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ASSIGNMENT OF PROXIMAL HISTIDYL IMIDAZOLE EXCHANGEABLE PROTON NMR RESONANCES TO INDIVIDUAL SUBUNITS IN HEMOGLOBINS A, BOSTON, IWATE AND MILWAUKEE

Gerd N. La Mar, Kiyoshi Nagai*, Thomas Jue, David L. Budd, K. Gersonde†, H. Sick†, T. Kagimoto§, A. Hayashi** and F. Taketa††

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SUMMARY

The proton nmr spectra of the synthetic valency hybrids, $\alpha_2(\beta^+CN)_2$, $(\alpha^+CN)_2\beta_2$ of hemoglobin A and the natural valency hybrids of the mutant hemoglobins Boston, Iwate and Milwaukee have led to the unambiguous assignment of the two proximal histidyl imidazole exchangeable proton signals at 64 and 76 ppm to individual α and β subunits, respectively. New single non-exchangeable proton resonances detected in the extreme downfield region of the spectra of Hbs Boston and Iwate are tentatively assigned to the coordinated tyrosine of the mutated α chains.

The investigation by proton nmr of structure-function relationships in hemoglobins is considerably aided by the hyperfine interactions (in their paramagnetic forms) which lead to the resolution of many of the resonances arising from the iron ligands in the heme pocket (1,2). Although all early studies focused on the presumed heme resonances (1), later comparison of the nmr spectra of high spin ferrous model compounds with that of deoxy Hb A, $\alpha_2\beta_2$, has provided the assignment of two exchangeable proton resonances which,

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Department of Chemistry, University of California, Davis, CA 95616
* 2nd Department of Physiology, Nara Medical College, Kashihara 634, Japan
† Department of Physiological Chemistry, TH Aachen, 5100 Aachen, Germany
§ 2nd Department of Internal Medicine, Kumamoto University Hospital, Kumamoto 860, Japan
**3rd Department of Internal Medicine, Osaka University Hospital, Osaka 530, Japan
††Department of Biochemistry, Medical College of Wisconsin, Milwaukee, WI 53226.

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by the nature of their unique hyperfine shift, must originate from the exchangeable N_1H's of the proximal histidyl imidazoles of the α,β subunits (3). Similar peaks have been found in other high spin ferrous hemoproteins (2).

The resolution of two such N_1H signals in deoxy Hb A is direct evidence for slight structural differences in the α and β subunits, and recent nmr studies of a series of modified and mutant hemoglobins (Nagai, K., La Mar, G.N. and Jue, T., manuscript in preparation) have revealed that these two resonances are highly sensitive probes of the quaternary state of deoxy hemoglobins. In view of the fact that control of the reactivity of the heme iron is proposed to originate primarily in the nature of the proximal histidine-iron interaction (4), definitive assignments of the N_1H resonances to individual subunits are essential to any interpretation of the tertiary structures within the subunits.

We report here on such an assignment based on the comparison of the proton nmr spectra of the synthetic valency hybrids (5), $\alpha_2(\beta^+CN)_2$, $(\alpha^+CN)_2\beta_2$ and the mutant hemoglobins Boston, $(\alpha^+[E7his\rightarrow tyr])_2\beta_2$, (6), Iwate, $(\alpha[F8his\rightarrow tyr])_2\beta_2$, (7), and Milwaukee, $\alpha_2(\beta^+[E7his\rightarrow glu])_2$, (8).

