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Site mutations disrupt inter-helical H-bonds (α14W–α67T and β15W–β72S) involved in kinetic steps in the hemoglobin R→T transition without altering the free energies of oxygenation☆

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Abstract

Three recombinant mutant hemoglobins (rHbs) of human normal adult hemoglobin (Hb A), rHb (αT67V), rHb (βS72A), and rHb (αT67V, βS72A), have been constructed to test the role of the tertiary intra-subunit H-bonds between α67T and α14W and between β72S and β15W in the cooperative oxygenation of Hb A. Oxygen-binding studies in 0.1 M sodium phosphate buffer at 29 °C show that rHb (αT67V), rHb (βS72A), and rHb (αT67V, βS72A) exhibit oxygen-binding properties similar to those of Hb A. The binding of oxygen to these rHbs is highly cooperative, with a Hill coefficient of approximately 2.8, compared to approximately 3.1 for Hb A. Proton nuclear magnetic resonance (NMR) studies show that rHb (αT67V), rHb (βS72A), rHb (αT67V, βS72A), and Hb A have similar quaternary structures in the $\alpha_1\beta_2$ subunit interfaces. In particular, the inter-subunit H-bonds between α42Tyr and β99Asp and between β37Trp and α94Asp are maintained in the mutants in the deoxy form. There are slight perturbations in the distal heme pocket region of the α- and β-chains in the mutants. A comparison of the exchangeable ^{1}H resonances of Hb A with those of these three rHbs suggests that α67T and β72S are H-bonded to α14W and β15W, respectively, in the CO and deoxy forms of Hb A. The absence of significant free energy changes for the oxygenation process of these three rHbs compared to those of Hb A, even though the inter-helical H-bonds are abolished, indicates that these two sets of H-bonds are of comparable strength in the ligated and unligated forms of Hb A. Thus, the mutations at αT67V and βS72A do not affect the overall energetics of the oxygenation process.

Abbreviations: Hb, hemoglobin; Hb A, human normal adult hemoglobin; deoxy-Hb, deoxyhemoglobin; HbCO, carbononoxy-hemoglobin; rHb, recombinant hemoglobin; met-Hb, methemoglobin; UVRR, ultraviolet resonance Raman; NMR, nuclear magnetic resonance; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; 2,3-BPG, 2,3-bisphosphoglycerate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; 2-D, two-dimensional; HMQC, heteronuclear multiple-quantum coherence; HSQC, heteronuclear single-quantum coherence.

☆ We would like to dedicate this paper to our friend and teacher, John T. Edsall, whose numerous contributions in biophysical chemistry of amino acids and proteins during the past century have laid the foundation for the rapid advance of our understanding of the structure–function relationships in proteins and enzymes.

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The preserved cooperativity in the binding of oxygen to these three mutants also implies that there are multiple interactions involved in the oxygenation process of Hb A.

Keywords: Site mutations; Inter-helical H-bonds; Hemoglobin

1. Introduction

Human normal adult hemoglobin (Hb A) is one of the most studied proteins and has also served as a model for the structure–function relationship in multimeric, allosteric proteins. Hb A binds oxygen cooperatively with a Hill coefficient of approximately 3.0. The cooperative ligand binding of Hb A has been interpreted by the sterochemical model of Perutz [1] on the basis of a detailed comparison of X-ray crystallographic structures of the deoxy- and liganded-Hb [2–4]. While the end states of the allosteric mechanism of Hb have been well characterized by the T (low affinity) and R (high affinity) structures [1], the T-to-R (or vice versa) transition pathway has not been clearly defined and remains a subject of great interest [5].

Time-resolved ultraviolet resonance Raman (UVRR) spectroscopy has been used to probe the R-to-T transition pathway, which can be induced by photolysis of HbCO A [6–9]. This pathway has been proposed to involve the displacement of the helices E and F, which sandwich the heme prosthetic group. These displacements would weaken the H-bonds connecting the E helix to the A helix, from α67T to α14W and from β72S to β15W, on the one hand, and the F helix to the H helix, from the backbone carbonyl groups of α93V to α140Y and from β98V to β145Y, on the other. These inter-helical H-bonds are re-formed by subsequent motions of the A and H helices, which serve to move the amino and carboxyl termini into positions from which they establish the inter-subunit salt-bridges that stabilize the T structure. Though UVRR studies of rHb (αT67V) and rHb (βS72A) have shown that α14W and β15W participate in the initial step (150 ns) of this pathway [10], the effect of these two H-bonds on the equilibrium oxygenation is not known. The question arises whether the influence of the H-bonds on the observable intermediates is connected to energetically significant changes in the equilibrium oxygenation process of Hb A.

One-dimensional (1-D) proton nuclear magnetic resonance (NMR) spectroscopy has been an excellent tool for investigating the tertiary and quaternary structures of Hbs in solution [11]. In particular, the chemical shift changes of the exchangeable protons due to the presence of H-bonds can be observed directly by NMR studies [12–18]. Recent developments in NMR spectroscopy together with isotopic labeling techniques have made it possible to gain new information on the structure–function relationship in Hb A [18–20]. Specifically, the assignments of α14W, β15W and β37W by two-dimensional (2-D) NMR spectroscopy [17,20] allow us to make a direct comparison between NMR and UVRR results on the structural changes upon the cooperative oxygenation of Hb A and rHbs.

To understand the role of inter-helical H-bonds between α14W and α67T and between β15W and β72S in the oxygenation process of Hb, we have further constructed a double mutant, rHb (αT67V, βS72A). Here, we report the oxygen-binding properties of rHb (αT67V), rHb (βS72A), and rHb (αT67V, βS72A) as a function of pH in the absence and presence of 2,3-bisphosphoglycerate (2,3-BPG). ^{1}H-NMR studies have been conducted to investigate the tertiary and quaternary structures of these three rHbs. We have also carried out 2-D NMR studies of ^{15}N-labeled rHb (αT67V) and rHb (αT67V, βS72A). We have found a reasonable correlation between the downfield shift of the amide proton resonances and the strength of H-bonds [12,15–17]. Hence, the amide proton resonances of α14W and β15W are expected to move upfield and closer to water resonances in rHb (αT67V) and rHb (βS72A), respectively, due to the loss of the H-bond partners.

2. Materials and methods

2.1. Construction of expression plasmids

The *Escherichia coli* Hb expression plasmid, pHE2, was constructed in our laboratory [21] and forms the basis for constructing other plasmids for expressing mutant Hbs. The plasmids, pHE209 and pHE210, for the expression of rHb (αT67V) and rHb (βS72A), were reported previously [10]. The plasmid pHE211 for the expression of rHb (αT67V, βS72A) was constructed by ligation of the 0.51 kb BstBI-NheI fragment of pHE210 and the 6.23 kb NheI-BstBI fragment of pHE209. Chemicals and restriction enzymes were purchased from major suppliers, such as Fisher, Sigma, Bio-Rad, Boehringer Mannheim, New England Bio-Labs, Pharmacia, Promega, United States Biochemicals, Inc., and Cambridge Isotope Laboratories, and were used without further purification.

