Effects of Substitutions of Lysine and Aspartic Acid for Asparagine at β108 and of Tryptophan for Valine at α96 on the Structural and Functional Properties of Human Normal Adult Hemoglobin: Roles of $\alpha_1\beta_1$ and $\alpha_1\beta_2$ Subunit Interfaces in the Cooperative Oxygenation Process†

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ABSTRACT: Using our *Escherichia coli* expression system, we have produced five mutant recombinant (r) hemoglobins (Hbs): r Hb (αV96W), r Hb Presbyterian (βN108K), r Hb Yoshizuka (βN108D), r Hb (αV96W, βN108K), and r Hb (αV96W, βN108D). These r Hbs allow us to investigate the effect on the structure−function relationship of Hb of replacing β108Asn by either a positively charged Lys or a negatively charged Asp as well as the effect of replacing α96Val by a bulky, nonpolar Trp. We have conducted oxygen-binding studies to investigate the effect of several allosteric effectors on the oxygenation properties and the Bohr effects of these r Hbs. The oxygen affinity of these mutants is lower than that of human normal adult hemoglobin (Hb A) under various experimental conditions. The oxygen affinity of r Hb Yoshizuka is insensitive to changes in chloride concentration, whereas the oxygen affinity of r Hb Presbyterian exhibits a pronounced chloride effect. r Hb Presbyterian has the largest Bohr effect, followed by Hb A, r Hb (αV96W), and r Hb Yoshizuka. Thus, the amino acid substitution in the central cavity that increases the net positive charge enhances the Bohr effect. Proton nuclear magnetic resonance studies demonstrate that these r Hbs can switch from the R quaternary structure to the T quaternary structure without changing their ligation states upon the addition of an allosteric effector, inositol hexaphosphate, and/or by reducing the temperature. r Hb (αV96W, βN108K), which has the lowest oxygen affinity among the hemoglobins studied, has the greatest tendency to switch to the T quaternary structure. The following conclusions can be derived from our results: First, if we can stabilize the deoxy (T) quaternary structure of a hemoglobin molecule without perturbing its oxy (R) quaternary structure, we will have a hemoglobin with low oxygen affinity and high cooperativity. Second, an alteration of the charge distribution by amino acid substitutions in the $\alpha_1\beta_1$ subunit interface and in the central cavity of the hemoglobin molecule can influence the Bohr effect. Third, an amino acid substitution in the $\alpha_1\beta_1$ subunit interface can affect both the oxygen affinity and cooperativity of the oxygenation process. There is communication between the $\alpha_1\beta_1$ and $\alpha_1\beta_2$ subunit interfaces during the oxygenation process. Fourth, there is considerable cooperativity in the oxygenation process in the T-state of the hemoglobin molecule.

It is known that the hemoglobin (Hb)[1] molecule has a lower oxygen affinity in the deoxy (or T) quaternary structure than in the oxy (or R) quaternary structure. The transition from the T- to the R-state involves a considerable change in the free energy of oxygen binding, which manifests itself in the cooperativity of oxygen binding. Based on a comparison of the detailed structural features of human normal adult hemoglobin (Hb A) in deoxy and oxy forms, Perutz and colleagues (*1*−*4*) have shown that during the transition from the deoxy to the oxy state, the $\alpha_1\beta_2$ subunit interface undergoes a sliding movement, while the $\alpha_1\beta_1$ subunit interface remains essentially unchanged. Both subunit interfaces are characterized by specific hydrogen bonds and noncovalent interactions. Using proton nuclear magnetic resonance (NMR) spectroscopy, we can observe some of these H-bonds which can be used for conformational studies (*5*). In particular, the resonance appearing at ∼14 ppm downfield from 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) in the deoxy form has been assigned to the intersubunit H-bond between α42Tyr and β99Asp in the $\alpha_1\beta_2$ interface in deoxy-Hb A (*6*), a characteristic feature of the deoxy (T) quaternary structure of Hb A (*1*). By observing this T structural marker in both the deoxy and oxy forms of Hbs under various experimental conditions, we can assess the

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[1] Abbreviations: Hb, hemoglobin; Hb A, human normal adult hemoglobin; met-Hb, methemoglobin; NMR, nuclear magnetic resonance; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; IHP, inositol hexaphosphate; 2,3-BPG, 2,3-bisphosphoglycerate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

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stability of the T conformation as well as monitor the T to R quaternary structural transition (*5*, *7*−*10*).

A desirable characteristic for an Hb-based blood substitute (or oxygen carrier) is having low oxygen affinity and normal cooperativity (*10*, *11*). It has been found that human Hbs with mutations in the $\alpha_1\beta_2$ subunit interface exhibit altered oxygen affinity and cooperativity (*12*−*14*). Upon reviewing the subunit interactions in the $\alpha_1\beta_2$ interface, Kim et al. (*8*) designed a novel r Hb (αV96W) with low oxygen affinity and high cooperativity (p_{50} = 12.8 mmHg, n_{max} = 2.8 in 0.1 M sodium phosphate at pH 7.4) compared to those of Hb A (p_{50} = 8.0 mmHg and n_{max} = 3.0 at pH 7.4). Structural studies by ^{1}H NMR spectroscopy show that by adding a strong allosteric effector, such as inositol hexaphosphate (IHP), and/or by reducing the temperature, the R-state of this r Hb can be switched to the T-state conformation without changing its ligation state (as evidenced by the appearance of the T-state marker at 14 ppm). The tendency of a ligated Hb to switch into the T-state conformation demonstrates that the equilibrium between the R- and the T-states of this r Hb has been shifted toward the T-state due to the mutation at the $\alpha_1\beta_2$ interface (*8*). According to the crystal structure of r Hb (αV96W) in the T-state, there is a novel water-mediated H-bond between the indole nitrogen of α96Trp and β101Glu in the $\alpha_1\beta_2$ subunit interface (*15*). This new H-bond is believed to be the structural basis for the lowered oxygen affinity of this r Hb (*15*).

Hb Presbyterian (βN108K) and Hb Yoshizuka (βN108D) are naturally occurring low oxygen affinity mutants (*16*, *17*). β108 (G10) is located in the $\alpha_1\beta_1$ subunit interface and in the central cavity of the Hb molecule (*12*). ^{1}H-NMR investigation of r Hb Presbyterian has demonstrated that the R-state conformation of this r Hb can also be switched to the T-state conformation without changing its ligation state by adding IHP and/or by lowering the temperature (*10*). r Hb (αV96W, βN108K) was constructed and expressed in our laboratory (*10*). It has the lowest oxygen affinity among the three low oxygen affinity mutant r Hbs previously studied; e.g., in 0.1 M sodium phosphate buffer at pH 7.4 and 29 °C, for r Hb Presbyterian, p_{50} = 24.5 mmHg and n_{max} = 2.9; for r Hb (αV96W), p_{50} = 12.8 mmHg and n_{max} = 2.8; and for r Hb (αV96W, βN108K), p_{50} = 38.1 mmHg and n_{max} = 2.1. ^{1}H-NMR studies of r Hb (αV96W, βN108K) show that the T-state marker at 14 ppm has a higher intensity compared to the single mutants studied. The structure of these low oxygen affinity r Hbs, r Hb (αV96W), r Hb Presbyterian (βN108K), and r Hb (αV96W, βN108K), as measured by ^{1}H-NMR spectroscopy, is consistent with the conclusion that these r Hbs prefer to return to the deoxy state even when they are still ligated (*10*). This implies that there is cooperativity in the T-state during the oxygenation process.

Based on the role of the central cavity in modulating the oxygen affinity of Hb, Bonaventura et al. (*18*) proposed that additional cationic groups located in the central cavity of the Hb molecule would destabilize the T quaternary structure by increasing electrostatic repulsion and thereby increase the oxygen affinity. O'Donnell et al. (*17*) have discussed the issue regarding the mechanism of the low oxygen affinity of Hb Presbyterian and Hb Yoshizuka, in light of their own results on these two mutants and the suggestion of Bonaventura et al. (*18*). Their results have prompted us to investigate the electrostatic interactions in the central cavity region of the Hb molecule which cause the equilibrium between the T- and R-states to shift, resulting in different oxygen affinity and cooperativity of the oxygenation process. However, it is interesting to note that both Hb Presbyterian and Hb Yoshizuka exhibit low oxygen affinity and high cooperativity, but the amino acid substitutions of these two mutants have an opposite charge, i.e., Lys vs Asp.

