A ^{1}H NMR comparative study of human adult and fetal hemoglobins

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Abstract The affinities of the individual subunits in human adult and fetal hemoglobins to azide ion have been determined from the combined analysis of NMR and optical titration data. Structural and functional non-equivalence of the constituent subunits, i.e. α and β subunits in human adult hemoglobin and α and γ subunits in human fetal hemoglobin, has been confirmed. The function of the α subunits, which are common to both hemoglobins, is essentially identical in these hemoglobins and, in spite of the substitutions of 39 amino acid residues between β and γ subunits, they exhibit similar azide ion affinities. The present study also demonstrates that the NMR spectral comparison between the two proteins provides signal assignments to the individual subunits in intact tetramer.

Key words: Azide ion affinity; Heme; Hemoglobin; NMR; Paramagnetic shift; Subunit interaction

1. Introduction

Functional properties of tetrameric hemoglobin (Hb) are controlled by the subunit interaction [1]. Cooperative ligand binding property emerges when the heterotetramer, i.e. in the case of human adult Hb (Hb A), $\alpha_2\beta_2$, is formed. X-ray crystallographic study has revealed that the intersubunit contacts at the α_1-β_2 and α_2-β_1 subunit interfaces undergo a significant alteration upon ligation of the protein [1–4] and the cooperativity of Hb has been interpreted on the basis of the two possible alternate quaternary structures of the molecule which exhibit considerably different ligand affinity [5]. Although X-ray study has provided a detailed description of the allosteric mechanism in Hb in terms of small displacements of specific atoms or groups of the protein accompanied by the alteration of these interfaces, the regulation of the reactivity of heme ion in the individual subunits through the tertiary and quaternary structures is not fully understood. Roles of amino acid residues located in the intersubunit contacts to both function and structure of Hb have been examined from the studies of abnormal Hbs [5,6] as well as genetic mutants [7–9].

Human fetal Hb (Hb F) possesses the same α subunit as Hb A, but its β subunit is replaced by similar, but not identical, γ subunits [10]. The homology of the primary structure between β and γ subunits is 73%. The comparison of structural properties between the α subunits in Hb A and Hb F is expected to provide useful information about the influence of intersubunit contacts on the electronic structure of the heme active site (structure in Fig. 1). NMR has played a key role in delineating the structure-function relationship in Hb [11,12]. The ability to detect structural perturbations in the heme cavity which results from the alteration of quaternary structure is largely enhanced if the protein is paramagnetic because of the high sensitivity of paramagnetically shifted signals to electronic/molecular structure of heme active site. NMR spectra of Hb F in its various oxidation and ligation states have been reported by Ho and coworkers [13–17]. Unfortunately, limited resolution of the signals did not allow detailed analysis of the spectra in terms of the structure of heme active sites. We report here the results of ^{1}H NMR comparative study between met-azido Hb A and Hb F, which demonstrate not only structural and functional non-equivalence of the two constituent subunits in these Hbs, but also the potential utility of Hb F in the assignment of NMR signals for Hb A. The fact that spectral properties of the α subunits in Hb A and Hb F are almost superimposable indicates that the structural coupling between subunit contacts and heme electronic structure of α subunit in met-azido Hb is relatively weak.

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Abbreviations: Hb A, human adult hemoglobin; Hb F, human fetal hemoglobin; Me, methyl group

2. Materials and methods

Hb A was prepared from blood obtained from the Medical Center at the University of Tsukuba using the reported procedure [18]. Hb F was isolated and purified in the carbonmonoxy form from the blood withdrawn from the cord of a patient, who agreed to the donation, in the Medical Center according to the method previously described [19]. Met-aquo Hb was prepared from carbonmonoxy Hb under a stream of oxygen with strong illumination in the presence of 5-fold molar excess of potassium ferricyanide (Wako Chemical Co.). The protein was separated from the residual chemicals using a Sephadex G-50 (Sigma Chemical Co.) column equilibrated with 50 mM Bis-Tris (Sigma Chemical Co.), pH 6.5. Met-azido Hb was prepared by the addition of 10-fold molar excess of sodium azide (Wako Chemical Co.) to met-aquo Hb. The Hb solution was concentrated to about 0.5 mM and the solvent was exchanged to ^2H_2O in an ultrafiltration cell (Amicon). The pH of the sample was adjusted using 0.2 M NaO^2H or 0.2 M ^{2}HCl, and the value was measured using a Horiba F-22 pH meter equipped with a Horiba type 6069-10C electrode. NMR spectra were recorded with a Bruker AC-400P FT NMR spectrometer operating at the ^{1}H frequency of 400 mHz. The spectra were obtained with about 3k transients, a spectral width of 20 kHz, 16k data points, a 9.5 μs 90° pulse, and a recycle time of 0.5 s. In the NMR titration of met-aquo Hb with azide ion, the spectra of Hb in the presence of a series of different concentrations of the ligand were obtained with the same experimental and data processing parameters. Chemical shifts are given in ppm downfield from sodium 2,2-dimethyl-2-silapentane-5-sulfonate with the residual H^{2}HO as an internal reference. Optical spectra were recorded with a Beckman DU 640 spectrophotometer.

