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Functional Defects of Hb Kempsey (β99Asp→Asn) Can be Compensated by Insertion of a New Intersubunit Hydrogen Bond at the $\alpha_1\beta_2$ Subunit Interface

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X-ray crystallographic studies of the deoxy form of human adult hemoglobin (Hb A) have shown that β99Asp is hydrogen bonded to both α42Tyr and α97Asn in the $\alpha_1\beta_2$ subunit interface, suggesting that the essential role of β99Asp is to stabilize the deoxy-Hb by creating the intersubunit hydrogen bond. In particular, for Hb Kempsey (β99Asp→Asn), molecular dynamics simulation indicated that a new hydrogen bond involving β99Asn can be induced by replacing α42Tyr with a strong hydrogen-bond acceptor such as Asp. Designed mutant recombinant (r) Hb (β99Asp→Asn, α42Tyr→Asp) have been produced in the *Escherichia coli* expression system and have shown that functional defects of Hb Kempsey could be compensated by the α42Tyr→Asp substitution. However, as the α42Tyr→Asp mutation has never been reported before, it is still possible that the functional properties of r Hb (β99Asp→Asn, α42Tyr→Asp) may be due to the mutation itself. Thus, it is required to produce r Hb (α42Tyr→Asp) and r Hb Kempsey (β99Asp→Asn) as controls, and to compare their properties with those of r Hb (β99Asp→Asn, α42Tyr→Asp). r Hb (α42Tyr→Asp) could not be purified because it is an unstable hemoglobin which forms Heinz bodies. r Hb Kempsey (β99Asp→Asn) exhibits very high oxygen affinity and greatly reduced cooperativity. Thus, r Hb (β99Asp→Asn) and r Hb (α42Tyr→Asp) compensate each other.

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Introduction

Human abnormal Hbs with amino acid substitutions in the $\alpha_1\beta_2$ subunit interface generally have a high oxygen affinity and reduced cooperativity in oxygen binding compared to human normal adult hemoglobin (Hb A) (Dickerson and Geis, 1983), suggesting the importance of the $\alpha_1\beta_2$ subunit interface to the functional properties of Hb. In particular, mutant human Hbs with an amino acid substitution at the β99Asp, such as Hb Kempsey (β99Asp→Asn or β:D99N) (Reed *et al.*, 1968), Hb Yakima (β99Asp→His) (Jones *et al.*, 1967), Hb Radcliff (β99Asp →Ala) (Weatherall *et al.*, 1977), Hb Hôtel Dieu (β99Asp →Gly) (Thillet *et al.*, 1981), and Hb Ypsilanti (β99Asp →Tyr) (Glynn *et al.*, 1968), possess greatly reduced cooperativity and increased oxygen affinity relative to those exhibited by Hb A. X-ray crystallographic studies of Hb A (Fermi *et al.*, 1984) have shown that β99Asp is hydrogen-bonded to both α42Tyr and α97Asn in the $\alpha_1\beta_2$ subunit interface of deoxy-Hb A, suggesting that the essential role of β99Asp is to stabilize the deoxy-Hb molecule by forming intersubunit hydrogen bonds. Recent investigation of r Hb (α97Asn→Ala) showed that the hydrogen bond between β99Asp and α97Asn is not as crucial as the role of the hydrogen bond between β99Asp and α42Tyr. However, the presence of both hydrogen bonds at the $\alpha_1\beta_2$ subunit interface of the deoxy form is essential to maintain the normal cooperative oxygenation process (Kim *et al.*, 1996).