An understanding of the molecular basis for Hbs with low oxygen affinity and high cooperativity is essential in applying protein engineering methodology to design novel Hbs as blood substitutes (i.e., Hb-based oxygen carriers). In this paper, we attempt to assess (i) the relative effect of substituting a positively charged Lys or a negatively charged Asp for Asn at β108 and (ii) the effect of the mutations at β108Asn in combination with the mutation at αV96W on the structure−function relationship in these r Hbs. We have applied site-directed mutagenesis to our *Escherichia coli* expression plasmid (pHE2) (*19*) to produce two new r Hbs, r Hb Yoshizuka (βN108D) and r Hb (αV96W, βN108D). A comparison of the ^{1}H-NMR spectra of r Hb Yoshizuka and r Hb (αV96W, βN108D) with those of the previously constructed low oxygen affinity r Hbs, r Hb (αV96W), r Hb Presbyterian (βN108K), and r Hb (αV96W, βN108K), suggests that these r Hbs also prefer to return to the T-state even when they are still ligated. A series of oxygen-binding studies was conducted in an attempt to investigate the factors that regulate the oxygen affinity and cooperativity of oxygen binding in these r Hbs. We have investigated the allosteric effects of chloride, inorganic phosphate, and 2,3-bisphosphoglycerate (2,3-BPG) as a function of pH and the effect of chloride concentration on the oxygen-binding properties of r Hb Presbyterian, r Hb Yoshizuka, r Hb (αV96W), r Hb (αV96W, βN108K), and r Hb (αV96W, βN108D) in comparison with those of Hb A. We hope to provide information on the molecular basis for the low oxygen affinity and high cooperativity found in these r Hbs and the effect of amino acid substitution at α96 (in the $\alpha_1\beta_2$ subunit interface), and at β108 (in the $\alpha_1\beta_1$ subunit interface and in the central cavity of the Hb molecule), on the structure−function relationship in the hemoglobin molecule.

MATERIALS AND METHODS

Construction of Expression Plasmids. The *E. coli* Hb expression plasmid, pHE2, was constructed in our laboratory (*19*), and forms the basis for constructing other plasmids for expressing mutant Hbs. The plasmid (pHE202) for the expression of r Hb (αV96W) was reported previously (*8*). The mutation βN108K and the construction of plasmids pHE240 and pHE249 for the expression of r Hb Presbyterian (βN108K) and r Hb (αV96W, βN108K) were described by Ho et al. (*10*). The mutation βN108D was made in a similar manner, except that the synthetic oligonucleotide used was 5′-ACGCAAACTAGGACGTCACCCAGCAG-3′ to produce pHE267 needed for the expression of r Hb Yoshizuka (βN108D). The plasmid pHE268 for the expression of r Hb (αV96W, βN108D) was constructed by ligation of the 5.51-kb *Sca*I−*Pst*I fragment of pHE202 and the 1.35-kb *Sca*I−*Pst*I fragment of pHE267.

Chemicals and restriction enzymes were purchased from major suppliers, such as Fisher, Sigma, Bio-Rad, Boehringer Mannheim, New England BioLabs, Pharmacia, Promega, and

United States Biochemicals, Inc., and were used without further purification. A site-directed in vitro mutagenesis kit was purchased from BIORAD.

The growth and purification of r Hbs followed the procedures described previously (*19*, *20*). In the first step after the cell lysis procedure, the supernatant from the lysate was left at 4 °C for two nights and then put into an incubator at 30 °C overnight after which the color of the supernatant became very red. Following the procedure developed in our laboratory (*19*, *20*), the r Hb fraction collected after the Q-Sepharose Fast-Flow column (Pharmacia anion exchanger) was oxidized and reduced, and converted to the CO form. This Hb solution was then purified by eluting through a fast protein liquid chromatography Mono-S column (Pharmacia Cation Exchanger, HR 16/10).

The electrospray ionization mass spectrometric analyses were performed on a VG Quattro-BQ (Fissons Instruments, VG Biotech, Altrincham, U.K.) as described in Shen et al. (*19*). 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). These two analytical procedures were used to assess the quality of our r Hbs. All r Hbs used in this study had the correct molecular weights and contained less than 3% methionine at the amino termini.

Preparation of Chloride-Free HEPES Buffer. The preparation of 0.1 M *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES, Sigma) at different pH values (chloride free) was described in Busch et al. (*21*).

Oxygen-Binding Properties of r Hbs. Hb samples were exchanged with deionized water by using a Centricon centrifugal concentrator with a molecular mass cutoff of 30 000 daltons (Centricon-30, Amicon, Inc.) and freed from organic phosphate and other ions by passing through a mixed-bed ion exchange column (BIORAD AG501-X8). The oxygen dissociation curves of r Hbs were measured by a Hemox Analyzer (TCS Medical Products, Huntington Valley, PA) at 29 °C as a function of pH (from 6.6 to 8.6) in (i) 0.1 M HEPES buffer, (ii) 0.1 M HEPES in the presence of 0.1 M chloride, (iii) 0.1 M sodium phosphate, and (iv) 0.1 M HEPES in the presence of 2 mM 2,3-BPG. We have also measured the oxygen-binding properties as a function of chloride concentration (from 0 to 0.5 M) in 0.1 M HEPES buffer at pH 7.4 and 29 °C. 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 (*22*). A visible absorption spectrum of each Hb sample was recorded immediately after oxygen equilibrium measurement, and the met-Hb content was estimated by using the extinction coefficients for Hb reported by Antonini (*23*). Oxygen equilibrium parameters were derived by fitting the Adair equations to each equilibrium oxygen-binding curve by a nonlinear least-squares procedure. p_{50}, a measure of oxygen affinity, was obtained at 50% O_2 saturation. The Hill coefficient (n_{max}), a measure of cooperativity, was determined from the maximum slope of the Hill plot by linear regression. n_{max} was derived between 60% and 65% oxygen saturation. The accuracy of p_{50} measurements in mmHg is ±5% and that for n_{max} is ±7%.

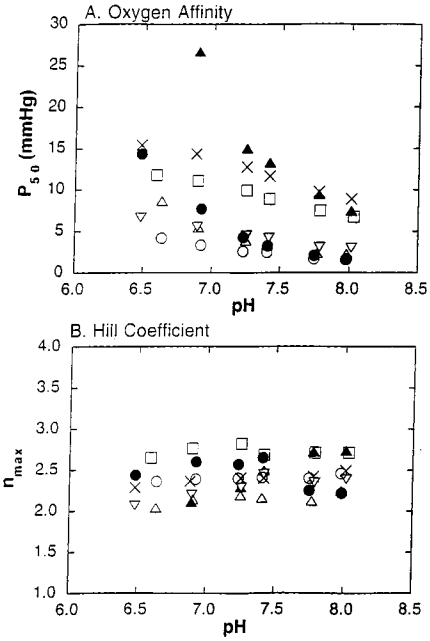

FIGURE 1: pH-dependence of the oxygen affinity (p_{50}) (A) and the Hill coefficient (n_{max}) (B) in 0.1 M chloride-free HEPES buffer at 29 °C in the absence and presence of a reductase system: (○ and ●) Hb A; (▽) r Hb (αV96W); (△) r Hb Presbyterian (βN108K); (□) r Hb Yoshizuka (βN108D); (▲) r Hb (αV96W, βN108K); (×) r Hb (αV96W, βN108D). Open symbols are for Hb samples in the absence of a reductase system, and closed symbols are for Hb samples in the presence of a reductase system (*22*). Oxygen-dissociation data were obtained with 0.1 mM Hb.

^{1}H-NMR Spectroscopy Investigation of Tertiary and Quaternary Structure of r Hbs. ^{1}H NMR spectra of r Hbs were obtained from Bruker AVANCE DRX-300 and/or AVANCE DRX-500 NMR spectrometers. All Hb samples were in 0.1 M sodium phosphate or 0.1 M HEPES with or without chloride in 100% water, and the Hb concentration was about 5% (∼3 mM). The water signal was suppressed by using a jump-and-return pulse sequence (*24*). Proton chemical shifts are referenced to the methyl proton resonance of DSS indirectly by using the water signal, which occurs at 4.76 ppm downfield from that of DSS at 29 °C, as the internal reference.