2.2. Growth of cells

The expression vectors pHE209, pHE210 and pHE211, were individually transformed into *E. coli* strain JM109. The transformed cells were grown in Terrific-Broth (TB) medium plus 100 μg/ml ampicillin at 32 °C in a 10-l Microferm fermentor (New Brunswick Scientific, Model BioFlow 3000), and the production of mutant rHbs was carried out as described previously [21,22]. For the ^{15}N-labeled rHb (αT67V) and rHb (αT67V, βS72A), TB medium was replaced by modified DM-1 and DM-4 media [19] and NH_4Cl was replaced by $^{15}NH_4Cl$ in the seed culture and 5-l fermentor, respectively.

2.3. Isolation and purification of rHbs

The purification of rHbs followed the procedures described previously [21–23].

2.4. Characterization of mutant rHbs

The N-terminal residues were determined from Edman degradation and the molecular weights of rHbs were determined by mass spectrometric analyses as detailed in our previous studies [21]. These two analyses were carried out to certify the quality of purified rHbs. All purified rHbs had correct molecular weights and retained less than 2% methionine at the N-termini.

2.5. Oxygen-binding properties of rHbs

The oxygen dissociation curves of rHbs were measured on a Hemox-Analyzer (TCS Medical Products, Huntington Valley, PA, USA) at 29 °C as a function of pH in 0.1 M sodium phosphate buffer in the presence and absence of 5 mM 2,3-BPG. The concentration of Hb used in this study was approximately 0.1 mM per heme. The met-Hb reductase system was used if needed to reduce the amount of met-Hb in the sample [24]. Partial oxygen pressure at 50% saturation (P_{50}) and the Hill coefficient (n_{max}) were determined from each oxygen dissociation curve (for details see Tsai et al. [23]). The accuracy for P_{50} measurements (in mmHg) is ±5% and that for n_{max} is ±7%.

2.6. ^{1}H-NMR investigation of rHbs

^{1}H-NMR spectra of rHbs were obtained from Bruker AVANCE DRX-300, DRX-500 and DRX-600 NMR spectrometers. All Hb samples were in 0.1 M sodium phosphate buffer in 100% H_2O or in a mixture of 90% H_2O/10% D_2O, and the Hb concentration was between 6 and 8%. The water signal was suppressed by using a jump-and-return pulse sequence [25]. Proton chemical shifts are referenced to the methyl proton resonance of 2,2-dimethyl-2-silapentane-5-sulfonate (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. HMQC and HSQC experiments were performed on ^{15}N-labeled rHb (αT67V), rHb (αT67V, βS72A), and rHb A as previously described [20].

3. Results

3.1. Oxygen-binding studies

Fig. 1 shows the oxygen-binding measurements for rHb (αT67V), rHb (βS72A), rHb (αT67V, βS72A), and Hb A in 0.1 M sodium phosphate

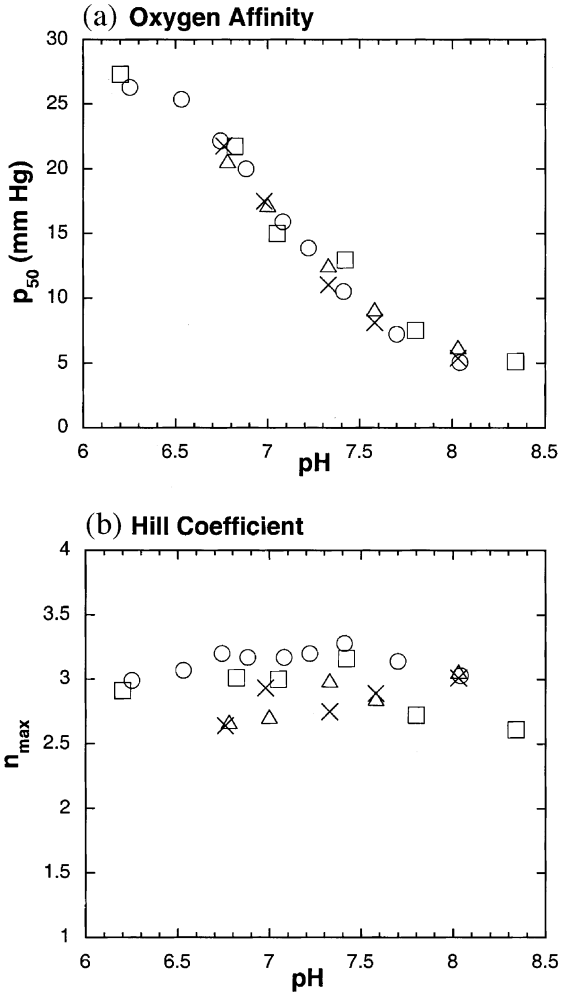

Fig. 1. The pH dependence of the oxygen affinity (a) and the Hill coefficient (b) of rHbs and Hb A in 0.1 M sodium phosphate buffer at 29 °C: (○) Hb A; (Δ) rHb (αT67V); (×) rHb (βS72A); and (□) rHb (αT67V, βS72A). P_{50} and n_{max} were determined from each oxygen dissociation curve, and Hb concentration was 0.1 mM heme. The oxygen-binding properties of the mutant rHbs were measured in the presence of a reductase system [24] to reduce the amount of met-Hb formed (less than 2%) during the oxygenation process.

buffer as a function of pH at 29 °C. rHb (αT67V), rHb (βS72A), and rHb (αT67V, βS72A) exhibit oxygen affinities similar to that of Hb A. The oxygenation process of rHb (αT67V), rHb (βS72A), and rHb (αT67V, βS72A) is very cooperative with an n_{max} value of approximately 2.6–3.0 depending on the pH, compared to approximately 3.0–3.2 for Hb A. Fig. 2 shows the decrease in oxygen affinity of these rHbs induced by the presence of 2,3-BPG. Over the pH range in which we have investigated the effect of 2,3-BPG,