MD simulations have recently been used to design mutant Hbs which have altered $\alpha_1\beta_2$ subunit interfaces

(Kim *et al.*, 1994). The approach has been to design compensatory mutant Hbs by introducing additional mutations in the local environment of the β99 mutation in order to create new hydrogen bonds to compensate for the missing ones. For Hb Kempsey (β99Asp→Asn), which has a high oxygen affinity and exhibits essentially no cooperativity in binding oxygen (Kim *et al.*, 1994), computer simulations indicated that a new hydrogen bond involving β99Asn can be induced in the deoxy form by replacing α42Tyr by a stronger hydrogen-bond acceptor, such as Asp. To test the validity of MD simulation, r Hb (β99Asp→Asn, α42Tyr→Asp) have been produced using the *Escherichia coli* (*E. coli*) expression system in which synthetic human α- and β-globin genes are coexpressed with *E. coli* aminopeptidase (Kim *et al.*, 1994). The oxygen binding properties of this r Hb showed that α42Tyr→Asp replacement can substantially compensate for the functional defect of Hb Kempsey (β99Asp→Asn). Proton nuclear magnetic resonance (^{1}H-NMR) spectroscopic data also showed that the deoxy form of r Hb (β99Asp→Asn, α42Tyr→Asp) was very stable, unlike that of Hb Kempsey (β99Asp→Asn).

As mutation α42Tyr→Asp has never been reported before, we cannot exclude the possibility that the functional properties of r Hb (β99Asp→Asn, α42Tyr→Asp) may result from the α42Tyr→Asp mutation itself. Thus, it is essential to produce r Hb (α42Tyr→Asp) and r Hb Kempsey (β99Asp→Asn) as controls, and then compare their functional properties with those of r Hb (β99Asp→Asn, α42Tyr→Asp). We have determined the oxygen binding properties of r Hbs and used ^{1}H-NMR spectroscopy to investigate the tertiary structures around the heme groups and the 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 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). Synthetic oligonucleotides 5′-CGGGAAGTCGGTC TTAGTGGTCG-3′ and 5′-GTTTTCCGGGTTAACGTGCAG TT-3′ were used for the mutations α42Tyr→Asp and β99Asp→ Asn, respectively.

Growth and purification of r Hb Resulting plasmid was transformed into *E. coli* JM109, and the cells were grown in TB medium in a 10-liter Microferm fermentor (New Brunswick Scientific, model BioFlo 3000, Brunswick, USA) at 30°C until the optical density at 600 nm reached 10. Expression was 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 frozen at −80°C until needed for purification. The r Hb Kempsey and r Hb (β99Asp→Asn, α42Tyr→Asp) were purified as previously described (Kim *et al.*, 1995; Choi *et al.*, 1998). We used two columns in the final purification process: (i) A Q-Sepharose fast-flow column (Pharmacia anion exchanger, Uppsala, Sweden) 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 at pH 6.8 to 20 mM sodium phosphate/0.1 mM EDTA at pH 8.3 was used to purify r Hbs.

Oxygen binding of Hb sample For the r Hb (β99Asp→Asn, α42Tyr→Asp), r Hb Kempsey, 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 at pH7.0. 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 and Discussion

r Hb (α42Tyr→Asp) and r Hb Kempsey (β99Asp→Asn) have been expressd in *E. coli*. However, r Hb (α42Tyr→Asp) could not be purified because it is an unstable hemoglobin which appears to lose hemes, i.e., the formation of Heinz bodies. The production of r Hb Kempsey (β99Asp→Asn) from Mono S column chromatography produced a major component with a visible optical spectrum (over the range 350–700 nm) in the carbonmonoxy form identical to Hb A.

^{1}H-NMR spectroscopy has been shown to be an excellent tool to investigate the tertiary and quaternary structural features of Hb (Ho, 1992; Ho and Perussi, 1994). The ferrous hyperfine-shifted proton and exchangeable resonances of r Hb (β99Asp→Asn, α42Tyr→Asp), r Hb Kempsey (β99Asp→Asn), Hb Kempsey, and Hb A in the deoxy form are shown in Fig. 1. r Hb Kempsey and Hb Kempsy showed almost similar resonances, indicating structural identity. The hyperfine-shifted proton resonances of both deoxy-r Hb Kempsey and deoxy-Kempsey observed at 15 to 20 ppm downfield from DSS show significant changes from those of deoxy-Hb A. However, the addition of the allosteric effector, inositol

