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Mutant Recombinant Hemoglobin (α96Val→Tyr) Exhibits Low Oxygen Affinity and High Cooperativity

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To investigate conformational information of a low oxygen affinity recombinant hemoglobin (rHb) containing 96Val→Trp mutation at the α96 position, we have produced rHb (α96Val→Phe) and rHb (α96Val→Tyr), using the *Escherichia coli* expression system and site-directed mutagenesis. The oxygen affinity of rHb (α96Val→Phe) is similar to that of human normal adult hemoglobin (Hb A). However, the oxygen affinity of rHb (α96Val→Tyr) showed much lower oxygen affinity than Hb A which is similar to that of rHb (α96Val→Trp), providing an opportunity as a potential candidate for a hemoglobin-based blood substitute. Both rHb (α96Val→Phe) and rHb (α96Val→Tyr) showed high cooperativity in oxygen binding. ^{1}H-NMR spectroscopy shows that both rHb (α96Val→Phe) and rHb (α96Val→Tyr) have very similar tertiary structure around the heme pockets and quaternary structure in the $\alpha_1\beta_2$ subunit interface compared to Hb A. The low oxygen affinity of rHb (α96Val→Tyr) has been suggested to be due to a hydrogen bond caused by an extra hydroxyl group not present in rHb (α96Val→Phe). However, investigation of the carbonmonoxy form of rHb (α96Val→Phe) and (α96Val→Tyr) in the presence of inositol hexaphosphate at low temperature suggests that low oxygen affinity of (α96Val→Tyr) may arise from a mechanism different to that of rHb (α96Val→Trp).

Keywords: ^{1}H-NMR, Low oxygen affinity hemoglobin, Quaternary structure, Recombinant hemoglobin.

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Introduction

Recently, a low oxygen affinity rHb (α96Val→Trp) has been produced using an *Escherichia coli* (*E. coli*) expression plasmid in which synthetic human α- and β-globin genes are coexpressed with the *E. coli* methionine aminopeptidase gene (Kim *et al.*, 1995). This artificial hemoglobin shows a low oxygen affinity, but high cooperativity in oxygen binding, and exhibits no unusual subunit dissociation when ligated. These functional properties provide an opportunity as a potential candidate for hemoglobin-based blood substitute.

Despite the replacement of a small amino acid residue, valine by a large tryptophan residue, this artificial hemoglobin shows very similar tertiary structure around the heme pockets and quaternary structure in the $\alpha_1\beta_2$ subunit interface compared to those of human normal adult hemoglobin (Hb A). Another unique feature of this hemoglobin is that the ligated form, e.g., carbonmonoxy (CO) form, of this hemoglobin in the oxy-quaternary structure can be converted to the deoxy-like quaternary structure by the addition of the allosteric effector, inositol hexaphosphate (IHP), without changing its ligation state, suggesting a very stable deoxy-quarternary structure. Preliminary molecular dynamics (MD) simulation using stochastic boundary methods suggested that the unique oxygen binding properties of rHb (α96Val→Trp) may be due to an extra hydrogen bond between α96Trp and β99Asp in the $\alpha_1\beta_2$ subunit interface of the deoxy form (Kim *et al.*, 1995).

To investigate the conformational information of rHb (α96Val→Trp) at the α96 position, we have produced rHb (α96Val→Phe) and rHb (α96Val→Tyr), using the *E. coli* expression system and site-directed mutagenesis. We have determined its oxygen binding properties and used ^{1}H-NMR spectroscopy to investigate the structures of rHb

(α96Val→Phe) and rHb (α96Val→Tyr), and compared these with those of rHb (α96Val→Trp) and Hb A.

Materials and Methods

Production of mutant rHb The Hb A expression plasmid PHE2 containing synthetic α- and β-globin genes and the *E. coli* methionine aminopeptidase gene was used to produce mutant Hbs. Phagemid pTZ18U and *E. coli* JM109 were purchased from Bio-Rad and Promega, respectively. Synthetic human α- and β-globin genes were inserted into phagemid pTZ18U. Site-directed mutagenesis was performed as previously described (Shen *et al.*, 1993).