RESULTS

Functional Studies

We have carried out studies of oxygen binding of r Hb Presbyterian (βN108K), r Hb Yoshizuka (βN108D), r Hb (αV96W), r Hb (αV96W, βN108K), r Hb (αV96W, βN108D), and Hb A as a function of pH at 29 °C under various experimental conditions. The results will give new insights into (i) the structure−function relationships of these five r Hbs, (ii) the molecular basis for the low oxygen affinity of these mutant r Hbs, and (iii) the Bohr effects of these r Hbs and Hb A. Chloride, even at low concentrations, can mask the functional differences of certain r Hbs. Thus, a chloride-free HEPES buffer (*25*) was used to isolate and investigate the effects of anions on the functional properties of these r Hbs and Hb A.

Oxygen-Binding Studies in Chloride-Free HEPES. Figure 1 shows the oxygen-binding measurements of r Hbs and Hb A in the absence of chloride as a function of pH. At pH 7.4

and above, r Hb Presbyterian has a p_{50} essentially the same as that of Hb A; however, when the pH is decreased, the difference in oxygen affinity becomes more evident. r Hb Yoshizuka, on the other hand, has an intrinsically lower oxygen affinity over the pH range (from pH 6.6 to 8.0) measured in chloride-free HEPES buffer and also exhibits significant cooperativity in binding of oxygen in chloride-free HEPES buffer as manifested by the Hill coefficient, n_{max}. r Hb (αV96W) has slightly lower oxygen affinity than that of r Hb Presbyterian, but higher than that of r Hb Yoshizuka in 0.1 M chloride-free HEPES buffer. r Hb (αV96W, βN108K), which has the lowest oxygen affinity, tends to oxidize more readily than the other r Hbs. To carry out oxygen-binding measurements, met-Hb reductase (*22*) was used to reduce the amount of met-Hb formed to less than 4% during the oxygenation process. It has been reported by Imaizumi et al. (*26*) that the oxygen-binding properties of Hb A are affected by the presence of the met-Hb reductase system at chloride concentration ≤0.03 M, presumably due to the presence of glucose 6-phosphate and nicotinamide adenine dinucleotide in the reductase system. We have also found this to be true, especially at low pH, when met-Hb reductase was added to the chloride-free HEPES buffer (Figure 1). Hence, the values of the oxygen affinity of r Hb (αV96W, βN108K) measured in the presence of the reductase system in chloride-free HEPES buffer are not completely comparable to those of the other r Hbs measured in the absence of the reductase system. r Hb (αV96W, βN108D) has higher oxygen affinity than that of r Hb (αV96W, βN108K), but it was found to have no met-Hb formed during the oxygenation process in 0.1 M chloride-free HEPES buffer.

Oxygen-Binding Studies in 0.1 M HEPES plus 0.1 M Chloride, in 0.1 M Phosphate, and in 2 mM 2,3-BPG. Figures 2, 3, and 4 show the decrease in the oxygen affinity induced by the presence of allosteric effectors, such as chloride, inorganic phosphate, and 2,3-BPG. The n_{max} values of Hb A and r Hb Presbyterian (βN108K) increase by 0.5 unit when an allosteric effector is added to the chloride-free HEPES buffer or in 0.1 M phosphate buffer (Figures 2B, 3B, and 4B). However, the n_{max} value of r Hb Yoshizuka (βN108D) remains essentially the same with or without allosteric effectors (Figures 2B, 3B, and 4B). This suggests that the heme−heme pathway in r Hb Yoshizuka is not affected by the presence of an allosteric effector. Figures 2−4 show that the alkaline Bohr effect (which in Hb A results in a decrease in oxygen affinity with a lowering of the pH) is enhanced by the mutation of Asn to Lys at β108 and reduced by the mutation of Asn to Asp at β108. The Bohr effect of r Hb (αV96W) is reduced by introducing the bulky side chain Trp at α96 as compared to Hb A. In the presence of allosteric effectors, the cooperativity for the oxygenation of r Hb Presbyterian approaches the normal value for Hb A. Under the various experimental conditions investigated, the oxygen affinity of all the mutants used is lower than that of Hb A, especially r Hb (αV96W, βN108K) and r Hb (αV96W, βN108D). The oxygenation of r Hb (αV96W, βN108K) and r Hb (αV96W, βN108D) is quite cooperative with an n_{max} value of 2.2−2.4. Based on the linkage equation (*27*, *28*), the total amount of H^+ ions released per heme upon oxygenation can be measured as $\Delta H^+ = -\partial \log p_{50}/\partial \mathrm{pH}$, where p_{50} is the oxygen pressure at 50% saturation. Table 1 summarizes the number of H^+ released per heme over the pH range from 6.6 to 8.0 under various experimental conditions. r Hb Presbyterian has an enhanced Bohr effect compared to that of Hb A in 0.1 M HEPES buffer with or without allosteric effectors. r Hb Yoshizuka and r Hb (αV96W) have a reduced Bohr effect compared to Hb A. The Bohr effects of r Hb (αV96W, βN108D) and r Hb (αV96W, βN108K) are less than that of Hb A, except for r Hb (αV96W, βN108K) having a larger Bohr effect in 2 mM 2,3-BPG.

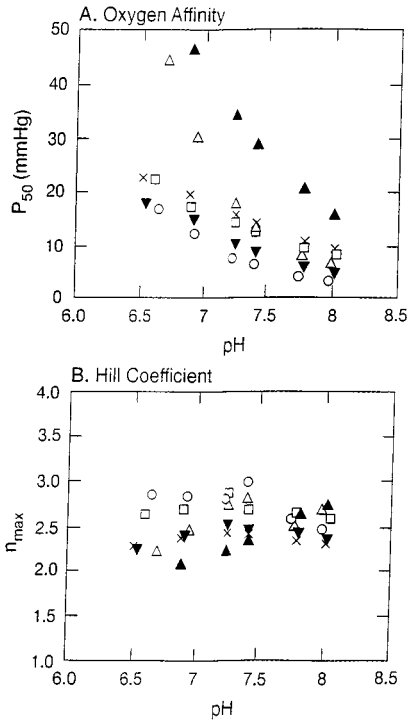

FIGURE 2: pH-dependence of the oxygen affinity (p_{50}) (A) and the Hill coefficient (n_{max}) (B) in 0.1 M HEPES buffer in the presence of 0.1 M chloride at 29 °C. (○) Hb A; (▼) r Hb (αV96W); (△) r Hb Presbyterian (βN108K); (□) r Hb Yoshizuka (βN108D); (▲) r Hb (αV96W, βN108K); (×) r Hb (αV96W, βN108D). Oxygen-dissociation data were obtained with 0.1 mM Hb. The oxygen-binding properties of r Hb (αV96W, βN108K) were measured in the presence of a reductase system (*22*) to reduce the amount of met-Hb formed to less than 4% during the oxygenation process.

It should be noted here that the oxygen affinity of r Hb (αV96W, βN108K) reported in this paper is lower than that reported before (*10*); e.g., in 0.1 M sodium phosphate buffer at pH 7.4 and 29 °C, in the present paper, $p_{50} = 48.8$ mmHg and $n_{max} = 2.3$; in the previous studies (*10*), $p_{50} = 38.1$ mmHg and $n_{max} = 2.1$. We have found that the difference in the oxygen-binding properties between these two studies is due to a difference in the incubation temperature in the first step after the cell lysis procedure. The supernatants of r Hbs used in this study were left at 4 °C over two nights and then incubated at 30 °C overnight, while in the previous studies (*10*), the supernatants of r Hbs were left at 4 °C over three nights without being incubated at 30 °C. Purified r Hbs from both procedures all had the correct molecular weight and contained less than 1% methionine at the amino termini. Based on recent measurements of oxygen-binding properties of Hb A, r Hb (αV96W), r Hb Presbyterian, r Hb Yoshizuka, r Hb (αV96W, βN108D), and r Hb (αV96W, βN108K) (C.-H. Tsai, N. T. Ho, and C. Ho, unpublished results), it appears that incubating the supernatants at 30 °C only affects the

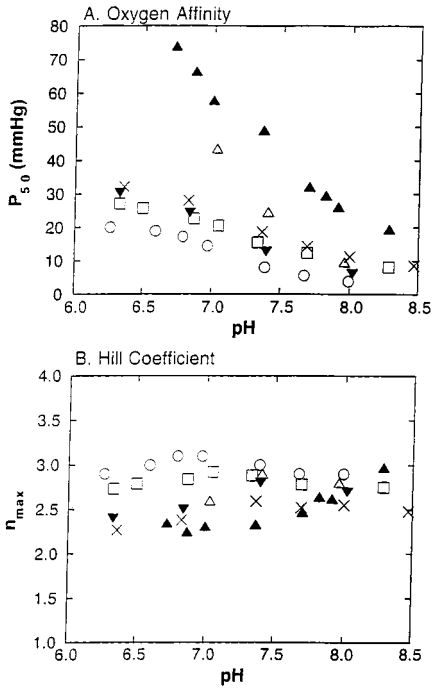

FIGURE 3: pH-dependence of the oxygen affinity (p_{50}) (A) and the Hill coefficient (n_{max}) (B) in 0.1 M phosphate buffer at 29 °C. (○) Hb A; (▼) r Hb (αV96W); (△) r Hb Presbyterian (βN108K); (□) r Hb Yoshizuka (βN108D); (▲) r Hb (αV96W, βN108K); (×) r Hb (αV96W, βN108D). Oxygen-dissociation data were obtained with 0.1 mM Hb.