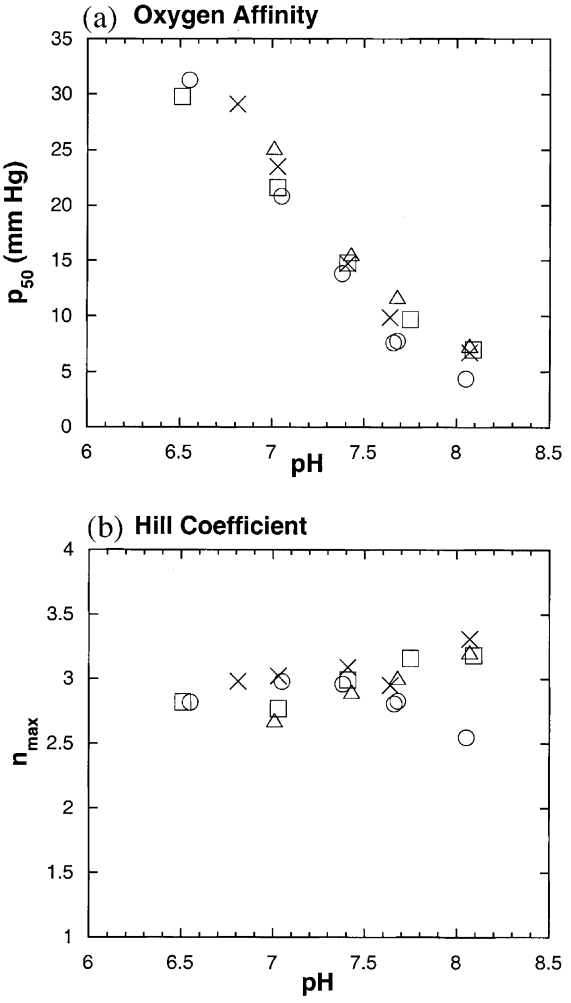

Fig. 2. The pH dependence of the oxygen affinity (a) and the Hill coefficient (b) of rHbs and Hb A in the presence of 5 mM 2,3-BPG in 0.1 M sodium phosphate buffer at 29 °C: (○) Hb A; (Δ) rHb (αT67V); (×) rHb (βS72A); and (□) rHb (αT67V, βS72A). P_{50} and n_{max} were determined from each oxygen dissociation curve, and Hb concentration was 0.1 mM heme. The oxygen-binding properties of the mutant rHbs were measured in the presence of a reductase system [24] to reduce the amount of met-Hb formed (less than 2%) during the oxygenation process.

Table 1
P_{50} and n_{max} values for Hb A and rHb (αT67V, βS72A) in the presence and absence of sodium chloride

[Chloride] (mM)	Hb A		rHb (αT67V, βS72A)	
	P_{50} (mmHg)	n_{max}	P_{50} (mmHg)	n_{max}
0	3.7	3.3	3.9	3.2
50	5.4	3.2	6.1	3.3
100	6.9	2.9	7.6	3.1
200	8.4	2.8	9.7	3.0

Data were obtained with 0.1 mM Hb in 0.1 M HEPES buffer at pH 7.4 and 29 °C; 30 nM catalase was used to prevent the formation of met-Hb (less than 2%) [24].

rHb (αT67V), rHb (βS72A), and rHb (αT67V, βS72A) exhibit similar oxygen affinity to Hb A. The intrinsic oxygenation properties of rHb (αT67V, βS72A) in HEPES buffer are also similar to those of Hb A, and rHb (αT67V, βS72A) exhibits a chloride effect similar to those of Hb A (Table 1). Figs. 1 and 2 also show that the alkaline Bohr effects of these three rHbs are similar to those of Hb A in the presence and absence of the allosteric effector, 2,3-BPG.

3.2. ^{1}H-NMR investigation

^{1}H-NMR is an excellent tool for monitoring changes in the tertiary and quaternary structures of Hb A and its variants [11,21,23,26–31]. Fig. 3a shows the exchangeable proton resonances in the 500-MHz NMR spectra of Hb A, rHb (αT67V), rHb (βS72A), and rHb (αT67V, βS72A) in the CO form. These exchangeable proton resonances arise from the exchangeable protons in the subunit interfaces. The resonances at 12.9 and 12.1 ppm from DSS have been

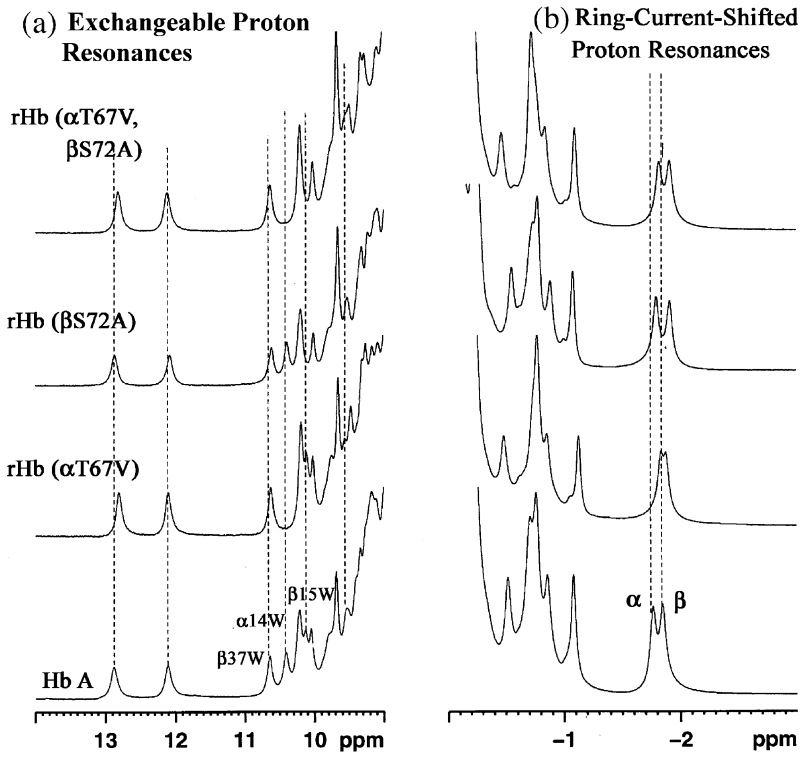

Fig. 3. The 500-MHz ^{1}H-NMR spectra of 4–6% solutions of Hb A, rHb (αT67V), rHb (βS72A), and rHb (αT67V, βS72A) in the CO form in 0.1 M sodium phosphate buffer at pH 7.0 and 29 °C: (a) exchangeable proton resonances; and (b) ring-current-shifted proton resonances.

assigned to the H-bonds between α122His and β35Tyr, and between α103His and β131Gln, respectively [20,32,33]. The resonance at 12.9 ppm is shifted upfield to 12.8 ppm in the spectra of rHbCO (αT67V) and rHbCO (αT67V, βS72A). The resonances at 10.2, 10.4 and 10.7 ppm have been assigned to the $^1H\varepsilon_1$ of β15W, α14W and β37W in the spectrum of HbCO A, respectively [17,20]. The chemical shift of β37W does not change in the spectra of these three rHbs in the CO form. In the spectra of rHbCO (αT67V) and rHbCO (αT67V, βS72A), the resonance at 10.4 ppm is missing and an additional resonance appears at 9.5 ppm as compared to the spectrum of HbCO A. In the spectra of rHbCO (βS72A) and rHbCO (αT67V, βS72A), the resonance at 10.2 ppm is missing.