3. Results

The downfield hyperfine shifted portion of 400 mHz ^{1}H NMR spectrum of met-azido Hb F in ^2H_2O, pH 7.8, at 45°C is compared with those of met-azido Hb A and cord blood Hb in Fig. 2. Six heme methyl proton resonances are resolved below 14 ppm in the spectrum of Hb A and have been assigned as indicated with the spectrum [20]. The same

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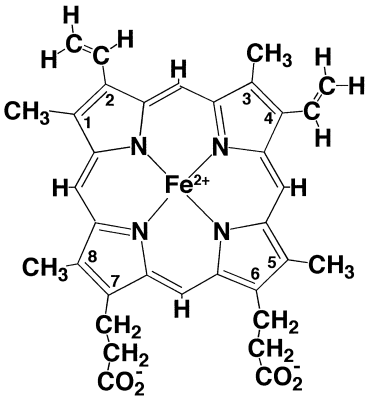

Fig. 1. The molecular structure and numbering system of heme.

number of heme methyl proton resonances are also resolved in the spectrum of Hb F. The shifts of three resonances in the spectrum of Hb F are essentially coincided with those of the signals for the α subunit in Hb A and hence they are assigned to the corresponding heme methyl protons of the α subunit in Hb F. The similarity in the shifts of the signals for the α subunits in both Hb F and Hb A indicates that the influence of subunit contact, i.e. the contact between α and β subunits in Hb A and that between α and γ subunits in Hb F, on the electronic structure of the heme in the α subunit is quite small. On the other hand, although the shifts of heme 5-methyl (5-Me) proton signals of the β and γ subunits are similar with each other, the shift differences of 0.92 and 1.26 ppm for 1- and 8-Me proton signals, respectively, at 45°C are observed between these subunits. Thus, as has been reported previously by Ho and coworkers [14], electronic structure of the constituent subunits in Hb A and Hb F can be clearly differentiated using NMR. The Curie plot, observed shift against reciprocal of absolute temperature, for the resolved heme methyl proton resonances of the individual subunits of met-azido Hb F and Hb A are compared in Fig. 3. The anti-Curie behavior observed for heme methyl proton signals of met-azido Hb has been analyzed quantitatively in terms of thermal spin equilibrium between high- and low-spin states [21]. In spite of a sizable difference in the shifts of 1- and 8-Me proton signals between the β and γ subunits, the slopes of their Curie plots are similar with each other.

Hb A is structurally inhomogeneous in terms of the orientation of heme relative to the protein and there are about 2% and 10% heme disorder in the α and β subunits, respectively [20,22]. Peaks a and b in the spectrum of met-azido Hb A arise from reversed heme in the β subunit. In the spectrum of met-azido Hb F, peak c is due to 8-Me proton of reversed heme in the α subunit and peaks d–f are likely to arise from such reversed heme in the γ subunit. The comparison of the intensities for the signals arising from two heme orientations in the individual subunits demonstrates that there are about 5% and 15% reversed heme in the α and γ subunits of Hb F, respectively. Therefore Hb F is structurally more disordered in terms of the heme orientation than Hb A. The spectrum of cord blood Hb reflects the presence of both Hb F and Hb A and the intensity of the signals for the β and γ subunits in the spectrum indicates that the ratio between the concentrations of Hb F and Hb A in cord blood is about 4.

The downfield portions, 14–33 ppm, of the 400 ^{1}H NMR spectra of met-aquo Hb F in ^2H_2O, pH 7.8, at 25°C in the

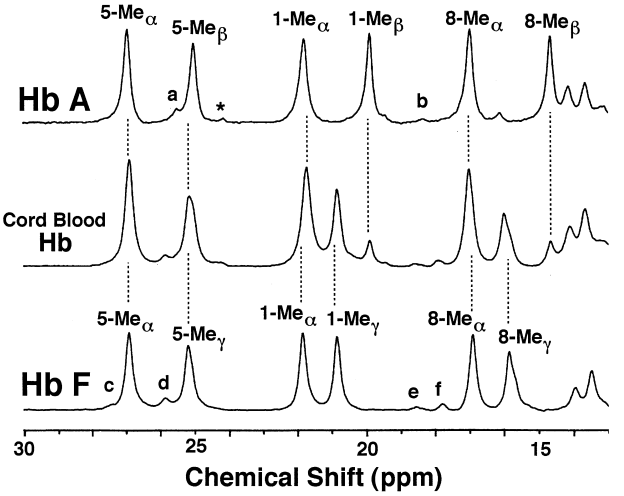

Fig. 2. Downfield hyperfine shifted portions of 400 mHz ^{1}H NMR spectra of met-azido forms of Hb F (lower trace), cord blood Hb (middle trace) and Hb A (upper trace) in ^2H_2O, pH 7.8, at 45°C. The assignments of heme methyl proton resonances are indicated with the spectra; subscript represents the subunit. Peaks a–f are due to reversed heme. Peaks labeled by * arise from impurity.