Growth and purification of rHb Resulting plasmid was transformed into *E. coli* JM109, and the cells were grown in TB medium in a 10-L Microferm fermentor (New Brunswick Scientific, model BioFlo 3000) at 30°C until the optical density at 600 nm reached 10. Expression of rHb (α96Val→Phe) and rHb (α96Val→Phe) were induced by adding isopropyl β-thiogalactopyranoside to 0.2 mM. The culture was then supplemented with hemin (50 mg/l), and the growth was continued for at least another 4 h. The cells were harvested by centrifugation and stored at −80°C until needed for purification. The rHb (α96Val→Phe) and rHb (α96Val→Phe) were purified as previously described (Kim *et al.*, 1996; Yeh *et al.*, 1998). We used two columns in the final purification process: (i) A Q-Sepharose fast-flow column (Pharmacia anion exchanger) was used to bind Hb. After the sample was loaded onto the column, it was washed throughly with the running buffer (20 mM Tris-HCl/0.1 mM EDTA at pH 8.3). Then, the Hb fraction was oxidized and reduced as described in Shen *et al.* (1993). (ii) A Mono S column (Pharmacia cation exchanger HR16/10) with a gradient of 10 mM sodium phosphate/0.1 mM EDTA (pH 6.8) to 20 mM sodium phosphate/0.1 mM EDTA (pH 8.3) was used to purify r Hbs.

Oxygen binding of Hb sample For the rHb (α96Val→Phe), rHb (α96Val→Phe), and Hb A (0.1 mM of each), oxygen-dissociation curves were measured by a Hemox-analyzer (TCS Medical Products, Huntington Valley, USA) at 29°C in 0.1 M sodium phosphate buffer of pH 7.0. Partial O_2 pressure at 50% saturation (P_{50}) and the Hill coefficient (n_{max}) were determined from each curve.

NMR measurements ^{1}H-NMR spectra were obtained on a Bruker AM-300 spectrometer operating at 300 MHz and 29°C. All Hb samples were in 0.1 M sodium phosphate buffer at pH 7.0. The Hb concentration was about 4%. The water signal was suppressed by a jump-and-return pulse sequence (Plateau and Guéron, 1982). Typically, 1024 scans were averaged to improve the signal-to-noise ratio. Proton chemical shifts are referenced to the methyl proton resonance of the sodium salt 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.

Results

The purification of rHbs from *E. coli* cells generally produces several peaks on a Mono S column, in which only one of them shows correct heme conformation. However, by oxidizing the Hb to the ferric state and then reducing it back to the ferrous state, and finally converting it back to either CO or the oxy form, incorrectly inserted heme could be converted to the correct conformation (Shen *et al.*, 1993; Kim *et al.*, 1995). In the present investigation, both rHb (α96Val→Phe) and rHb (α96Val→Tyr) were oxidized and reduced to the CO form before applying to the Mono S column. However, only fractions containing less than 10% methionine contents were used for both rHb (α96Val→Phe) and rHb (α96Val→Tyr).

The O_2 binding properties of rHb (α96Val→Phe) and rHb (α96Val→Tyr), rHb (α96Val→Trp), and Hb A in 0.1 mM sodium phosphate at 29°C are compared in Table 1. The oxygen affinity of rHb (α96Val→Phe) is similar to that of Hb A. However, the oxygen affinity of rHb (α96Val→Tyr) showed a much lower oxygen affinity than Hb A, which is similar to that of rHb (α96Val→Trp). Both rHb (α96Val→Phe) and rHb (α96Val→Tyr) showed high cooperativity in oxygen binding.

^{1}H-NMR spectroscopy has been shown to be an excellent tool to investigate the tertiary and quaternary structural features of Hb (Ho, 1992). Very low-field resonances of deoxy Hb A and deoxy rHbs, in which the valine α96 position is replaced with an aromatic amino acid, are compared in Fig. 1. The resonance at ~63 ppm from DSS has been assigned to the hyperfine-shifted $N_\delta H$ exchangeable proton of the proximal histidine residue (α87His) of the α chain of the deoxy-Hb A, and the one at ~77 ppm has been assigned to the corresponding residue of the β chain (β92His) of deoxy-Hb A (La Mar *et al.*, 1980; Takahashi *et al.*, 1980). The chemical shift positions of these two proximal histidyl resonances in rHb (α96Val→Phe), rHb (α96Val→Tyr), and rHb (α96Val→Trp), are exactly the same as those of deoxy-Hb A, indicating no perturbations around the proximal histidines of rHb (α96Val→Phe) and rHb (α96Val→Tyr).