To assign the additional proton resonances at 9.5 ppm in the ^{1}H-NMR spectra of rHb (αT67V) and rHb (αT67V, βS72A), heteronuclear two-dimensional (2-D) NMR studies of ^{15}N-labeled rHb (αT67V) and rHb (αT67V, βS72A) in the CO form have been performed (Fig. 4). The NH cross-peak at 10.4/129.5 ppm is missing in the 600-MHz HSQC spectra of ^{15}N-labeled rHb (αT67V) and rHb (αT67V, βS72A) in the CO form (Fig. 4), suggesting that α67Thr is H-bonded to α14Trp. However, no NH cross-peak in the proton dimension at 9.5 ppm has been observed in the HSQC spectra of ^{15}N-labeled rHb (αT67V) and rHb (αT67V, βS72A), suggesting that the extra resonance at 9.5 ppm observed in the 1D-NMR spectra of these two rHbs does not originate from α14Trp. The NH cross-peak at 10.2/127.7 ppm (assigned to β15Trp in HbCO A) is also missing in the HSQC spectrum of ^{15}N-labeled rHbCO (αT67V, βS72A) and a new NH cross-peak appears at 9.7/125.9 ppm. This suggests that the resonance of β15Trp shifts away from 10.2 to 9.7 ppm due to the loss of its H-bond partner β72Ser. To provide additional evidence for the β15Trp assignment at 9.7 ppm, HMQC spectra of ^{15}N-labeled rHbCO (αT67V, βS72A) have been acquired with a refocusing delay of 22 ms, which diminishes the intensity of the directly bonded ^{1}H-^{15}N cross-peaks, while retaining the cross-peak due to two- and three-bond correlation. As shown in Fig. 5a, we have observed the $^1H\delta_1$ cross-peak

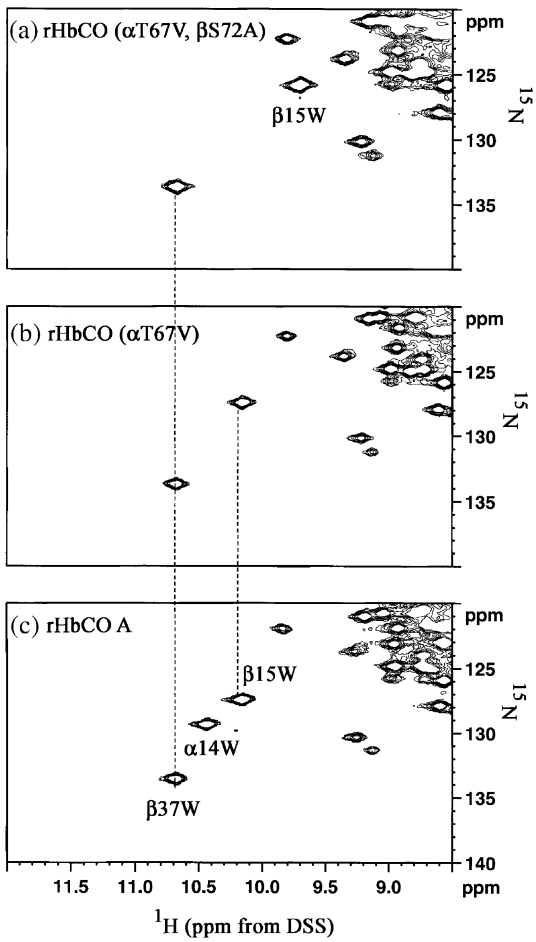

Fig. 4. The 600-MHz 2D-HSQC spectra of 6–8% solutions of ^{15}N-labeled rHb (αT67V, βS72A) (a), rHb (αT67V) (b), and rHb A (c) in the CO form in 90% H_2O/10% D_2O in 0.1 M sodium phosphate buffer at pH 7.0 and 29 °C.

at 7.1/125.9 ppm in the ^{15}N-labeled rHb (αT67V, βS72A), confirming the assignment of the cross-peak at 9.7/125.9 ppm to β15Trp. The present observation assigns the resonances at 10.4 and 10.2 ppm to the inter-helical H-bonds between α14W and α67T, and between β15W and β72S, respectively, in the CO form of Hb A.

Fig. 3b shows the ring-current-shifted proton resonances of Hb A, rHb (αT67V), rHb (βS72A), and rHb (αT67V, βS72A) in the CO form measured at 500 MHz. The ring-current-shifted proton resonances are sensitive to the orientation and/or

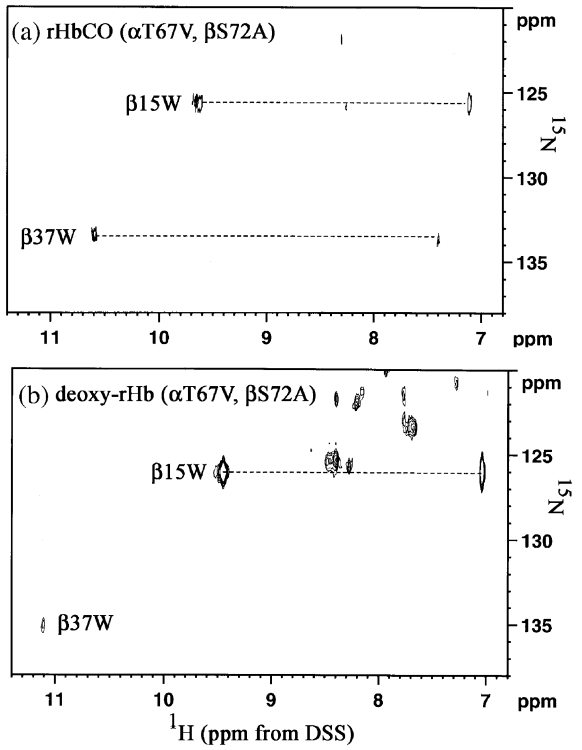

Fig. 5. The 600-MHz 2D-HMQC spectra of 6–8% solutions of ^{15}N-labeled rHb (αT67V, βS72A) in the CO (a) and deoxy (b) form in 90% H_2O/10% D_2O in 0.1 M sodium phosphate buffer at pH 7.0 and 29 °C.

conformation of the heme group relative to the amino acid residues in the heme pockets, i.e. the tertiary structure of the Hb molecule [11]. The resonances at approximately −1.7 and −1.8 ppm have been assigned to the γ_2-CH_3 of the E11Val of the α-chain and β-chain of HbCO A, respectively [34,35]. The resonance assigned to the γ_2-CH_3 of the αE11Val of rHbCO (αT67V) is shifted upfield to −1.9 ppm, suggesting that the γ_2-CH_3 group of the αE11 valine residues in rHbCO (αT67V) is located closer to the normal of the heme than in HbCO A. The resonances assigned to the γ_2-CH_3 of the αE11Val and βE11Val of rHbCO (βS72A) are shifted upfield to approximately −1.75 and −1.95 ppm, respectively. The resonances assigned to the γ_2-CH_3 of the αE11Val and βE11Val of rHbCO (αT67V, βS72A) are shifted upfield to approximately −1.8 and −1.95 ppm, respectively. This result suggests that the γ_2-CH_3 group of the α- and βE11 valine residues in both rHbCO (βS72A) and rHbCO (αT67V, βS72A) is located closer to the normal of the heme than in HbCO A. Since α67T (E16) and β72S (E16) are in proximity to E11Val, the amino acid substitutions, αT67V and βS72A, are expected to alter the conformation of the distal heme pocket site of the α- and β-chains, respectively. There are some other changes in the ring-current-shifted resonances among these rHbs. Our experience has been that minor differences in the intensity and positions of ring-current-shifted resonances are common features in many rHb mutants that we have studied [21,23,26–30,36]. 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 mutations.

