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Short communication

Characteristic of Aromatic Amino Acid Substitution at α96 of Hemoglobin

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Replacement of valine by tryptophan or tyrosine at position α96 of the α chain (α96Val), located in the $\alpha_1\beta_2$ subunit interface of hemoglobin leads to low oxygen affinity hemoglobin, and has been suggested to be due to the extra stability introduced by an aromatic amino acid at the α96 position. The characteristic of aromatic amino acid substitution at the α96 of hemoglobin has been further investigated by producing double mutant r Hb (α42Tyr → Phe, α96Val → Trp). r Hb (α42Tyr → Phe) is known to exhibit almost no cooperativity in binding oxygen, and possesses high oxygen affinity due to the disruption of the hydrogen bond between α42Tyr and β99Asp in the $\alpha_1\beta_2$ subunit interface of deoxy Hb A. The second mutation, α96Val → Trp, may compensate the functional defects of r Hb (α42Tyr → Phe), if the stability due to the introduction of trypophan at the α96 position is strong enough to overcome the defect of r Hb (α42Tyr → Phe). Double mutant r Hb (α42Tyr → Phe, α96Val → Trp) exhibited almost no cooperativity in binding oxygen and possessed high oxygen affinity, similarly to that of r Hb (α42Tyr → Phe). ^{1}H NMR spectroscopic data of r Hb (α42Tyr → Phe, α96Val → Trp) also showed a very unstable deoxy-quaternary structure. The present investigation has demonstrated that the presence of the crucible hydrogen bond between α42Tyr and β99Asp is essential for the novel oxygen binding properties of deoxy Hb (α96Val → Trp).

Keywords: *Escherichia coli*, ^{1}H NMR, Interfacial hydrogen bonding, Low oxygen affinity, Recombinant mutant hemoglobin

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Introduction

Low oxygen affinity recombinant (r) Hb (α96Val → Trp) and r Hb (α96Val → Tyr) have been produced (Kim *et al*., 1995; Choi *et al*., 1998) using an *Escherichia coli* expression plasmid in which synthetic human α- and β-globin genes were coexpressed with the *Escherichia coli* methionine aminopeptidase gene. These artificial hemoglobins show low oxygen affinity, but high cooperativity in oxygen binding, and exhibit no unusual subunit dissociation when ligated. These novel 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 aromatic amino acid residue, both r Hb (α96Val → Trp) and r Hb (α96Val → Tyr) show very similar tertiary structures around the heme pockets and quaternary structures in the $\alpha_1\beta_2$ subunit interface compared to those of human normal adult hemoglobin (Kim *et al*., 1995; Choi *et al*., 1998). Another unique feature of this hemoglobin is that the ligated form, e.g. carbonmonoxy form, in its oxy-quaternary structure can be converted to the deoxy-like quaternary structure by the addition of an allosteric effector, inositol hexaphosphate (IHP), without changing its ligation state, suggesting a very stable deoxy quaternary structure.

Recent MD simulation, using whole hemoglobin coordinates and x-ray crystallography of r Hb (α96Val → Trp), revealed that the indole side chain of tryptophan is directed toward the central cavity (Puise *et al*., 1998). In the structure, the indole nitrogen makes water mediated hydrogen bonds with β101Glu, and was proposed as the structural basis for the low oxygen affinity of the r Hb (α96Val → Trp).

X-ray crystallographic studies of deoxy-Hb A show that β99Asp is hydrogen-bonded to both α42Tyr and α97Asn in the $\alpha_1\beta_2$ (or $\alpha_2\beta_1$) interface of deoxy-Hb A (Fermi *et al*., 1984), suggesting that the essential role of β99Asp is to stabilize the deoxy-Hb molecule by making intersubunit hydrogen bonds; and thus, could provide the free energy of cooperativity in binding oxygen. Recent studies using site directed mutagenesis have shown that the hydrogen bond

between α42Tyr and β99Asp plays a key role in stabilizing the deoxy quaternary structure (Imai *et al*., 1991; Kim *et al*., 1994), while the hydrogen bond between α97Asn and β99Asp has only a supporting role (Kim *et al*., 1996).

