The artificial α1β1-contact mutant hemoglobin, Hb Phe-35β, shows only small functional abnormalities

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Abstract It was previously reported that Hb Philly with a mutation of Phe for Tyr at 35(C1)β showed non-cooperative oxygen binding with a very high affinity and instability leading to hemolysis. Further, it lacked the ^{1}H-NMR signal at 13.1 ppm from 2,2-dimethyl-2-silapentane-5-sulfonate in normal hemoglobin (Hb A), so that this signal was assigned to a hydrogen bond formed by Tyr-35(C1)β. Surprisingly, our artificial mutant hemoglobin with the same mutation as Hb Philly showed slightly lowered oxygen affinity, almost normal cooperativity, the ^{1}H-NMR signal at 13.1 ppm and no sign of instability. Our results indicate that the mutation reported for Hb Philly and the assignment of the 13.1 ppm signal need reexamination.

Key words: Hemoglobin; Mutagenesis; Nuclear magnetic resonance; Oxygen affinity

1. Introduction

The atomic-level structure of the intersubunit contacts in hemoglobin was extensively examined by X-ray crystallography. The functional importance of the α1β2 interface has been well documented [1,2]. This α1β2 interface undergoes an extensive relative movement and rearrangement of contact amino acid residues upon ligand binding [3,4]. On the other hand, the subunit interactions across the α1β1 interface have been assigned minor importance compared to those across the α1β2 interface. The α1β1 interface is so rigid as to be used as the reference frame when the structures of deoxy and liganded forms are compared [5].

The different contributions of these two types of subunit interface to the structure and function of hemoglobin have been well manifested in abnormal hemoglobins [6–9]. Generally, hemoglobin variants with amino acid substitutions at the α1β2 interface showed markedly altered oxygen binding properties but normal stability. On the other hand, variants with amino acid substitutions at the α1β1 interface usually showed normal or only mildly altered oxygen binding properties whereas stability was reduced, causing various degrees of hemolysis in many cases [9,10]. The rate of the amino acid substitution at the α1β1 interface among vertebrate hemoglobins is higher than that at the α1β2 interface [11]. These facts are consistent with the idea that the α1β1 interface is rigid enough to accommodate mutations without extensive changes of the structure whereas mutations at the α1β2 interface often result in crucial functional defects. However, it does not necessarily mean that there is no functionally important interaction across the α1β1 interface in hemoglobin.

Rieder et al. [12] reported that Hb Philly had a mutation at the α1β1 contact where Tyr-35β was replaced by Phe, and, as a result, the intersubunit hydrogen bond between Asp-126α and Tyr-35β at the α1β1 contact in normal human hemoglobin (Hb A) was removed in that mutant. It binds oxygen non-cooperatively with very high affinity and shows instability leading to hemolysis. It shows nearly twice as many titratable SH groups as Hb A. It lacks the exchangeable proton nuclear magnetic resonance (NMR) signal at 13.1 ppm from 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) and this has been the basis for the assignment of this signal to the hydrogen bond described above [13]. The drastic change in oxygen binding properties of Hb Philly has been only the exception to the rule on the functional role of the α1β1 interface.

We therefore intended to investigate this apparent inconsistency of the functional properties of Hb Philly, as well as the exact role of Tyr-35β in Hb A. Since natural Hb Philly is not available to us, we prepared an artificial mutant Hb Phe-35β, in which Tyr-35β was replaced by Phe, by site-directed mutagenesis. We studied its oxygen binding properties, proton NMR and the number of reactive SH groups.

2. Materials and methods

2.1. Preparation of mutant and normal hemoglobins

The mutant β-globin, in which Tyr-35β was replaced by Phe, was prepared as described by Jessen et al. [15]. The mutated DNA sequence of the whole β chain was confirmed twice on the mutation introduction vector M13 *mp*18 *c*IIFXβ-globin and the expression vector *p*T7*c*IIFXβ-globin using a DNA sequencer (model 373S, Applied Biosystems). Since the properties of Hb Phe-35β were quite inconsistent with those of the natural mutant, Hb Philly [12], and this was the key to our study, we further checked the amino acid sequence of the synthesized mutant β globin by means of fast-atom bombardment mass spectrometry on tryptic digests [16]. The spectrum showed disappearance of the peak with the mass to charge ratio of 1274.7 accompanied by appearance of a new peak with the ratio of 1258.8. No other mass spectrum changes were noted for other peptide peaks. The disappeared peak corresponded to the tryptic peptide from Leu-31β to Arg-40β which includes Tyr-35β. The difference of 15.9 agreed with what is expected for the replacement of Tyr by Phe. Although the replacement of Leu at 31β or 32β by Pro could be another possibility giving nearly the same difference by a single nucleotide replacement, the DNA sequence data excluded this possibility.