Fig. 6a shows the hyperfine-shifted $N_\delta H$ resonances of proximal histidines in the 300-MHz spectra of rHb (αT67V), rHb (βS72A), rHb (αT67V, βS72A), and Hb A in the deoxy form. The spectra for the hyperfine-shifted $N_\delta H$ resonances of proximal histidines of the mutant rHbs in the deoxy form are very similar to that of Hb A. Fig. 6b shows the hyperfine-shifted and exchangeable proton resonances in the 300-MHz NMR spectra of Hb A and rHbs in the deoxy form. The hyperfine-shifted proton resonances arise from the protons on the heme groups and on nearby amino acid residues due to the hyperfine interactions between these protons and unpaired electrons of Fe(II) in the heme pocket [11]. There are no noticeable differences in the resonances from 10 to 25 ppm between deoxy-Hb A and deoxy-rHbs, except that the resonance at 17 ppm is shifted upfield to 16.7 ppm in the spectra of deoxy-rHb (αT67V) and deoxy-rHb (αT67V, βS72A). The hyperfine-shifted resonance at 17.0 ppm has been assigned to the hyperfine interaction originating from the α-chain [11]. The results suggest that the mutation of Val in the E16 (α67T) position of the α-chain changes the environment of the heme pocket of the α-chain.

Fig. 6c shows the exchangeable proton resonances in the 500-MHz spectra of rHbs and Hb A in the deoxy form. The resonance at 13.0 ppm

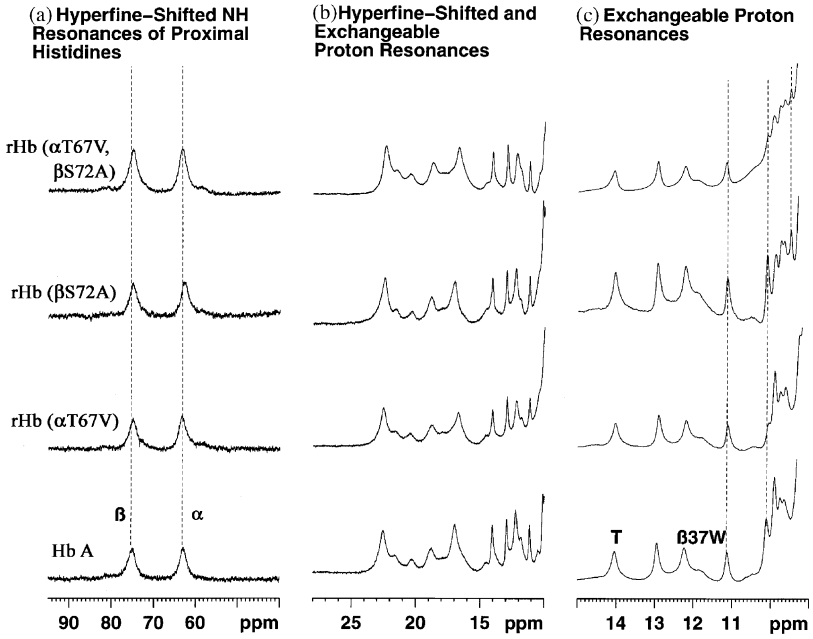

Fig. 6. The ^{1}H-NMR spectra of 4–6% solutions of Hb A, rHb (αT67V), rHb (βS72A) and rHb (αT67V, βS72A) in the deoxy form in 0.1 M sodium phosphate buffer at pH 7.0 and 29 °C: (a) hyperfine-shifted $N_\delta H$ resonances of proximal histidines at 300 MHz; (b) hyperfine-shifted and exchangeable proton resonances at 300 MHz; and (c) exchangeable proton resonances at 500 MHz.

(assigned to α122His) is shifted slightly upfield to 12.9 ppm in the spectra of rHb (αT67V) and rHb (αT67V, βS72A) in the deoxy form. The shift of the resonance assigned to α122His in the spectra of rHb (αT67V) and rHb (αT67V, βS72A) is the same in the CO form as in the deoxy form (compare Fig. 3a with Fig. 6c). The ^{1}H resonance at ~14 ppm has been identified as the inter-subunit H-bond between α42Tyr and β99Asp in the $\alpha_1\beta_2$ interface in deoxy-Hb A [13], a characteristic feature of the deoxy (T)-quaternary structure of Hb A [1]. Another characteristic feature of the T structure is the appearance of the resonance at 11.2 ppm downfield from DSS, which has been assigned to the H-bond between β37Trp and α94Asp in the $\alpha_1\beta_2$ interface [13,20,37]. The chemical shifts of these two resonances are not changed in the spectra of the rHbs in the deoxy form. The $^1H\varepsilon_1$ for β15W and α14W are observed at 9.9 and 10.1 ppm, respectively, in the deoxy form of Hb A [38]. The resonance at 10.1 ppm in the spectra of rHb (αT67V) and rHb (αT67V, βS72A) in the deoxy form is shifted slightly upfield to 10.0 ppm. The chemical shift of the resonance at 9.9 ppm in the spectra of rHb (βS72A) and rHb (αT67V, βS72A) is similar to that of Hb A in the deoxy form, except that the intensity of this resonance appears to be much smaller. An extra resonance is observed at 9.5 ppm in the spectra of rHb (βS72A) and rHb (αT67V, βS72A) in the deoxy form.

Fig. 7 shows HSQC spectra of ^{15}N-labeled rHb (αT67V), rHb (αT67V, βS72A), and rHb A in the deoxy form. The NH cross-peak at 10.1/129.4 ppm is missing in the 600-MHz HSQC spectra of ^{15}N-labeled rHb (αT67V) and rHb (αT67V, βS72A) in the deoxy form, suggesting that the resonance of α14Trp shifts away from 10.1 ppm due to the loss of the H-bond partner, α67Thr. The NH cross-peak at 9.9/127.4 ppm (assigned to β15Trp in deoxy-Hb A) is also missing in the HSQC spectrum of ^{15}N-labeled deoxy-rHb (αT67V, βS72A) and a new NH cross-peak appears at 9.5/126.2 ppm. This suggests that the resonance of β15Trp shifts away from 9.9 ppm to 9.5 due to the loss of the H-bond partner β72Ser.