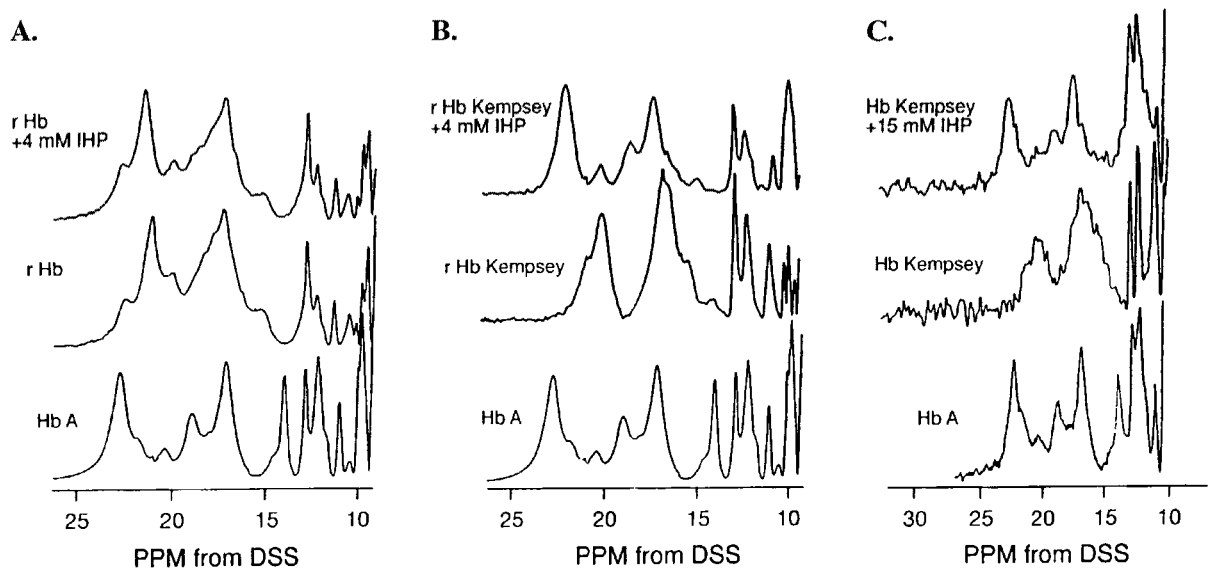

Fig. 1 Hyperfine-shifted and exchangeable proton resonances of Hb in the deoxy form: A. r Hb (β:D99N; α:Y42D) and Hb A; B. r Hb Kempsey (β:D99N); C. Hb Kempsey with and without IHP. ^{1}H-NMR spectra of A and C were obtained from Fig. 4 of Kim *et al.* (1994).

hexaphosphate (IHP), to r Hb Kempsey converted the spectrum to one similar to that of deoxy-Hb A in the hyperfine-shifted proton resonance region. This result can be compared to the spectra of deoxy-r Hb (β99Asp→Asn, α42Tyr→Asp), of which the addition of IHP did not cause any noticeable changes in the exchangeable resonances and ferrous hyperfine-shifted proton resonances (Kim *et al.*, 1994).