MATERIAL AND METHODS

Hb A, its isolated chains, Hb M Boston, Hb M Milwaukee, and Hb M Iwate were purified in the CO form as previously described (9,10). Carbon monoxide was removed in a rotary evaporator under a stream of oxygen and strong illumination. All Hb solutions were deionized by passage through Amberlite AG-501-X8. Solutions for NMR measurements were prepared in either ^2H_2O or 85% H_2O/15% ^2H_2O 0.1 M in bis Tris buffer, pH 6.5. The pH of the sample was determined using a Beckman 3550 pH meter equipped with an Ingold micro-combination electrode; no correction was made for isotope effect of ^2H_2O. The Hb solutions were deoxygenated by repeated evacuation and flushing with N_2 under gentle shaking. Cyano-met hybrid Hbs, $(\alpha^+CN)_2\beta_2$, $\alpha_2(\beta^+CN)_2$, were obtained by mixing deoxy α or β chain with a slight excess of the complementary chain in the cyano-met form just prior to the NMR measurement. Proton nmr spectra were obtained at 25°C and/or 35°C on a Nicolet NT-200 200 MHz FTNMR spectrometer. Typical spectra were obtained using a 20-30 kHz bandwidth, 4K data points, a 7 μs 90° pulse, and 3-6K scans. The water peak was suppressed with a ~30 ms presaturation pulse and the signal-to-noise improved by exponential apodization which introduced 50 Hz line broadening. Chemical shifts were referenced to the H_2O signal which in turn was calibrated against 2,2-dimethyl-2-silapentane-5-sulfonate, DSS.

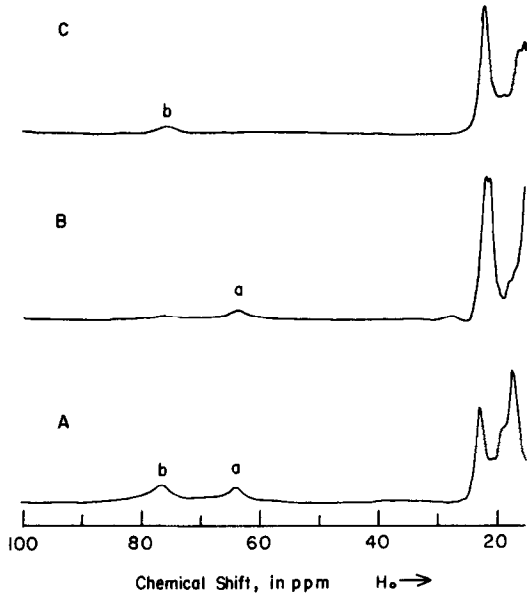

Figure 1: Downfield portions of the 200 MHz proton nmr spectra of Hb A, (A), and the valency hybrids $\alpha_2(\beta^+CN)_2$, (B), and $(\alpha^+CN)_2\beta_2$, (C), in 85% H_2O/15% ^2H_2O 0.1 M in bis-Tris, pH 6.5 at 25°C. The proximal histidyl imidazole N_1H peaks are a (73.7 ppm) and b (76.0 ppm) in Hb A, a (62.4 ppm, α subunit) in $\alpha_2(\beta\ CN)_2$, and b (74.7 ppm, β subunit) in $(\alpha\ CN)_2\beta_2$; chemical shifts are referenced to DSS.

RESULTS

The downfield regions of the 25°C proton nmr spectra in H_2O of deoxy Hb A and the synthetic valency hybrids, $\alpha_2(\beta^+CN)_2$ and $(\alpha^+CN)_2\beta_2$ in the presence of IHP are illustrated in Figure 1. Each of the valency hybrids exhibits primarily one of the two exchangeable N_1H peaks found in Hb A, leading directly to the assignment of peaks a (63.6 ppm) and b (76.7 ppm) to the α and β subunits, respectively. The presence of some residual intensity of the β chain N_1H in B is due to a small amount of heme exchange which leads to an equilibration with time of the reduced state between the two subunits.