This mutant β-globin was reconstituted into hemoglobin tetramer as described by Jessen et al. [15]. The Hb A which was used as the control in our experiments was purified from human adult red blood cells [14].

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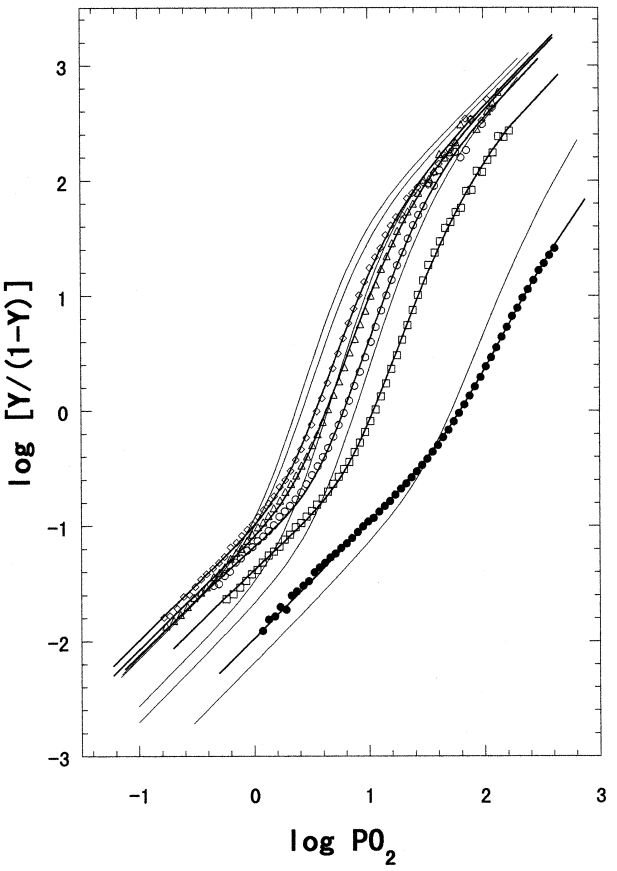

Fig. 1. Hill plots of oxygen binding to Hb Phe-35β. Y, fractional saturation of hemoglobin with oxygen; PO_2, partial pressure of oxygen (mm Hg). Symbols express observed points, and lines were calculated from the best-fit Adair constant values. □, pH 6.9; ○, pH 7.4; △, pH 7.9; ◇, pH 8.4; ●, pH 7.4+2 mM IHP. Hemoglobin concentration, 60 μM on a heme basis; in 50 mM Tris (pH 8.4 and 7.9) or 50 mM Bis-Tris (pH 7.4 and 6.9) containing 0.1 M Cl^-, in the presence of 0.1 μM catalase and 0.1 μM superoxide dismutase; 25°C. Hill plots for Hb A in the same experimental conditions are presented by thin lines without symbols. They are shown in the order of decreasing pH from the left to the right and the rightmost is pH 7.4+2 mM IHP.

2.2. Titration of SH groups

The free SH groups for Hb A and Hb Phe-35β were titrated with *p*-hydroxy-mercuribenzoate (PMB). Absorbance changes during titration were monitored at 250 nm [17].

2.3. Oxygen equilibrium experiments and analysis

Oxygen equilibrium curves were measured by using the method developed by Imai et al. [18,19]. The light absorbance at 560 nm was used to determine oxygen saturation of hemoglobin. The experimental conditions were: 60 μM protein concentration on a heme basis; in 50 mM Tris buffer (pH 8.4 and pH 7.9) or 50 mM Bis-Tris buffer (pH 7.4 and pH 6.9) containing 0.1 M Cl^-; at 25°C. The pH value was adjusted with a concentrated NaOH solution at the same temperature as that for oxygen equilibrium measurements (at 25°C). To minimize the autoxidation of hemoglobin during the measurement, catalase and superoxide dismutase were added to each sample (approximately 0.1 μM as the final concentrations for both of them)[20,21]. The methemoglobin content of the hemoglobin samples as measured before and immediately after oxygen equilibrium measurements ranged from 1.5% to 6.5% and 6.0% to 8.5%, respectively. Although both the deoxygenation and reoxygenation curves were recorded for each sample, the former one was adopted for the analysis since the methemoglobin content was lower for the former. They are comparatively low and do not influence the conclusions of this paper.

P_{50} (partial pressure of oxygen at half saturation), the Hill coefficient n_{max} (the maximum slope of the Hill plot) and K_1 and K_4 (the intrinsic association equilibrium constants for the first and fourth oxygen binding steps, respectively, the first and fourth Adair constants) were evaluated from the oxygen equilibrium data by a least-squares curve-fitting method [22].