The ferrous hyperfine-shifted and exchangeable proton resonances of rHb (α96Val→Phe), rHb (α96Val→Tyr), and rHb (α96Val→Trp) in the deoxy form are shown in Fig. 2. The hyperfine-shifted resonances arise from the protons on the heme groups and their nearby amino acid residues due to the hyperfine interactions between protons and unpaired electrons of Fe(II) in the heme iron atoms (Ho, 1992). The hyperfine-shifted resonances of rHb

Table 1. P_{50} and n_{max} values of Hb A and rHbs.

	P_{50} (mmHg)	n_{max}
Hb A	8.0	3.0
rHb (α96Val→Phe)	8.9	2.8
rHb (α96Val→Tyr)	12.2	2.7
rHb (α96Val→Trp)	11.6[a]	2.6[a]

[a] Kim *et al.* (1995)

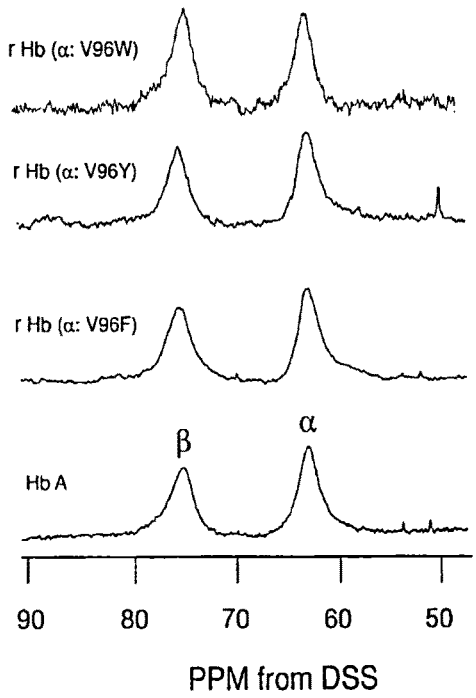

Fig. 1. The 300 MHz-hyperfine-shifted $N_\delta H$ exchangeable proton resonances of the proximal histidine residues of the deoxy form of rHb (α96Val→Phe), rHb (α96Val→Tyr), rHb (α96Val→Trp), and Hb A in 0.1 M phosphate in H_2O at pH 7.0 and 29°C.

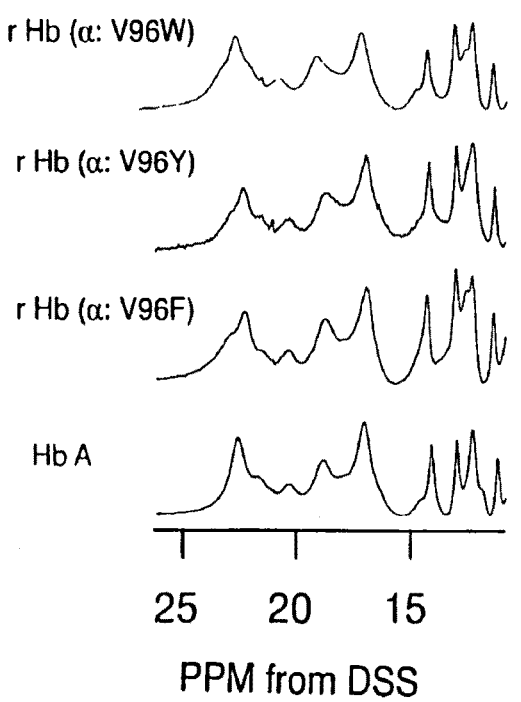

Fig. 2. The 300 MHz-hyperfine-shifted and exchangeable proton resonances of the deoxy form of rHb (α96Val→Phe), rHb (α96Val→Tyr), rHb (α96Val→Trp), and Hb A in 0.1 M phosphate in H_2O at pH 7.0 and 29°C.

(α96Val→Phe), rHb (α96Val→Tyr), and rHb (α96Val→Trp) are relatively similar to those of Hb A. The exchangeable ^1H resonances over the spectral region from ~11 to ~14 ppm are known as excellent markers for the deoxy-quaternary structure and of $\alpha_1\beta_2$ subunit interface of Hb A. The resonance at ~14 ppm has been assigned to the intersubunit hydrogen bond between α42Tyr and 99Asp (Fung and Ho, 1975), a characteristic feature of the deoxy-quaternary structure. There is no noticeable difference in the resonance at ~14 ppm between Hb A and rHbs, indicating that these $\alpha_1\beta_2$ interfaces are not perturbed by the mutations.