presence of different concentrations of azide ion are illustrated in Fig. 4. With increasing the concentration of the ligand, the intensity of the signals for met-azido form increases at the expense of the signals for met-aquo form. Since no signal from met-aquo form appears in the shift region of 23–33 ppm, the ratio of the concentrations of the individual subunits coordinated to the ligand, which in turn reflects relative affinity of the individual subunits to the ligand, can be estimated from the analysis of the 5-Me proton signal intensities of the two subunits. The preferential binding of the ligand to the γ subunit is apparent in Fig. 4 and this result is consistent with the earlier finding [14]. The value of 2.2 was obtained for the ratio of the affinity between the α and γ subunits in Hb F. The affinity of azide ion to Hb tetramer can be conveniently determined from optical titration study. Therefore the combined analysis of NMR and optical titration data permits the determination of the affinity of the individual subunits in in-

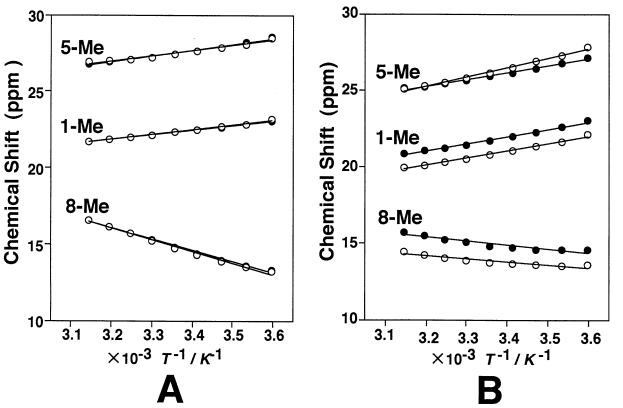

Fig. 3. The Curie plot, observed shift versus reciprocal of absolute temperature, of the resolved heme methyl proton signals for the (A) α subunit of Hb A (open circles) and Hb F (filled circles) and (B) β subunit of Hb A (open circles) and γ subunit of Hb F (filled circles). In A, the plots for the α subunits of both Hbs are almost superimposable.

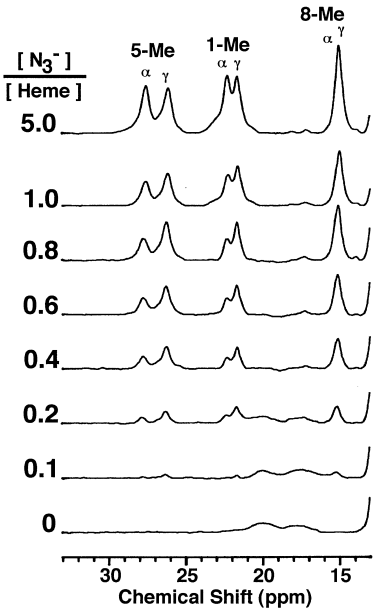

Fig. 4. Titration of met-aquo Hb F with azide ion in 0.1 M phosphate buffer, pH 8.0, at 25°C. The ratio of the concentration between Hb F and the ligand is indicated with the spectra.

tact Hb tetramer. The optical titration of met-aquo Hb F with azide ion yielded the equilibrium constant (K) of 2.8×10^{-6} M for the dissociation reaction in 0.1 M phosphate buffer, pH 6.8, at 25°C. Hence the K values of 4.4×10^{-6} and 2.0×10^{-6} M for the α and γ subunits, respectively, in intact Hb F were obtained. Similarly, the optical titration study of met-aquo Hb A with azide ion yielded the K value of 2.5×10^{-6} M and this value is smaller than the value reported earlier, i.e. 6.7×10^{-6} M [23]. From our K value, together with the value of 3.0 obtained for the ratio of the affinity between the α and β subunits of Hb A, determined by NMR titration, the K values of 4.8×10^{-6} and 1.6×10^{-6} M are obtained for the α and β subunits, respectively, in Hb A. The K values of the α subunits in both Hbs are essentially unchanged, indicating that subunit interaction does not affect the azide ion affinity of the α subunit. Moreover, the affinities of the β and γ subunits are also similar with each other and therefore the replacements of 39 out of 146 amino acid residues between these subunits does not influence their affinities.