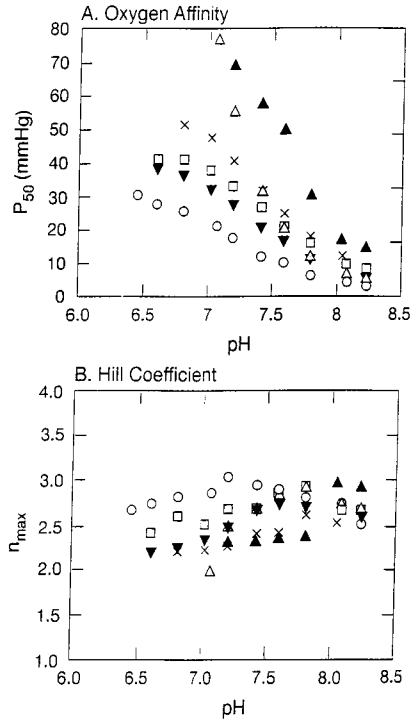

FIGURE 4: pH-dependence of the oxygen affinity (p_{50}) (A) and the Hill coefficient (n_{max}) (B) in 0.1 M HEPES buffer in the presence of 2 mM 2,3-BPG at 29 °C. (○) Hb A; (▼) r Hb (αV96W); (△) r Hb Presbyterian (βN108K); (□) Hb Yoshizuka (βN108D); (▲) r Hb (αV96W, βN108K); (×) r Hb (αV96W, βN108D). Oxygen-dissociation data were obtained with 0.1 mM Hb. The oxygen-binding properties of r Hb (αV96W, βN108K) were measured in the presence of a reductase system (*22*) to reduce the amount of met-Hb formed to less than 4% during the oxygenation process.

oxygen-binding properties of r Hb (αV96W, βN108K) among all the mutants and Hb A that we have studied here. Further investigation is under way to understand how the incubation temperature of the supernatant can perturb the protein conformation of this very low oxygen affinity r Hb.

Oxygen-Binding Studies as a Function of Chloride Concentration. The dramatic difference between the Bohr effect of r Hb Presbyterian (βN108K) and that of r Hb Yoshizuka (βN108D) has prompted us to investigate the effect of chloride. Figure 5 compares the effect of chloride on the oxygen-binding properties of Hb A and the five r Hbs in 0.1 M HEPES buffer at pH 7.4. In the absence of any chloride, r Hb Presbyterian and r Hb (αV96W) have essentially the same p_{50} as that of Hb A. However, r Hb Yoshizuka, r Hb (αV96W, βN108K), and r Hb (αV96W, βN108D) have an intrinsically lower oxygen affinity even in the absence of chloride. Upon increasing the chloride concentration, the oxygen affinity of r Hb Presbyterian dramatically decreases in comparison to that of Hb A. In the presence of excess chloride, the p_{50} values of r Hb Presbyterian and r Hb (αV96W, βN108K) continue to increase proportionally to chloride concentration. On the other hand, there is little or no chloride effect for r Hb Yoshizuka or r Hb (αV96W, βN108D), even at concentrations far exceeding those under physiological conditions. Table 2 gives an account of the number of chloride ions bound upon deoxygenation based on Wyman's linkage equation (*28*). For Hb A, about 1.4 chloride ions are bound upon deoxygenation per Hb tetrameter. This value is in good agreement with the ^{35}Cl NMR results reported by Chiancone et al. (*29*, *30*). According to Chiancone and co-workers, there are two binding sites for chloride in deoxy-Hb A, and only the high-affinity site is oxygen linked. The chloride effect of r Hb (αV96W, βN108D) is even less than that of r Hb (βN108D) and r Hb (αV96W). The chloride effect of r Hb (αV96W, βN108K) is between that of r Hb (αV96W) and r Hb Presbyterian.

Structural Studies

^{1}H-NMR Investigation. ^{1}H-NMR spectroscopy is an excellent tool for monitoring changes in the tertiary and quaternary structures of Hb A and its variants (*5*) and of recombinant Hbs (*7*−*10*, *19*, *31*, *32*). Figure 6 shows the exchangeable proton resonances and the ring-current-shifted proton resonances of r Hb (αV96W), r Hb Presbyterian (βN108K), r Hb Yoshizuka (βN108D), r Hb (αV96W, βN108K), and r Hb (αV96W, βN108D) in the CO form compared to those of Hb A. The ring-current-shifted resonances are sensitive to the orientation and/or the conformation of the heme group relative to the amino acid residues in the heme pockets, i.e., the tertiary structure of the Hb molecule (*5*). The ring-current-shifted resonances of these r Hbs in the CO form differ only slightly from those of Hb A. Our experience has been that minor differences in the intensity and positions of ring-current-shifted resonances are common features in many recombinant Hb mutants that we have studied (*7*−*10*, *19*, *31*, *33*). These changes reflect slight adjustments of the conformation of the hemes and/or the amino acid residues in the heme pockets as a result of the mutation.

The exchangeable proton resonances of the Hb molecule arise from the exchangeable protons in the subunit interfaces. Of special interest to this study are the exchangeable proton

Table 1: Bohr Effects of Recombinant Hemoglobins in Different Buffer Conditions at 29 °C

hemoglobin	$\Delta \log p_{50}/\Delta$ pH[b,c] in 0.1 M HEPES	$\Delta \log p_{50}/\Delta$ pH[b,c] in 0.1 M HEPES + 0.1 M chloride	$\Delta \log p_{50}/\Delta$ pH[b,c] in 0.1 M phosphate	$\Delta \log p_{50}/\Delta$ pH[b,d] in 0. 1 M HEPES + 2 mM 2,3-BPG
Hb A	0.33 (pH 6.72−8.00) 0.63 (pH 6.50−8.00)[a]	0.54 (pH 6.75−8.00)	0.51 (pH 6.59−8.00)	0.65 (pH 6.8−8.23)
r Hb (αV96W)	0.24 (pH 6.48−8.02)	0.38 (pH 6.54−8.02)	0.51 (pH 6.84−8.02)	0.58 (pH 6.82−8.22)
rHb Presbyterian (βN108K)	0.42 (pH 6.63−8.00)	0.64 (pH 6.69−8.00)	0.71 (pH 7.00−7.96)	0.99 (pH 7.0−8.23)
rHb Yoshizuka (βN108D)	0.18 (pH 6.59−8.00)	0.30 (pH 6.59−8.00)	0.29 (pH 6.50−8.28)	0.52 (pH 6.82−8.2)
rHb (αV96W, βN108K)	0.47 (pH 6.88−8.02)[a] 0.40 (pH 7.23−8.02)[a]	0.41 (pH 6.88−8.02)[a] 0.43 (pH 7.20−8.00)[a]	0.37 (pH 6.72−8.28)	0.72 (pH 7.20−8.24)[a]
rHb (αV96W, βN108D)	0.16 (pH 6.48−7.98)	0.26 (pH 6.53−8.01)	0.34 (pH 6.83−8.00)	0.52 (pH 6.8−8.04)

[a] Data are taken in the presence of a reductase system (*22*). Met% formation in the presence of reductase over the pH range from 7.23 to 8.02 was under 2.9%. At pH 6.88, the met% formation was 9.0%. [b] The values of Bohr effects reported here are the best effort estimate of the maximum Bohr effect in the pH range indicated in parentheses. [c] Based on Wyman's linkage equation (*28*), the number of H^+ ions released per heme upon oxygenation is calculated from $-\partial \log p_m/\partial$ pH, where p_m is the medium oxygen partial pressure. With $p_m \cong p_{50}$, we can estimate the Bohr coefficient by calculating the best fit of $-\partial \log p_{50}/\partial$ pH. [d] The oxygen-binding curves of mutants and Hb A in 2,3-BPG buffer are less symmetric than in other buffer systems. The Bohr coefficients calculated from $-\partial \log p_m/\partial$ pH in 2,3- BPG buffer of Hb A, r Hb (αV96W), r Hb Presbyterian (βN108K), r Hb Yoshizuka (βN108D), r Hb (αV96W, ββN108K), and r Hb (αV96W, βN108D) are 0.62, 0.53, 1.04, 0.47, 0.83, and 0.48, respectively. The trend of the number of H^+ ions released upon oxygenation among all the mutants is not changed between those calcuated from $-\partial \log p_m/\partial$ pH and $-\partial \log p_{50}/\partial$ pH.