Hyperfine-shifted N_δH-exchangeable ^{1}H resonances of r Hb (β99Asp→Asn, α42Tyr→Asp), r Hb Kempsey, and Hb A are shown in Fig. 2. Like the spectra of ferrous hyperfine-shifted proton and exchangeable resonances, hyperfine-shifted N_δH-exchangeable resonances of r Hb Kempsey showed identical chemical shift positions and a very similar shape to that of Hb Kempsey (Takahashi *et al.*, 1982). The resonance at ~63 ppm from DSS has been assigned to the hyperfine-shifted N_δH-exchangeable proton of the proximal 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 (β92His) of deoxy-Hb A (Lar Mar *et al.*, 1980; Takahashi *et al.*, 1980). The chemical shift positions of the proximal histidyl resonances of r Hb Kempsey and r Hb (β99Asp→Asn, α42Tyr→Asp) all show downfield resonance shifts in the histidyl resonance of the α chain. In r Hb Kempsey, the presence of IHP causes the proximal histidyl resonance from the α chain to be shifted upfield to a chemical shift position similar to that of deoxy-Hb A. In contrast, IHP exerts no significant effect on r Hb (β99Asp→Asn, α42Tyr→Asp). This result of hyperfine-shifted N_δH-exchangeable resonances supports our original suggestion (Kim *et al.*, 1994) that, due to the presence of a new interfacial hydrogen bond between α42 Asp and β99Asn at the $\alpha_1\beta_2$ interface, deoxy-r Hb (β99Asp→Asn, α42Tyr→Asp) already has a new stable deoxy-like structure, so that the addition of IHP does not cause significant structural changes.

Figure 3 compares the exchangeable proton resonances of r Hb (β99Asp→Asn, α42Tyr→Asp), r Hb Kempsey, and Hb A in the CO form. The exchangeable resonances at ~12.1 ppm and at ~12.9 ppm assigned hydrogen bonds between α126Asp and β35Tyr, and between α103Asp and β108Asn (Russu *et al.*, 1987), respectively, at the $\alpha_1\beta_1$ interface, are essentially the same in r Hb (β99Asp→Asn, α42Tyr→Asp), r Hb Kempsey, and Hb A in the CO form, suggesting no structural perturbations in the $\alpha_1\beta_1$ subunit interfaces of these r Hbs. However, the resonance at ~10.7 ppm from DSS of Hb A, which has been assigned to the intersubunit hydrogen bond between α94Asp and β102Asn at the $\alpha_1\beta_2$ interface (Fung and Ho, 1975), a key marker of the oxy-quaternary structure, has disappeared for both r Hb (β99Asp→Asn, α42Tyr→Asp) and r Hb Kempsey, suggesting significant changes in the oxy-quaternary structures of r Hb (β99Asp→Asn, α42Tyr→Asp) as well as those of r Hb Kempsey. Thus, r Hb (β99Asp→Asn, α42Tyr→Asp) may have new quaternary structures not only for the deoxy form but also for the oxy form.

The oxygen binding properties of r Hb (β99Asp→Asn, α42Tyr→Asp), r Hb (α42Tyr→Asp), r Hb Kempsey, Hb Kempsey, and other mutant Hbs are shown in Table 1. In 0.1 M sodium phosphate at pH 7.2 and 29°C, both Hb Kempsey and Hb Kempsey (β99Asp→Asn) exhibit very high oxygen affinity and greatly reduced cooperativity. The slight difference in oxygen binding properties between Hb

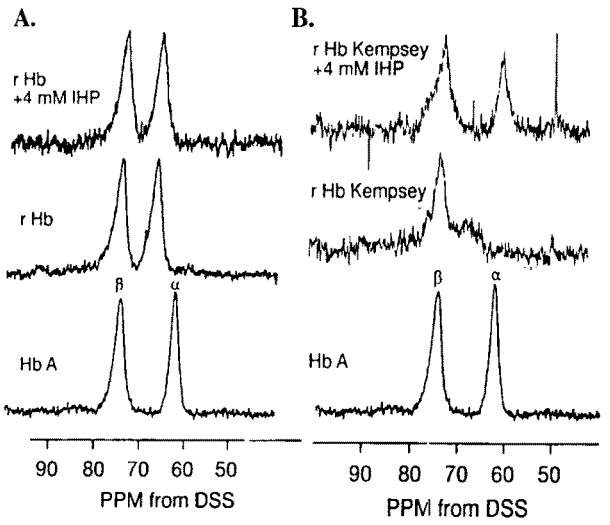

Fig. 2 Hyperfine-shifted $N_\delta H$ exchangeable proton resonances of the proximal histidine residues of Hb in the deoxy form: A. r Hb (β:D99N; α:Y42D) and Hb A; B. r Hb Kempsey (β:D99N) with and without IHP.