2.4. Proton NMR spectra

The ^{1}H-NMR spectra of Hb Phe-35β and Hb A were recorded at 500 MHz using Bruker Avance DRX 500 as previously described [23]. Chemical shifts were referenced with respect to the proton resonance of DSS. In this experiment, the proton resonance of H_2O was 4.89 ppm (at 17°C). The concentrations of Hb Phe-35β and Hb A were 2.0 mM and 2.9 mM on a heme basis, respectively.

3. Results and discussions

3.1. SH titration

The numbers of free SH groups obtained from titration with PMB for both carbonmonoxy form Hb Phe-35β and Hb A were 2.0 per tetramer. This result is in striking contrast to Hb Philly which showed twice as many free SH groups as Hb A.

3.2. Oxygen equilibrium curves

Oxygen equilibrium curves of Hb Phe-35β and Hb A were determined at four different pH values: 6.9, 7.4, 7.9 and 8.4 without inositol hexaphosphate (IHP) and at pH 7.4 with 2 mM IHP. They are presented in Fig. 1 by means of the Hill plot. Values of n_{max}, P_{50}, K_1 and K_4 obtained from these curves are listed in Table 1. The oxygen affinity measured by P_{50} for Hb Phe-35β was consistently lower (by 1.2–1.7-fold) than that for Hb A under all the experimental condition sets examined. However, the bottom asymptote of the Hill plot for Hb Phe-35β was shifted toward the left compared to that of Hb A under all the condition sets, suggesting that there were some contaminant hemoglobin components with high oxygen affinity. Those contaminants, which could be formed during protein expression in *Escherichia coli*, were

Table 1
Oxygen equilibrium parameters for normal hemoglobin and hemoglobin Phe-35β[a]

	P_{50}[b]	n_{max}[c]	K_1[d]	K_4[e]	δH^{+}[f]
Hb Phe35-β					
pH 6.9	11.0	2.60	0.044	2.3	
pH 7.4	6.1	2.80	0.076	3.9	−0.41
pH 7.9	4.3	2.68	0.085	4.4	
pH 8.4	3.5	2.67	0.10	4.6	
pH 7.4+2 mM IHP	62.4	1.85	0.010	0.15	
Hb A (normal Hb)					
pH 6.9	7.6	3.01	0.020	4.6	
pH 7.4	4.4	3.13	0.027	4.7	−0.46
pH 7.9	2.6	2.89	0.070	5.2	
pH 8.4	2.3	2.98	0.072	5.7	
pH 7.4+2 mM IHP	51.9	2.42	0.0064	0.85	

[a]Experimental conditions: Hb concentration, 60 μM on a heme basis; in 50 mM Bis-Tris (pH 6.9 and 7.4) or Tris (pH 7.9 and 8.4) containing 0.1 M Cl^-; 25°C.
[b]Partial pressure of oxygen at half saturation (in mm Hg).
[c]Maximum slope of the Hill plot (Hill coefficient).
[d]The first stepwise Adair constant (in mm Hg^{-1}).
[e]The fourth stepwise Adair constant (in mm Hg^{-1}).
[f]Bohr coefficient (= $\Delta\log P_{50}/\Delta$pH).

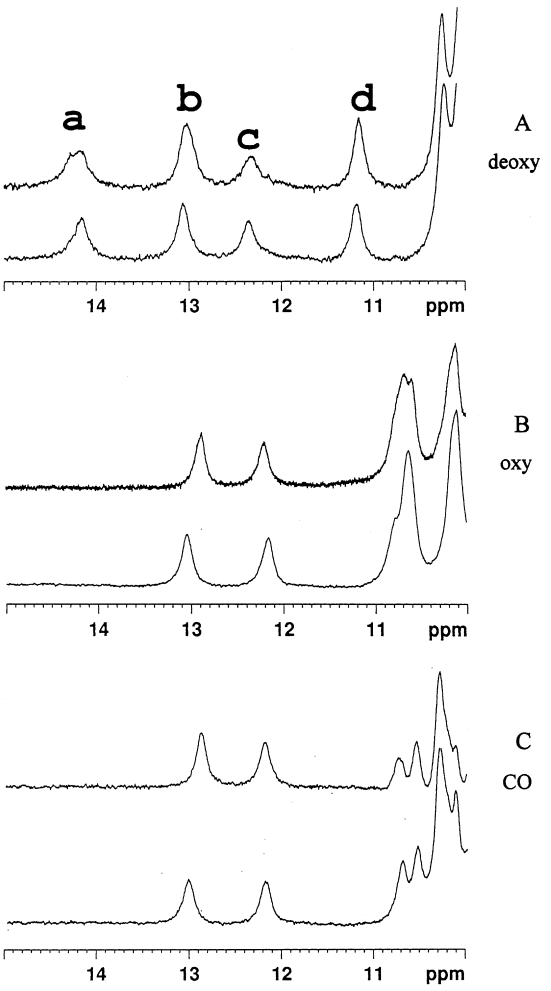

Fig. 2. Proton NMR spectra (500 MHz) for the deoxy form (A), the oxy form (B) and the CO form (C). The upper and lower spectra stand for Hb Phe-35β and Hb A, respectively. Chemical shift is given in ppm from DSS. Experimental conditions: protein concentration, 2.94 mM (Hb A) and 2.01 mM (Hb Phe-35β) on a heme basis; in 50 mM Bis-Tris (pH 7.4) containing 0.1 M Cl^-; 17°C.