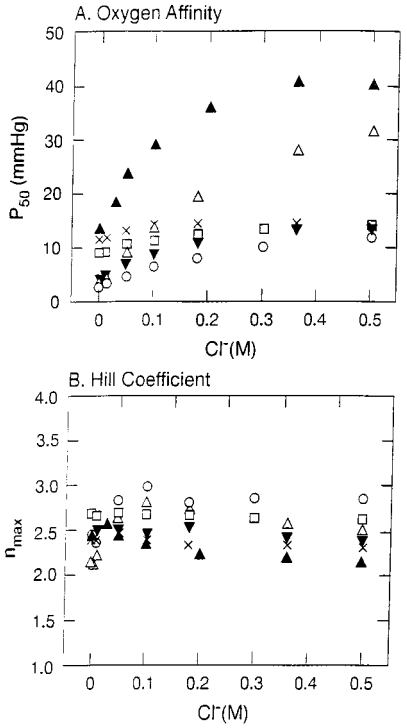

FIGURE 5: Chloride concentration dependence of the oxygen affinity (p_{50}) (A) and the Hill coefficient (n_{max}) (B) in 0.1 M HEPES buffer at pH 7.4 and at 29 °C. (○) Hb A; (▼) r Hb (αV96W); (△) r Hb Presbyterian (βN108K); (□) r Hb Yoshizuka (βN108D); (▲) r Hb (αV96W, βN108K); (×) r Hb (αV96W, βN108D). Oxygen dissociation data were obtained with 0.1 mM Hb. The oxygen-binding properties of r Hb (αV96W, βN108K) were measured in the presence of a reductase system (*22*) to reduce the amount of met-Hb formed to less than 4% during the oxygenation process.

resonances at 14.2, 13.0, 12.2, 11.2, and 10.7 ppm from DSS, which have been characterized as the intersubunit H-bonds in the $\alpha_1\beta_1$ and $\alpha_1\beta_2$ subunit interfaces in both deoxy (T) and/or oxy (R) states of Hb A (*5*, *6*, *34*). As shown in Figure 6, the resonance at 12.2 ppm obtained at 300-MHz ^{1}H NMR disappears at 29 °C in the spectra of r Hb Yoshizuka and r Hb (αV96W, βN108D) in the CO form. However, this resonance appears at 500-MHz ^{1}H NMR (Figure 7). It is estimated that the exchange rate for this H-bond with water is faster than 2235 s^{-1} (from the 300-MHz spectrum), but slower than 3635 s^{-1} (from the 500-MHz spectrum). When

Table 2: Effects of Chloride on Oxygen Binding of Recombinant Hemoglobins in 0.1 M HEPES at pH 7.4 and 29 °C

hemoglobin	$\Delta \log p_{50}/\Delta \log [Cl^-]$ in 0. 1 M HEPES[a]	no. of Cl^- bound upon deoxygenation per Hb tetramer
Hb A	0.34	1.4
r Hb (αV96W)	0.27	1.1
r Hb Presbyterian (βN108K)	0.42	1.7
r Hb Yoshizuka (βN108D)	0.12	0.5
r Hb (αV96W, βN108K)[b]	0.29	1.2
r Hb (αV96W, βN108D)	0.04	0.2

[a] Chloride concentration varied from 0 to 0.5 M. The numbers of chloride ion bound upon deoxygenation are the best effort estimate in the chloride concentration range between 0 and 0.5 M. [b] Data were taken in the presence of the met-Hb reductase system of Hayashi et al. (*22*).

lowering the temperature, this resonance reappears in the 300-MHz ^{1}H NMR (Figure 7), indicating that the effect of lowering temperature is to slow the exchange rate of this H-bond assigned at 12.2 ppm to less than 2235 s^{-1}. The restoration of this resonance at 12.2 ppm by lowering the temperature and/or increasing the magnetic field indicates that substituting Asp at β108Asn increases the exchange rate of the proton in this H-bond with the solvent. The resonance at 12.2 ppm was assigned to the H-bond between α103His and β108Asn in the $\alpha_1\beta_1$ interfaces (*34*). However, based on our recent ^{1}H NMR investigation of r Hb (βQ131E) (C.-K. Chang and C. Ho, unpublished results) as well as a highly refined crystal structure of deoxy-Hb A (J. Tame, unpublished results; personal communication), it appears that the 12.2 ppm resonance is due to the H-bond between α103His and β131Gln in the $\alpha_1\beta_1$ interfaces. Additional work is needed to ascertain the origin of this resonance.

Figure 8 shows the exchangeable and ferrous hyperfine-shifted proton resonances of r Hb (αV96W), r Hb Presbyterian (βN108K), r Hb Yoshizuka (βN108D), r Hb (αV96W, βN108K), and r Hb (αV96W, βN108D) in the deoxy form are similar to those of Hb A in the deoxy form. The resonance at 12.2 ppm is present in r Hb Yoshizuka and r Hb (αV96W, βN108D), but its relative intensity has changed. Figure 8 also shows the low-field hyperfine-shifted ^{1}H resonances of Hb A and the r Hbs in the deoxy form. The resonance at 63 ppm from DSS has been assigned to the hyperfine-shifted N^ϵH-exchangeable proton of the proximal

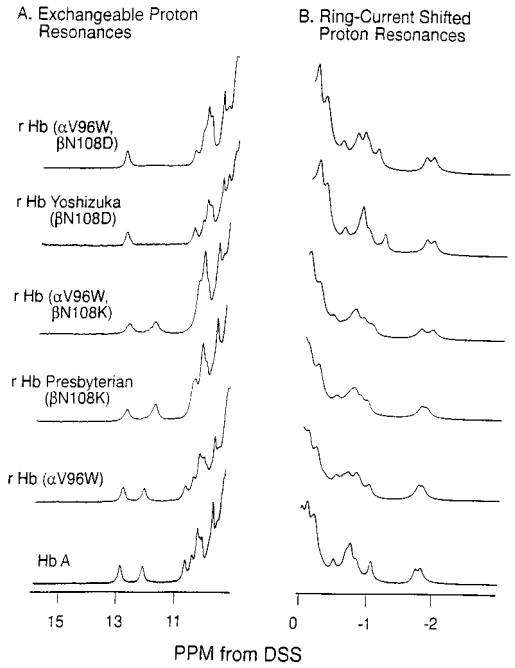

FIGURE 6: 300-MHz ^{1}H NMR spectra of 4−6% solutions of Hb A, r Hb (αV96W), r Hb Presbyterian (*β*N108K), r Hb (αV96W, *β*N108K), r Hb Yoshizuka (*β*N108D), and r Hb (αV96W, *β*N108D) in the CO form in H_2O in 0.1 M sodium phosphate at pH 7.0 and 29 °C.