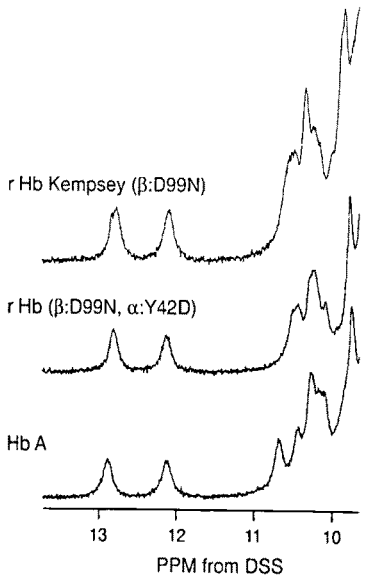

Fig. 3 Exchangeable proton resonances of r Hb (β:D99N; α:Y42D), r Hb Kempsey, and Hb A in the CO form.

Table 1. P_{50} and n_{max} values of Hb A and r Hbs in 0.1 M phosphate at pH 7.2 and 29°C.

Hb	P_{50}, mmHg		n_{max}		
	−IHP	+IHP[a]	−IHP	+IHP[a]	Reference
Hb A	10.5	46.7	3.0	2.6	present work
r Hb (α42Tyr→Asp, β99Asp→Asn)	2.5	12.9	1.7	2.5	Kim *et al.* (1994)
r Hb (α97Asn→Ala)[b]	1.1	5.2	1.4	2.6	Kim *et al.* (1996)
r Hb (α42Tyr→His)[c]	1.4	15	1.9	2.1	Imai *et al.* (1991)
r Hb Kempsey (β99Asp→Asn)	1.6	1.5	1.3	1.1	present work
Hb Kempsey (β99Asp→Asn)[d]	0.2	1.1e	1.1	1.7e	Bunn *et al.* (1974)

[a] IHP concentration was 2 mM unless otherwise specified.
[b] In 0.1 M phosphate at pH 7.4 and 29°C.
[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.

Kempsey and r Hb Kempsey may be due to the different conditions of measuring the oxygen dissociation curves. r Hb (β99Asp→Asn, α42Tyr→Asp) exhibits intermediate oxygen affinity. A marked change occurs when IHP is added to r Hb (β99Asp→Asn, α42Tyr→Asp). The oxygen affinity is reduced and the cooperative oxygenation process for this double mutant approaches the normal value for Hb A as manifested by the Hill coefficient, with an n_{max} value of 2.5. r Hb (α42Tyr→His) (Imai *et al.*, 1991) and r Hb (α97Asn→Ala) (Kim *et al.*, 1996), which are believed to have a weak hydrogen bond in the $\alpha_1\beta_2$ interface, show oxygen properties similar to those of r Hb (β99Asp→Asn, α42Tyr→Asp).

The restoration of cooperativity in our r Hb (β99Asp→Asn, α42Tyr→Asp) relative to Hb Kempsey (β99Asp→Asn) can most likely be explained by the presence of a new hydrogen bond between α42Asp and β99Asn in the $\alpha_1\beta_2$ subunit interface, introduced by the additional mutation α42Tyr→Asp. This new interfacial hydrogen bond can stabilize a deoxy-like (or T-type) structure, and thus, could provide the necessary free energy of cooperativity in binding oxygen.

r Hb Kempsey (β99Asp→Asn) exhibits very high oxygen affinity and greatly reduced cooperativity, and r Hb (α42Tyr→Asp) is an unstable hemoglobin which forms Heinz bodies. Thus, r Hb (β99Asp→Asn, α42Tyr→Asp) may be described as a case of "two wrongs making a right"!

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