The effect of temperature on the exchangeable proton resonances of rHbs in the CO form in the presence of IHP are compared in Fig. 3. It was shown for rHb (α96Val→Trp) that the disappearance of an exchangeable resonance at ~10.7 ppm, a characteristic feature of the oxy-quaternary structure (the intersubunit hydrogen bond between α94Asp and β102Asn), and the temperature dependent gradual appearance of an exchangeable resonance at ~14 ppm, a characteristic feature of the deoxy-quaternary structure (the intersubunit hydrogen bond between α42Tyr and β99Asp), might be interpreted as a switch from the oxy-like quaternary structure to the deoxy-like quaternary structure (Kim *et al.*, 1995). Unlike rHb (α96Val→Trp), an exchangeable resonance at ~10.7 ppm of rHb (α96Val→Phe) and rHb (α96Val→Tyr) is clearly visible at 29°C, and even at lower temperatures. An exchangeable resonance at ~14 ppm of rHb (α96Val→Phe) gradually appeared as temperature is decreased, although it is very weak compared to that of rHb (α96Val→Trp). In contrast, rHb (α96Val→Tyr) shows a new resonance at ~13.7 ppm at low temperature in addition to a resonance at ~14 ppm. As the appearance of these exchangeable resonances does not accompany the hyperfine-shifted resonances from ~11 to ~14 ppm, it is believed that the heme is still in the low-spin, diamagnetic state. (i.e., ligand-bound form). The appearance of the new exchangeable resonance at ~13.7 ppm of rHb (α96Val→Tyr) is likely to be from the new hydrogen bond formed while converted from the oxy-like quaternary structure to the deoxy-like quaternary structure without changing its ligation state.

Discussion

The $\alpha_1\beta_2$ subunit interface of hemoglobin is very sensitive to mutation. If a substituted aromatic amino acid at the α96 position directs toward the $\alpha_1\beta_2$ subunit interface, the $\alpha_1\beta_2$ interface must be perturbed. ^1H-NMR spectroscopy revealed that the $\alpha_1\beta_2$ subunit interface of rHb (α96Val→Trp) is well preserved without much

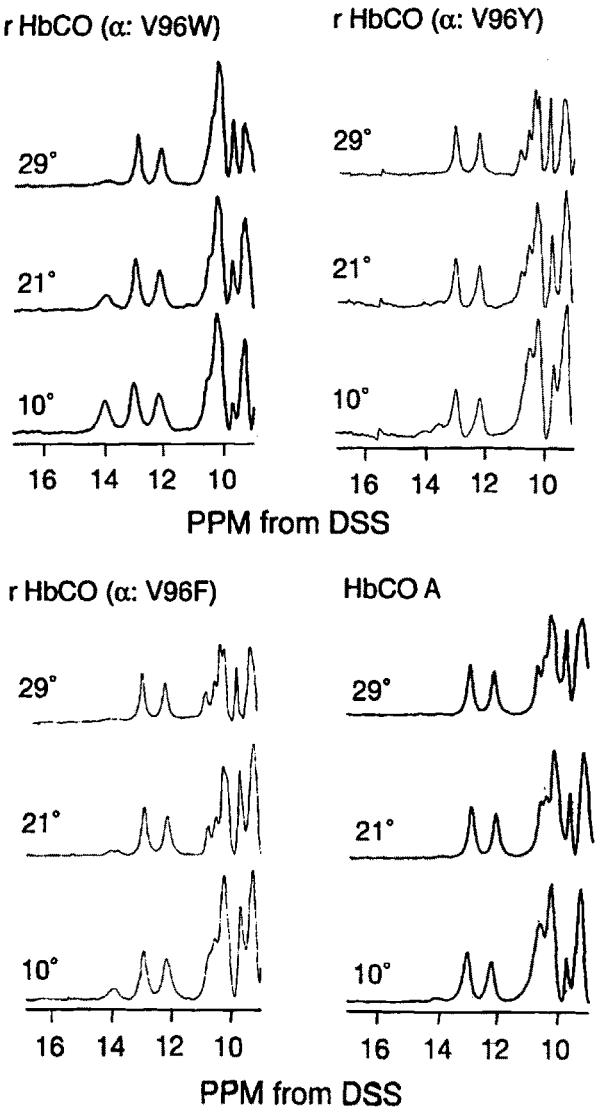

Fig. 3. Effect of temperature on the 300 MHz proton resonances of the CO form of r Hb (α96Val→Trp), r Hb (α96Val→Tyr), r Hb (α96Val→Phe), and Hb A in the presence of 10 mM IHP in 0.1 M phosphate in H_2O at pH 7.0.