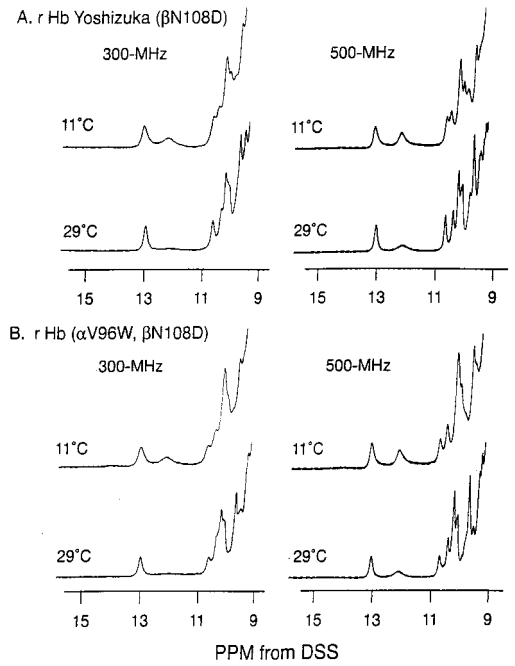

FIGURE 7: Effects of temperature on the 300-MHz and 500-MHz ^{1}H NMR spectra of 4−6% r HbCO (αV96W, *β*N108D) and r HbCO Yoshizuka (*β*N108D) in 0.1 M phosphate buffer in H_2O at pH 7.0 at 29 and 11 °C: (A) exchangeable proton resonances of r HbCO Yoshizuka (*β*N108D); (B) exchangeable proton resonances of r HbCO (αV96W, *β*N108D).

histidine residue (α87His) of the α chain of deoxy-Hb A, and the one at 77 ppm from DSS has been assigned to the corresponding residue of the *β* chain (*β*92His) of deoxy-Hb A (*35*, *36*). The chemical shift positions of these two proximal histidyl resonances in these five r Hbs are exactly the same as those of Hb A, indicating no perturbations around the proximal histidine residues of the r Hbs.

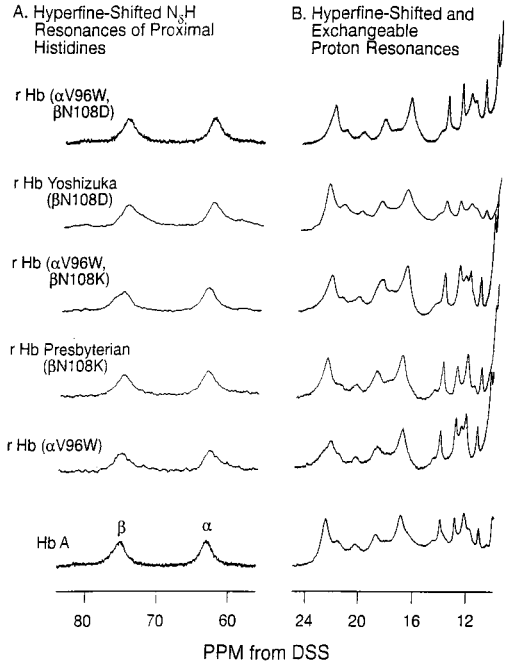

FIGURE 8: 300-MHz ^{1}H NMR spectra of 4−6% solutions of Hb A, r Hb (αV96W), r Hb Presbyterian (*β*N108K), r Hb (αV96W, *β*N108K), r Hb Yoshizuka (*β*N108D), and r Hb (αV96W, *β*N108D) in the deoxy form in H_2O in 0.1 M sodium phosphate at pH 7.0 and 29 °C.

Figures 9 and 10 show the exchangeable proton resonances of Hb A and the five r Hbs in the CO form under various experimental conditions in 0.1 M HEPES buffer at pH 7.0 as a function of temperature. The resonance at 14.2 ppm has been identified as the intersubunit H-bond between α42Tyr and *β*99Asp in the $\alpha_1\beta_2$ interface in deoxy-Hb A (*6*), a characteristic feature of the deoxy (T) quaternary structure of Hb A (*1*). The resonance at 10.2 ppm has been assigned to the intersubunit H-bond between α94Asp and *β*102Asn in the $\alpha_1\beta_2$ interface in oxy-Hb A (*6*, *34*), a characteristic feature of the oxy (R) quaternary structure (*1*). As discussed above (*8*, *10*), the T-marker at 14.2 ppm is present when the temperature is lowered and/or by the addition of IHP even when the hemes are still ligated (in the CO form or in the oxy form) for r Hb (αV96W) and r Hb (αV96W, *β*N108K). Studies on the temperature dependence of exchangeable proton resonances of r Hbs in the CO form can be used to assess the structural consequences of oxygen affinity. As shown in Figure 9, r Hb Presbyterian and r Hb (αV96W) have very stable T-states as indicated by the appearance of the T-marker in the R-state. The T-marker of the double mutant, r Hb (αV96W, *β*N108K), has a higher intensity compared to the single mutants. This suggests that the effect of the two mutations on the protein conformation might be complementary. As shown in Figure 10, the T-marker also appears when IHP is added to r Hb Yoshizuka and r Hb (αV96W, *β*N108D) at 11 °C. These results indicate that the T-states of Hb Yoshizuka and r Hb (αV96W, *β*N108D) are also more stable than that of Hb A.

DISCUSSION

r Hb (αV96W) constructed by Kim et al. (*8*) has been shown to have low oxygen affinity and high cooperativity. Kim et al. (*8*) demonstrated that r Hb (αV96W) can switch from the R structure into the T structure without changing

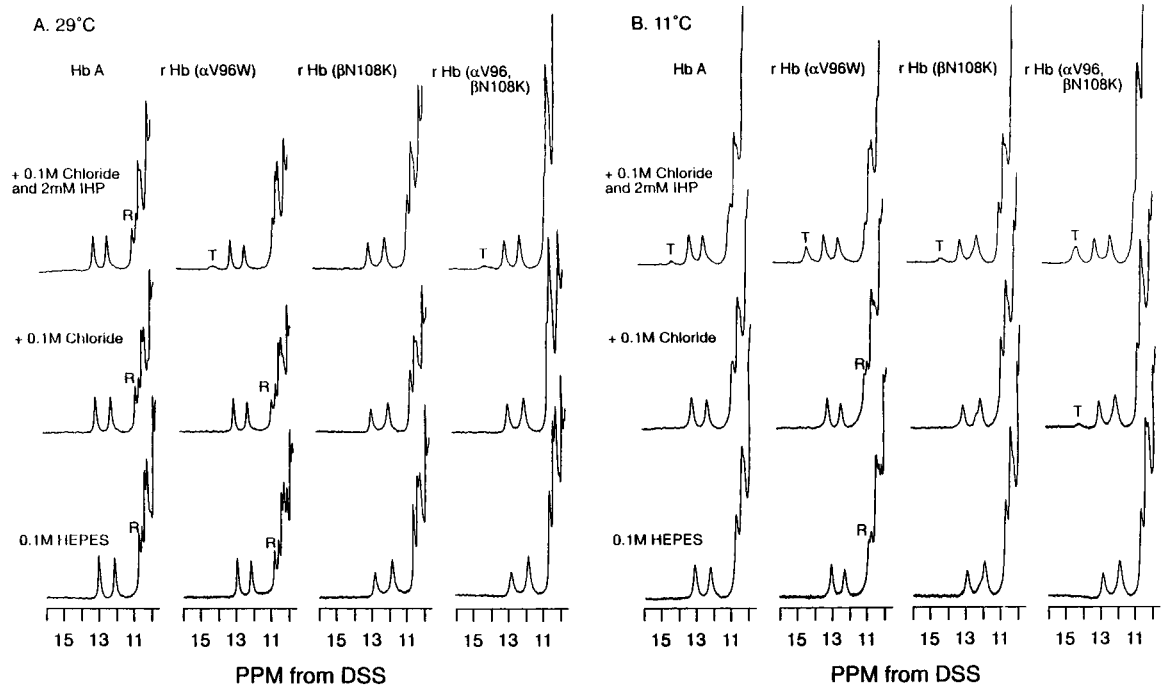

FIGURE 9: Effects of temperature on the 300-MHz ^{1}H NMR spectra of Hb A, r Hb (αV96W), r Hb Presbyterian (βN108K), and r Hb (αV96W, βN108K) in the CO form in 0.1 M HEPES in H_2O at pH 7.0, in 0.1 M HEPES plus 0.1 M chloride, and in 0.1 M HEPES plus 0.1 M chloride plus 2 mM IHP: (A) at 29 °C; (B) at 11 °C.