not thoroughly removed through subsequent purification procedures. Without these contaminants the mutant hemoglobin would show still lower oxygen affinity than the observed one. The n_{max} values for Hb Phe-35β were somewhat smaller than those for Hb A, probably due to the presence of the contaminants in Hb Phe-35β samples. The Bohr coefficient for Hb Phe-35β was −0.41 which was similar to that for Hb A (−0.46) (see Table 1). The oxygen equilibrium curves of Hb Phe-35β measured at pHs 7.4, 7.9 and 8.4 asymptotically approach to those of Hb A measured in the same pH range. In fact, K_4 values for Hb Phe-35β are not very different from those for Hb A at these pH values. On the other hand, at pH 6.9 the difference becomes larger, being nearly double (Table 1). In the presence of 2 mM IHP the difference is the largest among the five condition sets. Although K_1 values are most influenced by the high affinity contaminants other parameter values in Table 1 are less influenced because they were determined from the middle and upper portions of the oxygen equilibrium curve.

3.3. Proton NMR spectra

Fig. 2 shows the ^{1}H-NMR spectra in the exchangeable proton resonance region for Hb Phe-35β and Hb A. The signals at 14.2 ppm (a) and 11.2 ppm (d) (see Fig. 2A), which are present only in the spectra for the deoxy form, have been assigned to the hydrogen bonds between Tyr-42α and Asp-99β and between Asp-94α and Trp-37β, respectively, at the α1β2 interface [24–27]. These signals disappear in the oxy and carbonmonoxy forms because these hydrogen bonds are broken due to the conformational change at the α1β2 interface upon ligand binding. Thus, they have been used as spectroscopic probes for the deoxy quaternary structure [24,27]. The characteristic peaks around 13.1 ppm and 12.2 ppm are common to all the three forms of Hb A. A 0.2 ppm downward shift for the 12.2 ppm peak from the deoxy form (12.4 ppm) to the liganded form (12.2 ppm) is noted in accordance with Ho et al. [25,27]. These 13.1 ppm and 12.2 ppm peaks have been assigned to the intersubunit hydrogen bonds between Asp-126α and Tyr-35β and between His-103α and Asn-108β, respectively, at the α1β1 interface [13,25]. These assignments were based on the absence of the 13.1 ppm signal in Hb Philly and the nuclear Overhauser effect measurement. Russu et al. [25] concluded from a structural consideration that the nuclear Overhauser effect for the 13.1 ppm signal was consistent with the above assignment, and that the 12.2 ppm signal must have originated from the other hydrogen bond. Since isolated α and β chains showed none of these exchangeable proton signals and those signals of Hb A did not change significantly upon ligand binding, it appeared reasonable to conclude that they originated from the α1β1 interface. In the case of Hb Phe-35β both signals also show small downward shifts, of 0.2 ppm (from 13.1 ppm to 12.9 ppm) for the 13.1 ppm peak and of 0.1 ppm (from 12.3 ppm to 12.2 ppm) for the 12.2 ppm peak. If the assignment for the 13.1 ppm signal described above was correct, that peak must be absent in Hb Phe-35β. Thus, our recent results indicate that the previous assignment for the 13.1 ppm signal should be reexamined.

M. Nagai (personal communication) has shown that Hb Phe-35β provided by us gave ultraviolet resonance Raman spectra identical to those of Hb A, showing that the environments around Tyr-35β do not undergo large changes upon ligand binding.

In conclusion, our present oxygen equilibrium data for Hb Phe-35β accord with the rule that the amino acid substitutions at α1β1 interface exert little influence on oxygen equilibrium properties [28]. The most marked exception to this rule has been removed by our present study. The apparent inconsistency of the oxygen equilibrium data for Hb Philly, the presence of the 13.1 ppm ^{1}H-NMR peak in Hb Phe-35β and the disagreement of the number of free SH groups between Hb Phe-35β and Hb Philly strongly indicate that Hb Phe-35β is distinctly different from Hb Philly and, therefore, the amino acid substitution in Hb Philly [12] and the assignment of the 13.1 ppm ^{1}H-NMR peak [13] must be reexamined.

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