Hb A−NEM, quite different from the case of Hb A, where NO binds preferentially to the α-heme in the T-state, as shown in Figures 7−9. It has been suggested that the predominant species formed in the presence of physiological amounts of NO ([NO]/[heme] ≪ 0.5) is α-nitrosyl-Hb in both arterial and venous blood because the dissociation of NO is faster from the β-heme than from the α-heme (*59*, *60*). Electron paramagnetic resonance spectra further reveal that α-nitrosyl-Hb exhibits five-coordinate nitrosyl hemes in venous blood due to the breakage of the proximal histidine bond, but hemoglobin nitrosylated completely or α-nitrosyl-Hb in arterial blood is a six-coordinate species (*61*). This might be attributed to the allosteric change between the R- and T-states in the arterio-venous system. The increase of NO binding to the β-heme for Hb variants reported here can be interpreted by the environmental change of the β-heme as the result of mutation and modification at β93Cys, which is very close to the β-heme (10−12 Å) (*51*). The pathway through which these Hbs with modifications at β93Cys communicate with the β-heme might involve a perturbation of the Fe−His bond because the iron−proximal histidine stretching bond [ν(Fe−His)] has been shown to increase for Hb A modified at β93 in the deoxy form (*62*), reflecting a weaker barrier for ligand binding to the β-heme.

The present results also suggest that the effect of IHP on HbNO A is quite different from that on Hb A in the oxy or CO form. We have observed the two T-state markers, i.e., the exchangeable resonances at 14.2 and 11.2 ppm, for

HbNO A in the presence of IHP at 29 °C, consistent with the result reported by Huang (*44*). However, our previous studies (*35*) indicated that the addition of IHP results in the appearance of a weaker T marker at 14.2 ppm in HbCO A only at lower temperature, suggesting that HbNO A has more tendency to switch from the R- to the T-state as compared to HbCO A. This might be interpreted by a weakening of the Fe−His bond, because the breakage of the Fe−His bond in the α-heme is observed for HbNO A in the presence of IHP at pH 6.7 (*63*). It should be noted that the effect of IHP on the NO form of the Hb variants with modifications at β93Cys is quite different (Figure 10). For rHb (βC93L) and Hb A−NEM, addition of IHP does not result in the transition of the structure from the R- to the T-state, presumably due to their very high ligand affinity. Thus, we conclude that mutation and chemical modification at the β93 site might favor the R quaternary state. The present results also suggest that the behavior of NO in the reduction of the met-Hb variants is the same as with met-Hb A (Figure 11), while there are some alterations in the environment of the heme among met-Hbs. Previous kinetic results reported by Hoshino et al. (*57*) indicated that, for met-Hb A, the nitrosyl adduct (Hb^{III}-NO) undergoes nucleophilic attack by OH^- to produce Hb^{II}-NO in the presence of excess NO.

In conclusion, the experimental results reported in this paper show that the mutant rHbs and Hb As chemically modified at the β93 site have similar structural and functional features. The extent of the functional differences appears to be closely related to the steric effect of the introduction of a hydrophobic side chain at the β93 site. In a comparison of these Hb variants with modifications at β93Cys with Hb A, higher oxygen affinity might be attributed to the conformational changes of the β-heme pocket due to the finding that the α-heme pockets are essentially unaffected. We have also observed that the conformational changes propagated from the β93 site to the surface histidyl residues can lead to the impairment of the salt bridge formed between β146His and β94Asp, the decrease of binding ability of allosteric effectors, and the destabilization of the T-state. Thus, our studies presented here give further insight into the structure and function of β93Cys in hemoglobin.

ACKNOWLEDGMENT

We thank Dr. Ming F. Tam for carrying out both amino-terminal sequence and electrospray ionization mass spectrometric analyses to evaluate the quality of the recombinant hemoglobins used in this study. We also thank Dr. Chung-ke Chang, Dr. Yue Yuan, Dr. Ching-Hsuan Tsai, Ms. Nancy T. Ho, and Dr. E. Ann Pratt for helpful discussions and assistance in this study.

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BI0202880