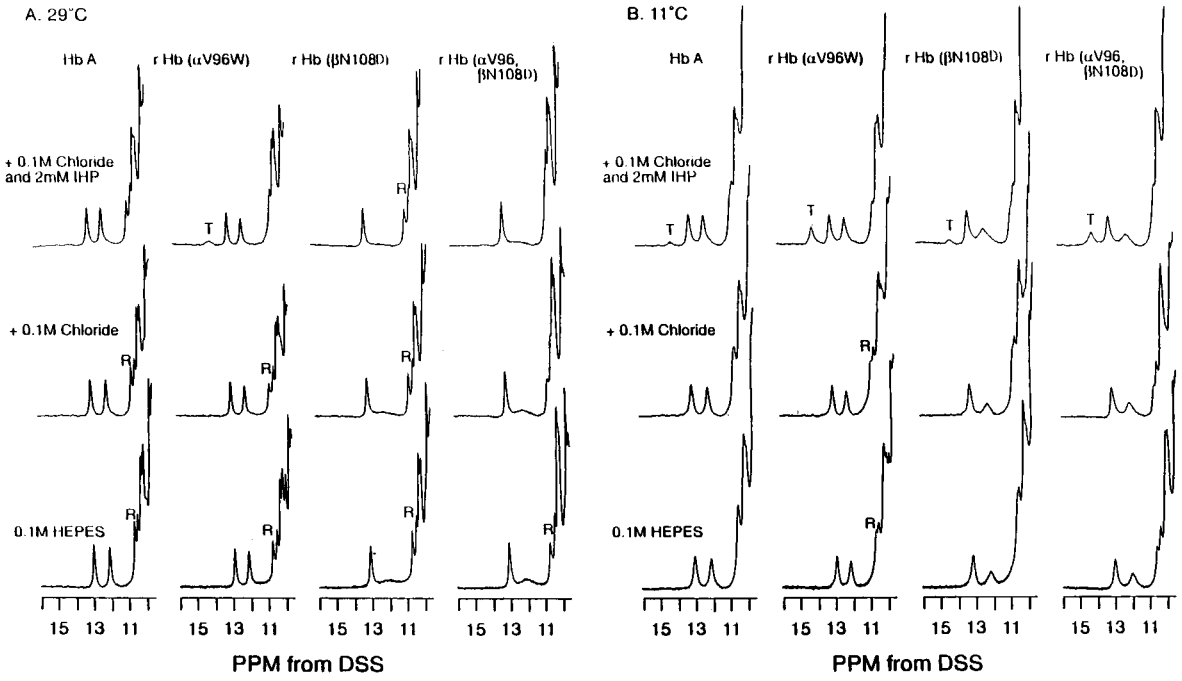

FIGURE 10: Effects of temperature on the 300-MHz ^{1}H NMR spectra of Hb A, r Hb (αV96W), r Hb Yoshizuka (βN108D), and r Hb (αV96W, βN108D) in the CO form in 0.1 M HEPES in H_2O at pH 7.0, in 0.1 M HEPES plus 0.1 M chloride, and in 0.1 M HEPES plus 0.1 M chloride plus 2 mM IHP: (A) at 29 °C; (B) at 11 °C.

its ligation state under low temperature and/or in the presence of IHP. This indicates that the molecular basis for the low oxygen affinity of r Hb (αV96W) is that it favors the T conformation as compared to Hb A. r Hb Presbyterian (βN108K) is a low affinity mutant with normal cooperativity (*16*, *17*, *37*−*39*). Ho et al. (*10*) showed that r Hb Presbyterian and r Hb (αV96W, βN108K) also have a similar mechanism for low oxygen affinity and high cooperativity. The T-marker of the double mutant, r HbCO (αV96W, βN108K), has a higher intensity compared to that of the single mutants (Figure 9). This suggests that the effect of the two mutations on the protein conformation is complementary or additive. We have further investigated the molecular basis for the low oxygen affinity and high cooperativity in r Hb Yoshizuka and r Hb (αV96W, βN108D). r Hb Yoshizuka is a mutant with a negative charge at the β108 site. Figure 10 shows that the R structure of r Hb Yoshizuka and r Hb (αV96W, βN108D) can also switch to the T structure with the ligands intact, by lowering the ambient temperature and adding IHP. r Hb (αV96W, βN108K), which has the lowest oxygen

Table 3: Oxygen Affinities of Recombinant Hemoglobins in 0.1 M HEPES and in 0.1 M HEPES plus 0.1 M Chloride or 2 mM 2,3-BPG at pH 7.4 and 29 °C[a]

	Hb A	r Hb Presbyterian (βN108K)	r Hb Yoshizuka (βN108D)	r Hb (αV96W)	r Hb (αV96W, βN108K)	r Hb (αV96W, βN108D)
p_{50} (mmHg)						
−chloride	2.49	3.42	8.91	4.24	13.32[b]	11.69
+chloride	6.5	13.49	12.66	9.07	29.03[b]	14.01
+2,3-BPG	11.87	31.54	26.96	20.68	57.58[b]	31.54
log [p_{50}(mutant)/p_{50}(Hb A)]						
−chloride		0.14	0.55	0.23	0.61[b]	0.67
+chloride		0.32	0.29	0.14	0.65[b]	0.33
+2,3-BPG		0.42	0.36	0.24	0.60[b]	0.42
$\Delta\Delta$ log p_{50}(chloride)		+0.18	−0.26	−0.09	+0.04	−0.34
$\Delta\Delta$ log p_{50}(2, 3-BPG)		0.28	−0.19	+0.01	−0.01	−0.25

[a] $\Delta\Delta \log p_{50} = \{\log [p_{50}(\text{mutant})/p_{50}(\text{Hb A})](+\text{chloride or 2,3-BPG})\} - \{\log [p_{50}(\text{mutant})/p_{50}(\text{Hb A})](-\text{chloride or 2,3-BPG})\} = \Delta \log p_{50}(\text{mutant}) - \Delta \log p_{50}(\text{Hb A})$. The buffers used were 0.1 M HEPES buffer at pH 7.4, the same with either 0.1 M Cl^- or 2 mM 2,3-BPG. The temperature was 29 °C, and the hemoglobin concentration was 0.1 mM heme. [b] The oxygen-binding properties of r Hb (αV96W, βN108K) were measured in the presence of a reductase system (*22*). In the presence of a reductase system, the formation of met-Hb can be reduced from more than 9% to less than 2.9%. The control data for Hb A were $p_{50} = 3.3$ mmHg measured in the presence of a reductase system (*22*) in the 0.1 M HEPES buffer and $p_{50} = 6.5$ mmHg in the 0.1 M chloride/HEPES and $p_{50} = 14.6$ mmHg in the presence of 2,3-BPG and reductase in 0.1 M HEPES.

affinity among the six Hbs studied, has the greatest tendency to switch to the T quaternary structure.

Ho et al. (*10*) reported that at 10 °C in 0.1 M phosphate at pH 7.4, the p_{50} and n_{max} values for r Hb (αV96W) are 2.3 mmHg and 2.2, respectively, and the corresponding values in the presence of 2 mM IHP are 13.3 mmHg and 2.1, respectively. They also reported the p_{50} and n_{max} values for r Hb (αV96W, βN108K); namely, in the absence of IHP, $p_{50} = 11.8$ mmHg and $n_{max} = 1.7$, and in the presence of 2 mM IHP, $p_{50} = 23.2$ mmHg and $n_{max} = 1.3$. The corresponding values for Hb A are the following: in the absence of IHP, $p_{50} = 1.3$ mmHg and $n_{max} = 1.8$; and in the presence of 2 mM IHP, $p_{50} = 10.4$ mmHg and $n_{max} = 2.5$ (*10*). As shown in Figure 9B, the T-markers for both r Hb (αV96W) and r Hb (αV96W, βN108K) at 11 °C are very prominent, and both r Hbs exhibit very considerable cooperativity in their oxygenation process ($n_{max} = 1.3-2.2$). The likely reason that the n_{max} value for r Hb (αV96W, βN108K) in the presence of IHP and at 10 °C is 1.3 is that this r Hb strongly prefers to remain in the T-state even when it is fully ligated as demonstrated by the presence of a strong resonance at 14 ppm, thus giving rise to a low value of n_{max}. It should be noted that in Figure 9B, the intensity of the 14 ppm resonance is barely detectable for Hb A in the presence of IHP at 11 °C. These results strongly suggest that there is considerable cooperativity in the oxygenation process in the T-state of the Hb molecule.