perturbation. It was suggested by MD simulation that due to the possible existence of a new hydrogen bond between α96Trp and β99Asp, the $\alpha_1\beta_2$ subunit interface of rHb (α96Val→Trp) could be preserved (Kim *et al.*, 1995). ^{1}H-NMR spectroscopic studies of this investigation shows that $\alpha_1\beta_2$ subunit interfaces of rHb (α96Val→Tyr) and rHb (α96Val→Phe) are well preserved and very similar to those of Hb A. As phenylalanine at the α96 position of rHb (α96Val→Phe) cannot form a hydrogen bond, if phenylalanine directs toward this interfacial surface like rHb (α96Val→Trp), it must perturb the $\alpha_1\beta_2$ subunit interface. The absence of such perturbation for rHb (α96Val→Phe) suggests that the direction of phenylalanine is not likely to be toward the $\alpha_1\beta_2$ interfacial surface. According to the rotamer library (Ponder and Richards, 1987), the order of preference for the side chain angles of rHb (α96Val→Tyr) and rHb (α96Val→Phe) in most of the known x-ray structures is exactly same. This could mean that phenylalanine and tyrosine at the same position of protein are likely to have the same conformation, both of which may not be toward the $\alpha_1\beta_2$ subunit interface.

While the oxygen affinity of rHb (α96Val→Phe) is similar to that of Hb A, rHb (α96Val→Tyr) shows quite a low oxygen affinity which is slightly lower than that of rHb (α96Val→Trp). Both rHb (α96Val→Tyr) and rHb (α96Val→Phe) show high cooperativity, comparable to Hb A. Functional properties of rHb (α96Val→Trp) and rHb (α96Val→Tyr), i.e., low oxygen affinity and high cooperativity in oxygen binding, provide an opportunity as a potential candidate for a hemoglobin-based blood substitute. As the conformational difference between rHb (α96Val→Tyr) and rHb (α96Val→Phe) is just the presence of a hydroxyl group in rHb (α96Val→Tyr), the oxygen affinity difference between rHb (α96Val→Tyr) and rHb (α96Val→Phe) must be due to the presence of the hydrogen bond caused by the hydroxyl group of tyrosine at the α96 position. This result also supports the idea of low oxygen affinity of rHb (α96Val→Trp) due to the presence of a hydrogen bond caused by tryptophan (Kim *et al.*, 1995).

Temperature-dependent ^{1}H-NMR spectral changes of rHbCO (α96Val→Trp) have been explained as reflecting the intermediate quaternary states during the oxygenation process (Kim *et al.*, 1995). Most of the proton resonances appearing from ~9 to ~14 ppm from DSS have been assigned to the exchangeable proton resonances from interfacial hydrogen bonds (Ho, 1992). An exchangeable resonance at ~14 ppm of rHbCO (α96Val→Phe), a characteristic feature of the deoxy-quaternary structure, gradually appeared at low temperature like that of rHbCO (α96Val→Trp), although very weakly. However, for rHb (α96Val→Tyr), a new exchangeable resonance at ~13.7 ppm also appears at low temperature in addition to an exchangeable resonance at ~14 ppm. This new exchangeable resonance at ~13.7 ppm is most likely to be from the new hydrogen bond caused while converted from oxy-like quaternary structure to deoxy-like quaternary structure without changing its ligation state. The existence of extra exchangeable resonances in the intermediate structures may be the reason for the low oxygen affinity of rHb (α96Val→Tyr). However, the absence of the exchangeable resonance at ~13.7 ppm in the exchangeable resonances region of the deoxy form (Fig. 2) indicates that the hydrogen bond disappeared in the real deoxy-quaternary structure. Further studies are expected in this regard.

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