In the present work, a unique feature of the tryptophan side chain at α96position has been further investigated by producing double mutant r Hb (α42Tyr → Phe, α96Val → Trp) using the *E. coli* expression system. r Hb (α42Tyr → Phe) is known to exhibit almost no cooperativity in binding oxygen, and possesses high oxygen affinity due to the missing crucial hydrogen bond between α42Tyr and β99Asp (Imai *et al*., 1991). r Hb (α96Val → Trp) has a very stable deoxy quaternary structure and shows low oxygen affinity. Thus, a α96ValTrp substitution is expected to compensate the functional defects of r Hb (α42Tyr → Phe). The oxygen binding properties of r Hb (α42Tyr → Phe, α96Val → Trp) have been determined and ^{1}H NMR spectroscopy used to investigate the tertiary and quaternary structures.

Materials and Methods

Production of mutant r Hb The Hb A expression plasmid, PHE2, containing synthetic α and β-globin genes, and the *E. coli* methionine aminopeptidase gene were used to produce mutant Hbs. The phagemid pTZ18U and *E. coli* JM109 were purchased form 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). Synthetic oligonucleotide 5'-CGGAAGTCGAAATTAGTG GTC-3' and 5'-TTTGAAGTTCCATGGATCAAC-3' were used for the mutations, α42TyrPhe and α96ValTrp, respectively. The normal human α- and β-globin genes in plasmid pHE2 were then replaced by the mutated globin genes.

Growth and purification of r Hb The resulting plasmid was transformed into *E. coli* JM109, and the cells grown in TB medium in a 10-liter Microferm fermentor (New Brunswick Scientific, model BioFlo 3000) at 30°C until the optical density at 600 nm reached 10. The expression of r Hb (α42Tyr → Phe, α96Val → Trp) was induced by adding isopropyl β-thiogalactopyranoside to a concentration of 0.2 mM. The culture was then supplemented with hemin (50 mg/L), and the growth continued for at least another 4 h. The cells were harvested by centrifugation and stored at -80 until needed for purification. The r Hb (α42Tyr → Phe, α96Val → Trp) was purified as previously described (Kim *et al*., 1996). Two columns were used in the final purification process: (i) a Q-Sepharose fast-flow column (Pharmacia anion exchanger) to bind Hb. After the sample had been loaded onto the column, it was thoroughly washed with the running buffer (20 mM Tris-HCl/0.1 mM EDTA at pH 8.3). The Hb fraction was then 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 at pH 6.8 to 20 mM sodium phosphate/0.1 mM EDTA at pH 8.3 to purify the r Hb (α42Tyr → Phe, α96Val → Trp).

Analytical procedures The mass spectrometric analyses were performed on a VG Quatttro-BQ (Fissions Instruments, VG Biotech, Altrincham, UK), as previously described (Shen *et al*., 1993).

Oxygen binding of Hb sample To measure the oxygen binding of the r Hb (α42Tyr → Phe, α96Val → Trp) and Hb A (0.1 mM of each), oxygen-dissociation curves were measured by a Hemox-analyzer (TCS Medical Products, Huntington Valley, PA) at 29°C in 0.1 M sodium phosphate buffer. The 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 (pH 7.0) at concentrations of about 4%. The water signal was suppressed by a jump-and-return pulse sequence (Plateau and Gueron, 1982). Typically 1024 scans were averaged to improve the signal-to-noise ratio. The proton chemical shifts are indirectly referenced to the methyl proton resonance of the sodium salt of 2, 2-dimethyl-2-silapentane-5-sulfonate (DSS) using the water signal occurring 4.76 ppm downfield from that of DSS at 29°C as the internal reference.

Results and Discussion

The purification of r Hb from *E. coli* cells generally produces several peaks on a Mono S column, with only showing the 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 the CO or oxy form, an incorrectly inserted heme can be converted to the correct conformation (Shen *et al*., 1993; Kim *et al*., 1995). In the present investigation, r Hb (α42Tyr → Phe, α96Val → Trp) was oxidized and reduced to the CO form before its application to the Mono S column. The r Hb (α42Tyr → Phe, α96Val → Trp) purified from *E. coli* JM109 showed two major peaks on Mono S column chromatography. Both r Hb (α42Tyr → Phe, α96Val → Trp) peaks from the Mono S column showed a correctly inserted heme conformation by ^{1}H NMR spectroscopy (results not shown), but differed in their N-terminal methionine contents. Mass spectrometric analyses of this purified r Hb (α42Tyr → Phe, α96Val → Trp) show that 47% of the combined α and β chains for the first peak contain N-terminally added methionine, whereas only 7% of the total α and β chains for the second peak contain N-terminal methionine. Thus, the r Hb (α42Tyr → Phe, α96Val → Trp) from the second peak was used for further experiments.