The β108 residue is located in the $\alpha_1\beta_1$ interface and in the central cavity of the Hb molecule. Bonaventura et al. (*18*, *40*) proposed that the repulsion among the excess positive charges in the central cavity of the Hb molecule can destabilize its T structure and thereby shifts the allosteric equilibrium toward the R-state which in turn increases the oxygen affinity of the Hb molecule. Perutz et al. (*41*, *42*) suggested that the repulsion among the positively charged groups in the central cavity is diminished by random delocalization of chloride ions. They proposed that the introduction of additional cationic groups into the central cavity should raise the oxygen affinity in the absence of chloride. If this hypothesis is correct, Hb Presbyterian should have a higher oxygen affinity by further destabilization of the T-state in preference to the R-state. As shown in Figure 1, r Hb Presbyterian (βN108K) has essentially the same oxygen affinity as that of Hb A at pH >7.4 in chloride-free HEPES buffer. It appears that excessive positive charges in the central cavity can give rise to low oxygen affinity for r Hb Presbyterian. This indicates that mechanism(s) other than the build-up of excess positive charge in the central cavity is (are) responsible for the oxygenation property of r Hb Presbyterian. r Hb Yoshizuka (βN108D) has an intrinsically low oxygen affinity as compared to Hb A in chloride-free HEPES buffer, which is in accordance with the above-mentioned central cavity mechanism.

A marked difference of the oxygen binding properties between r Hb Presbyterian (βN108K) and r Hb Yoshizuka (βN108D) occurs when an allosteric effector is added, such as chloride, inorganic phosphate, or 2,3-BPG, as shown in Figures 2−5. Quantitatively, the effect of anions on the oxygen affinity can be observed by comparing a change in the free energy of oxygen binding, ΔG, in buffers with anions and in chloride-free HEPES buffer: $\Delta G = -RT \ln K_4 = 4RT \ln p_{50}$, where K_4 is the overall association constant. Changes in the free energy of the anion induced by mutations, therefore, can be quantified as proportional to $\Delta\Delta \log p_{50} = \Delta \log p_{50}(\text{mutant}) - \Delta \log p_{50}(\text{Hb A})$, where $\Delta \log p_{50}$ is the difference of free energy in buffers with anions and in chloride-free HEPES buffer. Table 3 summarizes the changes in free energy of the chloride effect induced by the mutation in 0.1 M HEPES buffer at pH 7.4. The change in the oxygen affinity of r Hb Presbyterian due to chloride is +0.18, and that of r Hb Yoshizuka is −0.26. This suggests that substituting a positively charged Lys at the β108 site enhances the chloride effect, while substituting a negatively charged Asp at the β108 site abolishes the chloride effect. The change in the oxygen affinity of r Hb (αV96W) is only −0.09 due to chloride. This further indicates that the sensitivity of oxygen affinity to chloride ions depends on the charge distribution on the Hb molecule. This is consistent with the conclusion reached by Perutz et al. (*42*), which predicts that the introduction of additional cationic groups into the central cavity should strengthen the chloride effect. The $\Delta\Delta \log p_{50}$ of r Hb (αV96W, βN108K) is close to the sum of $\Delta\Delta \log p_{50}$ of r Hb (αV96W) and $\Delta\Delta \log p_{50}$ of r Hb (βN108K); and the $\Delta\Delta \log p_{50}$ of r Hb (αV96W, βN108D) is the sum of $\Delta\Delta \log p_{50}$ of r Hb (αV96W) and

$\Delta\Delta\log p_{50}$ of r Hb Yoshizuka. This suggests that the effect of the mutations on the chloride effect is additive.

2,3-BPG reduces the oxygen affinity of hemoglobin by binding strongly to the deoxy structure and only weakly to the oxy structure (*43*,*44*). Thus, an increased 2,3-BPG effect, as in r Hb Presbyterian (Figure 4), indicates increased affinity of 2,3-BPG for the deoxy structure. As shown in Table 3, the free energy change on addition of 2,3-BPG in r Hb Presbyterian is +0.28, which suggests that the structural consequences of the presence of the new positive charges in the middle of the central cavity have been communicated to the $\beta\beta$ cleft (2,3-BPG-binding site) of the protein. This is consistent with the findings of Gottfried et al. (*45*) in which the binding of fluorescent analogues of the natural allosteric effector 2,3-BPG to Hb Presbyterian was stronger than that to Hb A under various experimental conditions. There is a change of −0.19 unit in the oxygen affinity due to 2,3-BPG binding in r Hb Yoshizuka and only +0.01 change in oxygen affinity due to 2,3-BPG in r Hb (αV96W).

Perutz et al. (*41*, *42*) suggested that the "invasion of positively charged ions into the central cavity" of the Hb molecule could influence the allosteric interactions without specific chloride binding. The results of oxygen-binding studies under various concentrations of chloride reported by Perutz et al. (*42*) indicate the sensitivity of oxygen binding to the ionic strength of the buffer (anion effect). These results support our early conclusion regarding the important role that electrostatic effects play in regulating the Bohr effect of Hb A (*21*, *33*, *46*, and references cited therein). To further illustrate the effect of charge on the β108 residue (the side chain of which interacts with the water molecules in the central cavity), we have measured the Bohr effects of r Hb Presbyterian, r Hb Yoshizuka, and r Hb (αV96W) and their respective double mutants and compared them to Hb A under various buffer conditions. As shown in Table 1, in the chloride-free HEPES buffer, r Hb Presbyterian has the largest Bohr effect, followed by Hb A and r Hb Yoshizuka. In the presence of different allosteric effectors, such as chloride, inorganic phosphate, and 2,3-BPG, the Bohr effect of all r Hbs increases, without changing this order. This indicates that the amino acid substitution in the central cavity that increases the net positive charge density can enhance the Bohr effect. r Hb (αV96W) has a smaller Bohr effect than Hb A, but has a larger Bohr effect than r Hb Yoshizuka in the chloride-free HEPES buffer as well as in the presence of allosteric effectors. Recent X-ray crystallographic studies of r Hb (αV96W) in the T-state show that the side chain of α96Trp makes a water-mediated H-bond with β101Glu in the $\alpha_1\beta_2$ interfaces and also interacts with the central cavity water (*15*). The presence of a bulky side chain (i.e., Trp) could mediate the repulsive forces in the central cavity, hence reduce the Bohr effect.

The results from our recombinant hemoglobins with amino acid substitutions at α96 and β108 have allowed us to make the following conclusions: (i) If we can stabilize the deoxy (T) quaternary structure of an Hb molecule without perturbing its oxy quaternary structure, we will have an Hb molecule with low oxygen affinity and high cooperativity. (ii) A change in the ionic environment, i.e., an alteration of electrostatic interactions within the central cavity of the Hb molecule, has a striking effect on the modulation of oxygen affinity by the heterotropic effectors, such as Cl^-, inorganic phosphate, and 2,3-BPG. (iii) An alteration of the charge in the central cavity of an Hb molecule can influence the Bohr effect. This supports our model of the Bohr effect, in which the presence of anions alters the electrostatic distributions in the Hb molecule and thereby influences the microscopic mechanism of the Bohr effect (*21*, *33*, *46*, and references cited therein). (iv) The allosteric effect induced by the mutation at αV96W on the mutations at βN108K and βN108D is additive. (v) An amino acid substitution in the $\alpha_1\beta_1$ subunit interface can affect both the oxygen affinity and cooperativity in the oxygenation process. Thus, there is communication between the $\alpha_1\beta_1$ and $\alpha_1\beta_2$ subunit interfaces during the oxygenation process of the Hb molecule. This is another indication that allosteric interactions of allosteric proteins have multiple pathways for signal transmission (*32*, *33*). (vi) There is cooperativity in the T-state of hemoglobin in solution, consistent with our early conclusion (*47*) and that of Ackers et al. (*48*), and suggesting that there are substantial functional differences between T-state Hb in solution and in both crystals and encapsulated in silica gels, as T-state Hb in crystals and in silica gels has been shown to bind oxygen noncooperatively by Eaton, Mozzarelli, and co-workers (*49*−*51*). Thus, the detailed molecular mechanisms for the cooperative oxygenation process of Hb are now being revealed at the atomic level as a result of mutagenesis of the Hb gene, combined with NMR, X-ray crystallography, and thermodynamic and kinetic analyses.

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