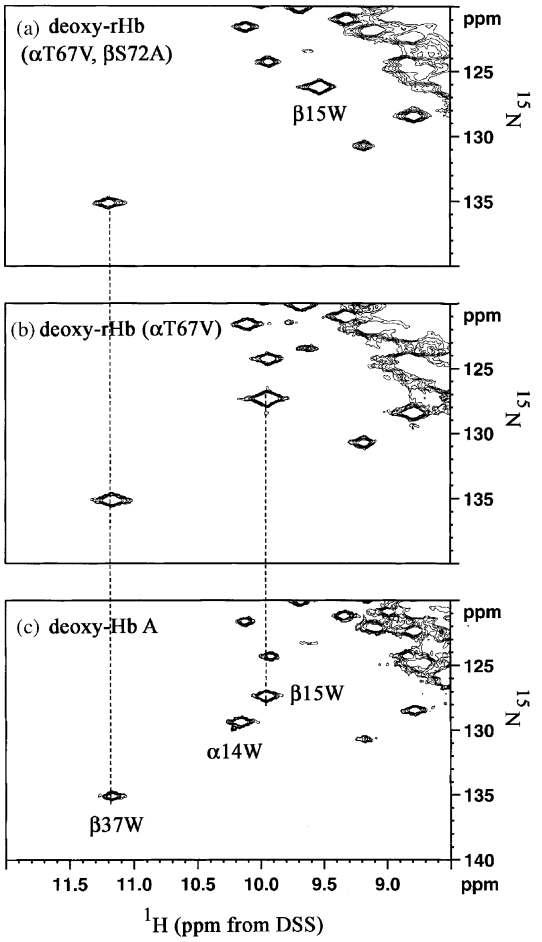

Fig. 7. The 600-MHz 2D HSQC spectrum of 6–8% solutions of ^{15}N-labeled rHb (αT67V, βS72A) (a), rHb (αT67V) (b), and rHb A (c) in the deoxy form in 90% H_2O/10% D_2O in 0.1 M sodium phosphate buffer at pH 7.0 and 29 °C.

In addition, we have observed the $^1H\delta_1$ cross-peak at 7.0/126.2 ppm in the HMQC spectrum of ^{15}N-labeled rHb (αT67V, βS72A), confirming the assignment of the resonance at 9.5/126.2 ppm to β15Trp (Fig. 5b). The present result assigns the resonances at 10.1 and 9.9 ppm to the inter-helical H-bonds between α14W and α67T, and between β15W and β72S, respectively, in the deoxy forms.

4. Discussion

We have chosen the αT67V and βS72A mutations to test the role of the inter-helical H-bonds that are donated by the indole-ring NH protons of the A helix residues, α14W and β15W, to the hydroxy groups of the E helix residues, α67T and β72S, respectively. These H-bonds are evident in crystal structures of both the CO and deoxy forms of Hb A [2,3,39]. The valine for threonine and alanine for serine substitutions are sterically conservative, and abolish the hydroxy H-bond acceptors. The mutant proteins, rHb (αT67V) and rHb (βS72A), are shown to fold properly, and to maintain the native-like T-state structures in the deoxy form, as assessed by the visible and ultraviolet resonance Raman probes [10]. The present NMR data confirm that the T-state inter-subunit contacts are unaffected by the mutations, i.e. the exchangeable ^{1}H resonances arising from the α42Tyr-β99Asp and β37Trp-α94Asp H-bonds are at the characteristic Hb A positions in rHb (αT67V) and rHb (βS72A), and also in the rHb (αT67V, βS72A) double mutant. Other NMR resonances show that the mutation-induced perturbations of the protein structure, in either the R or T states, are limited to minor adjustments of the E helix residues in the heme pocket.

Thus, the effect of the mutations is limited to disruption of the A-E inter-helical H-bonds, as intended. This disruption can be detected in both UVRR and NMR spectra. H-bond donation by indole augments the UVRR intensities of indole vibrational bands because of the red shift in the indole electronic transitions. This augmentation is readily apparent in the difference UVRR spectra between Hb A and the mutants [10]. The augmentation associated with the H-bonding is the same in the deoxy and CO forms, implying that the H-bond strengths are the same in the R and T states. However, the augmentation is greater when the α67 threonine is substituted than when the β72 serine is substituted. The implication that the A–E H-bond is stronger in the α- than the β-chains is supported by the observation of a longer distance between the donor and acceptor atoms in the β-chains [10].

The present results show that the NH proton resonance of the β15W indole ring is shifted upon the loss of the β72S H-bond, from 10.2 to 9.7 ppm in the CO form and from 9.9 to 9.5 ppm in the deoxy form. These assignments have been

confirmed via the HSQC spectra of ^{15}N-labeled protein. The similarity in the chemical shift difference again supports the view that the H-bond strengths are essentially the same in the R and T states. In Hb A, the α14W indole NH resonance is found at 10.4 and 10.1 ppm in the CO and deoxy forms, respectively. The 0.2 ppm upfield shifts, relative to the β15W resonances, are consistent with stronger H-bonds in the α- than in the β-chains, as has been inferred from the UVRR intensities. However, the α14W resonances could not be located in the α-chain mutants. No cross-peaks appear in the ^{15}N HSQC spectra, implying that the indole NH proton is in rapid exchange with solvent. The exchange rate must be much faster than that for J_{NH} coupling of Trp at 600 MHz, which is estimated to be 90 s^{-1}. Attempts to slow the rate down by lowering the temperature or altering the pH were unsuccessful; no cross-peaks have been observed at 11 °C or at pH 5.7 or 8.4. The rapid exchange is attributable to the loss of the H-bond, which allows the indole NH to interact with the solvent. That this does not happen for the β15W residue is attributable to lower solvent exposure. The β15W indole ring is surrounded by the contacting residues: 130Y, 75L, 72S and 68L, supplemented in the T state by 71F and 18V [40]. In contrast, the α14W indole contacts only 66L and 63A, supplemented by 70V in the T state [40]. Thus, α14W forms stronger H-bonds than β15W, but is also more accessible to the solvent when the H-bond is disrupted.