The oxygen binding properties of r Hb (α42Tyr → Phe, α96Val → Trp), Hb A and other mutant Hbs are compared in Table 1. In 0.1 mM sodium phosphate at 29°C, r Hb (α42Tyr → Phe, α96Val → Trp) exhibits higher oxygen affinity than Hb A and very low cooperativity in binding oxygen at neutral pH. The addition of IHP to r Hb (α42Tyr → Phe, α96Val → Trp) caused only slight changes in both oxygen affinity and cooperativity. These oxygen binding properties of r Hb

Table 1. P_{50} and n_{max} values of Hb A and r Hbs in 0.1 M Phosphate at pH 7.4 and 29

Hb	P_{50}, mmHg		n_{max}		Reference
	-IHP	+IHP[a]	-IHP	+IHP[a]	
Hb A	8.0	35.5	3.1	2.6	present work
r Hb (a96Val→Trp)	11.6	[b]	2.6	[b]	Kim *et al* (1995)
r Hb (α97Asn→Ala)	1.1	5.2	1.4	2.6	Kim *et al* (1996)
r Hb (α42Tyr→Asp, β99Asp→Asn)	1.9	10.0	1.7	2.4	Kim *et al* (1994)
r Hb (α42Tyr→His)[c] 1.4	15	1.9	2.1		Imai *et al* (1991)
r Hb (α42Tyr→Phe)[c]	0.6	1.0	1.1	1.4	Imai *et al* (1991)
Hb Kempsey (β99Asp→Asn)[c]	0.2	1.1[d]	1.1	1.7[d]	Bunn *et al* (1974)
r Hb (α42Tyr→Phe, α96Val→Trp)	1.2	1.7	1.1	1.4	present work

[a]IHP concentration was 2 mM unless otherwise specified.
[b]Not measured.
[c]In 0.05 M Bis-Tris (pH 7.4) containing 0.1 M Cl^- at 25°C.
[d]In 0.01 M Bis-Tris (pH 7.2) containing 0.1 M Cl^- at 20°C.
[e]IHP concentration was 1 mM.

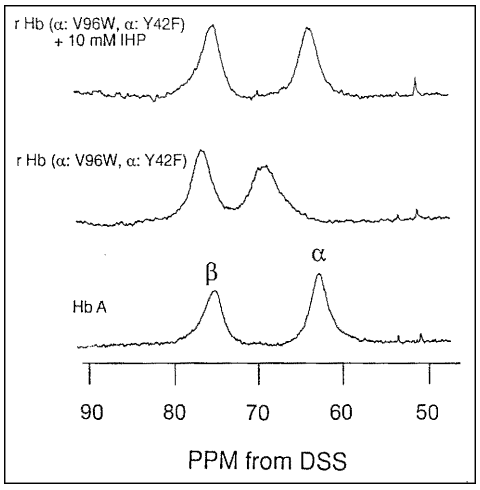

Fig. 1. 300-MHz Hyperfine-shifted $N_\delta H$ exchangeable proton resonances of the proximal histidine residues of deoxy r Hb (α42Tyr→Phe, α96Val→Trp) and deoxy Hb A in 0.1 M phosphate in H_2O at pH 7.0 and 29°C.

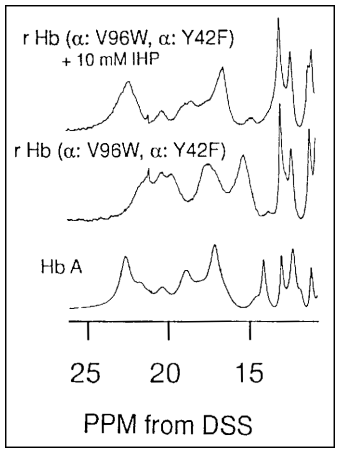

Fig. 2. 300-MHz Hyperfine-shifted and exchangeable proton resonances of deoxy r Hb (α42Tyr→Phe, α96Val→Trp) and deoxy Hb A in 0.1 M phosphate in H_2O at pH 7.0 and 29°C. The spike at 21 ppm was due to an instrumental artifact.

(α42Tyr → Phe, α96Val → Trp) are rather similar to those of Hb Kempsey and r Hb (α42TyrPhe), which are known to have no hydrogen bond in the $\alpha_1\beta_2$ subunit interface of their deoxy forms, compared to those of r Hb (α42Tyr → His)(Imai *et al*., 1991), r Hb(α97Asp → Ala)(Kim *et al*., 1996) and r Hb (α42Tyr → Asp, β99Asp → Asn)(Kim *et al.*, 1994), which are believed to have intermediate strength hydrogen bonds in the $\alpha_1\beta_2$?subunit interface of their deoxy forms.