4. Discussion

Owing the non-equivalence in the heme electronic structure of these three subunits, the spectral comparison between met-azido Hb F and Hb A provides unambiguous assignments of signals to the individual subunits of intact tetramer. The spectral similarity of the α subunits in both Hb complexes indicates that the electronic structure of heme active site in this subunit is not largely perturbed by subunit interaction. This result is consistent with the previous finding that there is no significant difference in the molecular structure between the α subunits of Hb A and Hb F [24]. There are replacements of four amino acid residues in the α_1-β_1 (or γ_1) interface between Hb A and Hb F and these replacements result in a considerable conformational change in this interface between these Hbs [10]. On the other hand, there is a single replacement, namely Glu CD2(43) in the β subunit is substituted to Asp in the γ subunit, in the α_1-β_2 (or γ_2) interface [10]. The side-chain of Glu CD2(43) in the β subunit forms a salt bridge to that of Arg FG4(92) in the α subunit in Hb A [24]. Due to the substitution of the residue at CD2(43) position in Hb F, the side-chain of Arg FG4(92) in the α subunit is bonded, via a salt bridge, to that of Gln C5(39) in the γ subunit. Therefore the loss of one intersubunit salt bridge is compensated by the formation of another in the α_1-γ_2 interface of Hb F. The similarity in the spectral properties of the α subunits in Hb A and Hb F dictates that the differences in these intersubunit contacts, due to the amino acid substitutions, between the two Hbs are not reflected in the electronic structure of the α subunit. Moreover, the absence of a strong subunit interaction in met-azido Hb has been demonstrated from the study of heme disorder, which revealed that the 10 possible structural isomers resulted from random heme orientation in subunits are not resolved through the heme electronic structure in the met-azido form, although partial resolution of these isomers, resulted from the fact that the environment of a given heme orientation in one subunit affects the heme electronic structure in another subunit, are allowed in other Hb derivatives [22].

In spite of the replacements of 39 amino acid residues between the β and γ subunits, the tertiary structures of these subunits have shown to be highly alike with each other, with some differences in the orientation of the N terminal and A helix relative to the other portion of the molecule. Therefore the observed shift differences of 1- and 8-Me proton signals between β and γ subunits might be attributed to the amino acid substitutions between them. The N terminal end of E helix is located near 1- and 8-Me in the subunits and seven amino acid residues in this region are substituted between the subunits [10]. In the β subunit, the distance between 1-Me proton and the center of the benzene ring of Phe E15(71) is <0.5 nm and, according to the crystal structure [25], an upfield shift bias by the ring current of Phe E15(71) is expected for 1-Me proton signal. This ring current shift diminishes and hence downfield shift of the signal is expected due to the replacement of Phe E15(71) by Leu in the γ subunit. On the other hand, 8-Me is located in the close proximity to the residue at E14(70) position. The replacement of Ala E14(70) in the β subunit to Ser in the γ subunit renders the chemical environment around 8-Me in the latter subunit relatively more polar than that in the former. Hence the shift difference observed for 8-Me proton signal between the β and γ subunits could be attributed to the difference in chemical environments. Although the sensitivity of paramagnetic shift to solvent polarity has been studied for model compounds [26], the effects of the polarity of the environments on the shift of heme methyl proton signal in paramagnetic hemoprotein have not been exploited. The present results suggest that the shifts are also influenced by local environments. The similarity in the slopes of Curie plots for 1- and 8-Me proton signals between the β and γ subunits, as witnessed in Fig. 3, also supports that the shift differences observed for these signals arise from diamagnetic contribution. Similar shifts for their 5-Me proton signals could be rationalized by the fact that amino acid residues around 5-Me are all conserved between the subunits.

The present study further confirmed that the constituent subunits in Hb tetramer are structurally and functionally non-equivalent. The azide ion affinities of the individual subunits in intact tetramer determined from the combined anal-

yses of NMR and optical titration data indicate that the functional property of the α subunit is essentially unchanged in both Hb A and Hb F and that the β and γ subunits are also functionally similar with each other. The functional similarity between these Hbs is correlated well with their structural resemblance [24].

The present study demonstrated the potential utility of Hb F in assigning NMR signals of Hb A to the individual subunits. The assignment strategy is based on the assumption that the molecular and electronic structures of the active site of the α subunits, which are common to both Hb A and Hb F, are almost identical in the two Hbs. Further comparative study between Hb A and Hb F in other oxidation and spin states may lead to the observation of effects of the alteration in subunit contacts on functional and structural properties of the α subunit.

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