There is no naturally occurring mutant reported at the α67 site, while the only naturally occurring mutant at β72, Hb Headington (βS72R), is reported to exhibit high oxygen affinity and reduced cooperativity [41]. As for mutations at the α14W and β15W sites, Hb Evanston (βW14R) and Hb Belfast (βW15R) are reported to be high-oxygen-affinity variants [41]. The only other naturally occurring mutant at the β15 site, Hb Randwick (βW15G) [41], as well as our mutants, rHb (αT67V), rHb (βS72A), and rHb (αT67V, βS72A), exhibit functional properties similar to those of Hb A. As compared to Arg, the side chains of Val, Ala and Gly can easily fit into the 'Trp pocket' formed between the A and E helices in both the CO and deoxy forms of Hb A and hence, preserve the hydrophobicity around the Trp pocket. None of the side chains of these mutants, i.e. Arg, Gly, Val or Ala, are able to preserve the H-bonds between α14W and α67T and between β15W and β72S. These results suggest that the van der Waals contacts around the 'Trp pocket' may be critical in stabilizing both the T and R quaternary structure of the Hb molecule in addition to the H-bonds between α14W and α67T and between β15W and β72S. A similar case was observed in rHb (βY145F) in which a 'Tyr pocket' stabilized by van der Waals interaction has been proposed to stabilize the T quaternary structure of deoxy-Hb in addition to the H-bond to β98Val [37].

The oxygen-binding data indicate that the αT67V and βS72V mutations have no significant effect on the free energies of binding. Within experimental error, neither the P_{50} values nor the Hill coefficients of these three mutants differ from those of Hb A, in the absence or the presence of allosteric effectors, 2,3-BPG or chloride. This finding may seem paradoxical, since the free energy changes should be affected by the addition or subtraction of H-bonds. The key to the apparent paradox is that the H-bond strength is essentially the same in the R and the T states, as gauged by both the UVRR intensities and the NMR chemical shifts. Thus, the free energies of the initial and final states are affected equally by the inter-helical H-bonds, resulting in no change in the free energy difference associated with ligation. On the other hand, the highly cooperative oxygen binding to rHb (αT67V, βS72A) could also indicate that this rHb is fully able to undergo the T-to-R transition upon oxygenation even after losing the four H-bonds. NMR relaxation of the amide proton of β37W has been shown to be sensitive to the R-to-T conformational exchange, which can be induced by adding IHP to HbCO A or by a single site mutation, rHb (αV96W), a low-oxygen-affinity mutant. The R-to-T conformational exchange in the micro- to millisecond time-scale motions can be monitored by the transverse relaxation rate constant in the laboratory frame, R_2, at different τ_{cp} values in the TROSY-CPMG experiment, in which τ_{cp} is the delay between 180° pulses in the CPMG pulse train [42]. Strong dependence of

$R_2(1/\tau_{cp})$ on τ_{cp} has been observed for β37W in rHbCO (αT67V, βS72A) in the presence of IHP, similar to that observed in rHbCO A (Tsai et al., unpublished results). These results suggest that after losing the four inter-helical H-bonds, the amide proton of β37W can still undergo the R-to-T conformational exchange in the $\alpha_1\beta_2$ subunit interface when IHP is added. These results indicate that there are multiple interactions supporting the R-to-T transition in the cooperative oxygenation of Hb.

Nevertheless, these inter-helical H-bonds are involved in the allosteric pathway between the initial and final states. Time-resolved UVRR spectra reveal a loss of tryptophan intensity upon forming the first protein intermediate following ligand photodissociation (R_{deoxy}, $\tau \sim 50$ ns), followed by the recovery of the intensity upon forming the second intermediate (S, $\tau \sim 1$ ms) [7]. 150-ns transient UVRR spectra of the αT67V and βS72V mutants show that the R_{deoxy} intensity loss is associated with the α14W and β15W H-bonds [10]. The interpretation of this finding is that the α14W and β15W H-bonds are broken transiently via the displacement of the distal E helix toward the heme upon the departure of the ligand from the heme pocket (R_{deoxy} intermediate); the H-bonds are then re-formed (S intermediate) by a subsequent motion of the A helix, which positions the N-terminus for salt-bridge formation in the final T state. Thus, the H-bonds guide the helices along the $R–T$ reaction co-ordinate without affecting the overall free energy change.

The next question that needs to be addressed is how the loss of the inter-helical H-bonds affects the allosteric pathway. This issue is currently under investigation with time-resolved spectroscopic studies of the mutant proteins.

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References

[1] M.F. Perutz, Stereochemistry of cooperative effects in haemoglobin, Nature 228 (1970) 726–739.
[2] G. Fermi, M.F. Perutz, B. Shaanan, R. Fourme, The crystal structure of human deoxyhaemoglobin at 1.74 Å resolution, J. Mol. Biol. 175 (1984) 159–174.
[3] J.M. Baldwin, The structure of human carbonmonoxy haemoglobin at 2.7 Å resolution, J. Mol. Biol. 136 (1980) 103–128.
[4] B. Shaanan, Structure of human oxyhaemoglobin at 2.1 Å resolution, J. Mol. Biol. 171 (1983) 31–59.
[5] C. Ho, J.A. Lukin, Haemoglobin: cooperativity in protein–ligand interaction, Encyclopedia of Life Sciences, Macmillan, London, 2000, (http://www.els.net).
[6] K.R. Rodgers, T.G. Spiro, Nanosecond dynamics of the R→T transition in hemoglobin: ultraviolet Raman studies, Science 265 (1994) 1697–1699.
[7] V. Jayaraman, K.R. Rodgers, I. Mukerji, T.G. Spiro, Hemoglobin allostery: resonance Raman spectroscopy of kinetic intermediates, Science 269 (1995) 1843–1848.
[8] X. Hu, K.R. Rodgers, I. Mukerji, T.G. Spiro, New light on allostery: dynamic resonance Raman spectroscopy of hemoglobin Kempsey, Biochemistry 38 (1999) 3462–3467.
[9] I.J. Juszczak, J.M. Friedman, UV resonance Raman Spectra of ligand binding intermediates of sol–gel encapsulated hemoglobin, J. Biol. Chem. 274 (1999) 30357–30360.
[10] D. Wang, X. Zhao, T.-J. Shen, C. Ho, T.G. Spiro, Role of interhelical H-Bonds [Wα14-Tα67 and Wβ15-Sβ72) in the hemoglobin allosteric reaction path evaluated by UV resonance Raman spectroscopy of site-mutants, J. Am. Chem. Soc. 121 (1999) 11197–11203.
[11] C. Ho, Proton nuclear magnetic resonance studies on hemoglobin: cooperative interactions and partially ligated intermediates, Adv. Protein Chem. 42 (1992) 153–312.
[12] M. Llinas, M.P. Klein, J.B. Neilands, Solution conformation of the ferrichromes. A comparative proton magnetic resonance study of glycine- and serine-containing ferrichromes, J. Mol. Biol. 68 (1972) 265–284.
[13] L.W.-M. Fung, C. Ho, A proton nuclear magnetic resonance study of the quaternary structure of human homoglobins in water, Biochemistry 14 (1975) 2526–2535.
[14] G. Viggiano, C. Ho, Proton nuclear magnetic resonance investigation of structural changes associated with cooperative oxygenation of human adult hemoglobin, Proc. Natl. Acad. Sci. USA 76 (1979) 3673–3677.