^{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 ^{1}H resonances of Hb A and r Hb (α42Tyr → Phe, α96Val → Trp) 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 that at ~77 ppm to the corresponding residue of the β chain (β92His) of deoxy-Hb A (Takahashi *et al*., 1980; La Mar *et al*., 1980). The chemical shift positions of these two proximal histidyl resonances in r deoxy-Hb (α42Tyr → Phe, α96Val → Trp) have been down-field shifted. However, the addition of IHP could convert the spectrum to one similar to that of deoxy-Hb A. This is a typical feature of mutant Hbs missing the crucial hydrogen bond between α42Tyr and β99Asp at the $\alpha_1\beta_2$ subunit interface.

The exchangeable and ferrous hyperfine-shifted proton resonances of r Hb (α42Tyr → Phe, α96Val → Trp) in the deoxy form are shown in Fig. 2. The resonance at ~14 ppm from DSS of the Hb A has been assigned to the intersubunit hydrogen bond between α42Tyr and β99Asp (Fung and Ho, 1975), and is a key marker for the deoxy-quaternary structure of Hb A. This resonance is completely absent from the spectra

of r Hb (α42Tyr → Phe, α96Val → Trp), and no new noticeable exchangeable resonance appeared. All the other exchangeable resonances assigned to the $\alpha_1\beta_2$ interfacial hydrogen bonds between the α126Asp and β35Tyr (~12.9 ppm) and between the α103 → Asp and β108 → Asn (~12.1 ppm) (Russu *et al*., 1987) were present in the ^{1}H NMR spectrum of r Hb (α42Tyr → Phe, α96Val → Trp). 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 the unpaired electrons of Fe(II) in the heme iron atoms. The hyperfine-shifted resonances of r Hb (α42Tyr → Asp, β99Asp → Asn) over the spectral region ~16 to ~24 ppm showed significant changes from those of Hb A. However, the addition of IHP can convert the spectrum to one similar to that of deoxy-Hb form. These results indicate that the deoxy form of r Hb (α42Tyr → Phe, α96Val → Trp) is very unstable i.e., exists in an oxy-like (or R type) quaternary structure. Mutant Hbs, which are known to have intermediate strength hydrogen bonds in the $\alpha_1\beta_2$ subunit interface, such as, r Hb (α42Tyr → His) (Imai *et al*., 1991) and r Hb (α97Asn → Ala) (Kim *et al*., 1996), all exhibited stable deoxy-like quaternary structures from the ^{1}H-NMR spectra.

Double-mutant recombinant hemoglobin, r Hb (α42Tyr → Asp, β99Asp → Asn), was recently produced by site-directed mutagenesis, in an expectation that a new hydrogen bond involving β99Asn could be induced by replacing α42Tyr by a strong hydrogen bond acceptor, such as Asp (Kim *et al*., 1994; Yeh *et al*.,1998). A modified Hb was expected to regain the cooperativites lost in Hb Kempsey (β99Asp → Asn). The oxygen affinity of r Hb (α42Tyr → Asp, β99Asp → Asn), while still high, was significantly lower than that of Hb Kempsey (β99Asp → Asn), with the restoration of substantial cooperativity. The ^{1}H NMR spectrum of deoxy-r Hb (α42Tyr → Asp, β99Asp → Asn) also showed a very stable new deoxy-like structure.

In the present investigation, r Hb (α42Tyr → Phe, α96Val → Trp) has been produced with the expectation of compensating for the functional defects of r Hb (α42Tyr → Phe), if the α96Trp should add extra stability to the deoxy structure of Hb (α42Tyr → Phe), as suggested by Kim *et al.* (1995). However, neither the oxygen binding properties nor ^{1}H spectroscopic results of r Hb (α42Tyr → Phe, α96Val → Trp) in the present investigation showed the expected compensation.

These results could mean that the extra stability of the deoxy form introduced by α96Val → Trp, either due to the water mediated hydrogen bonds between the indole ring of tryptophan and β101Glu (Puis *et al*., 1998), or due to the direction of the aromatic ring into the internal cavity (Kim *et al*., 2001; Choi *et al*., 1998), may not be strong enough to maintain the deoxy-like quaternary structure in the absence of the crucial hydrogen bond between α42Tyr and β99Asp. The present investigation has demonstrated that the presence of the crucial hydrogen bond between α42Tyr and β99Asp is essential for the novel oxygen binding properties of r Hb (α96Val → Trp).

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