[15] G. Wagner, A. Pardi, K. Wüthrich, Hydrogen-bond length and H-1-NMR chemical-shifts in proteins, J. Am. Chem. Soc. 105 (1983) 5948–5949.
[16] A.G. Redfield, M.Z. Papastratos, NMR study of the phosphoryl binding loop in purine nucleotide proteins: evidence for strong hydrogen bonding in human N-ras p21, Biochemistry 29 (1990) 3509–3514.
[17] T.-Y. Fang, V. Simplaceanu, C.-H. Tsai, N.T. Ho, C. Ho, An additional H-bond in the alpha $\alpha_1\beta_2$ interface as the structural basis for the low oxygen affinity and high cooperativity of a novel recombinant hemoglobin (βL105W), Biochemistry 39 (2000) 13708–13718.
[18] J.A. Lukin, V. Simplaceanu, M. Zou, N.T. Ho, C. Ho, NMR reveals hydrogen bonds between oxygen and distal histidines in oxyhemoglobin, Proc. Natl. Acad. Sci. USA 97 (2000) 10354–10358.
[19] J.M. Nocek, K. Huang, B.M. Hoffman, Extension of transverse relaxation-optimized spectroscopy techniques to allosteric proteins: CO- and paramagnetic fluoromet-hemoglobin [beta (15N-valine)], Proc. Natl. Acad. Sci. USA 97 (2000) 2538–2543.
[20] V. Simplaceanu, J.A. Lukin, T.-Y. Fang, M. Zou, N.T. Ho, C. Ho, Chain-selective isotopic labeling for NMR studies of large multimeric proteins: application to hemoglobin, Biophys. J. 79 (2000) 1146–1154.
[21] T.-J. Shen, N.T. Ho, V. Simplaceanu, et al., Production of unmodified human adult hemoglobin in *Escherichia coli*, Proc. Natl. Acad. Sci. USA 90 (1993) 8108–8112.
[22] T.-J. Shen, N.T. Ho, M. Zou, et al., Production of human normal adult and fetal hemoglobins in *Escherichia coli*, Protein Eng. 10 (1997) 1085–1097.
[23] C.-H. Tsai, T.-J. Shen, N.T. Ho, C. Ho, 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, Biochemistry 38 (1999) 8751–8761.
[24] A. Hayashi, T. Suzuki, M. Shin, Enzymic reduction system for metmyoglobin and methemoglobin, and its application to functional studies of oxygen carriers, Biochim. Biophys. Acta 310 (1973) 309–316.
[25] P. Plateau, M. Guéron, Exchangeable proton NMR without base-line distortion, using new strong-pulse sequences, J. Am. Chem. Soc. 104 (1982) 7310–7311.
[26] H.-W. Kim, T.-J. Shen, D.P. Sun, et al., Restoring allosterism with compensatory mutations in hemoglobin, Proc. Natl. Acad. Sci. USA 91 (1994) 11547–11551.
[27] H.-W. Kim, T.-J. Shen, D.P. Sun, N.T. Ho, M. Madrid, C. Ho, A novel low oxygen affinity recombinant hemoglobin (α96val→Trp): switching quaternary structure without changing the ligation state, J. Mol. Biol. 248 (1995) 867–882.
[28] H.-W. Kim, T.-J. Shen, N.T. Ho, M. Zou, M.F. Tam, C. Ho, Contributions of asparagine at α97 to the cooperative oxygenation process of hemoglobin, Biochemistry 35 (1996) 6620–6627.
[29] C. Ho, B.F. Willis, T.-J. Shen, et al., Roles of α114 and β87 amino acid residues in the polymerization of hemoglobin S: implications for gene therapy, J. Mol. Biol. 263 (1996) 475–485.
[30] C. Ho, D.P. Sun, T.-J. Shen, et al., Recombinant hemoglobins with low oxygen affinity and high cooperativity, in: E. Tsuchida (Ed.), Present and Future Perspectives of Blood Substitutes, Elsevier Science SA, Lausanne, Switzerland, 1998, pp. 281–296.
[31] D. Barrick, N.T. Ho, V. Simplaceanu, F.W. Dahlquist, C. Ho, A test of the role of the proximal histidines in the Perutz model for cooperativity in haemoglobin, Nat. Struct. Biol. 4 (1997) 78–83.
[32] I.M. Russu, N.T. Ho, C. Ho, A proton nuclear overhauser effect investigation of the subunit interfaces in human normal adult hemoglobin, Biochim. Biophys. Acta 914 (1987) 40–48.
[33] C.-K. Chang, V. Simplaceanu, C. Ho, Effects of amino acid substitutions at β131 on the structure and properties of hemoglobin: evidence for the communication between $\alpha_1\beta_1$ and $\alpha_1\beta_2$ subunit interfaces, Biochemistry 41 (2002) 5644–5655.
[34] T.R. Lindstrom, I.B.E. Norén, S. Charache, H. Lehmann, C. Ho, Nuclear magnetic resonance studies of hemoglobins. VII. Tertiary structure around ligand binding site in carbonmonoxyhemoglobin, Biochemistry 11 (1972) 1677–1681.
[35] C. Dalvit, C. Ho, Proton nuclear overhauser effect investigation of the heme pockets in ligated hemoglobin: conformational differences between oxy and carbonmonoxy forms, Biochemistry 24 (1985) 3398–3407.
[36] D.P. Sun, M. Zou, N.T. Ho, C. Ho, Contribution of surface histidyl residues in the alpha-chain to the Bohr effect of human normal adult hemoglobin: roles of global electrostatic effects, Biochemistry 36 (1997) 6663–6673.
[37] K. Ishimori, K. Imai, G. Miyazaki, et al., Site-directed mutagenesis in hemoglobin: functional and structural role of inter- and intrasubunit hydrogen bonds as studied with 37 beta and 145 beta mutations, Biochemistry 31 (1992) 3256–3264.
[38] Y. Yuan, V. Simplaceanu, J.A. Lukin, C. Ho, NMR investigation of dynamics of tryptophan side-chains in hemoglobins., J. Mol. Biol. 321 (2002) 863–878.
[39] M.M. Silva, P.H. Rogers, A. Arnone, A third quaternary structure of human hemoglobin A at 1.7-Å resolution, J. Biol. Chem. 267 (1992) 17248–17256.
[40] R.E. Dickerson, I. Geis, Hemoglobin: Structure, Function, Evolution, and Pathology, Benjamin/Cummings, Menlo Park, 1983.
[41] T.H.J. Huisman, M.F.H. Carver, G.D. Efemov, A Syllabus of Human Hemoglobin Variants, 2nd ed, The Sickle Cell Anemia Foundation, Augusta, GA, USA, 1998.
[42] J.P. Loria, M. Rance, A.G. Palmer, A TROSY CPMG sequence for characterizing chemical exchange in large proteins, J. Biomol. NMR 15 (1999) 151–155.