The downfield portions of the 35°C proton nmr spectra of the mutant hemoglobins Iwate, Boston, and Milwaukee in H_2O and ^2H_2O are shown in Figure 2. For both Hb Iwate and Boston, the traces in H_2O differ from those in

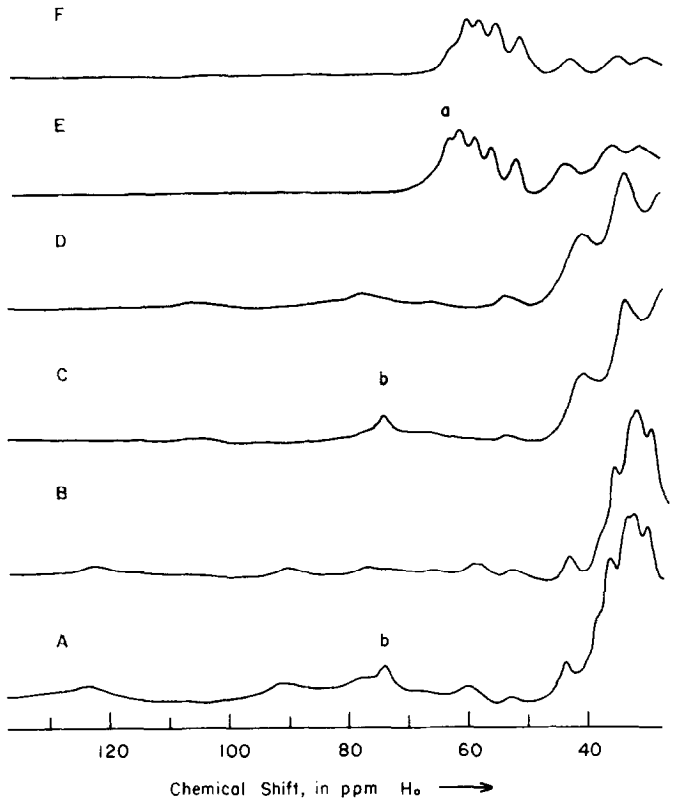

Figure 2: Downfield portions of the 200 MHz proton nmr spectra of Hb Iwate in H_2O, (A), and ^2H_2O, (B), Hb Boston in H_2O, (C), and ^2H_2O, (D), and Hb Milwaukee in H_2O, (E), and ^2H_2O, (F). All spectra are at 35°C, with the solution 0.1 M in bis-Tris at pH 6.5. The exchangeable resonances are marked b (β subunit) in Hb Iwate (73.9 ppm) and Hb Boston (73.6 ppm) and a (α subunit) in Hb Milwaukee (62.8 ppm); all chemical shifts are referenced to DSS.

^2H_2O primarily by the presence of an additional single exchangeable proton peak designated b. However, comparison of traces A-D also reveals the presence of a number of non-exchangeable single protons in the region 80-120 ppm present in both H_2O and ^2H_2O solution. The position of peak b (73.9 ppm in Hb Iwate, 73.6 ppm in Hb Boston) is completely consistent with its origin as the N_1H in the normal deoxy β subunit in Hb A (73.7 ppm at 35°C).

The traces for Hb Milwaukee fail to exhibit a clearly resolved exchangeable proton resonance. However, the shoulder (marked a) at 63 ppm has significantly higher intensity in H_2O (E) than ^2H_2O (F) solution, indicating

that a component of that resonance must arise from the N_1H of the deoxy subunit (62.2 ppm in Hb A at 35°C), which again is consistent with the assignments in Figure 1.

DISCUSSION

The comparison of the proton nmr spectra of the synthetic valency hybrids with that of deoxy Hb A leads to the unambiguous assignment of the 64 and 76 ppm signals to the N_1H of the α and β subunits, respectively. The larger hyperfine shift for the N_1H of the β subunit dictates that there is more extensive iron→imidazole spin transfer (3,11) in the β than α chains, and is consistent with the presence of slightly more strain in the histidine-iron linkage in the α than in the β subunits (12). Evaluation of the magnitude of this strain and its possible implications for control of oxygen affinity must await further studies on modified hemoglobins, which are in progress in these laboratories.

Our assignment of the N_1H resonances is consistent with conclusions drawn by Takahashi et al (13). However, their assignments, based solely on the comparison of the H_2O solution proton nmr spectra of Hb A, Hb Boston and Hb Milwaukee, did not recognize the presence of a resonance under peak b in Hb Boston which is present even in ^2H_2O.

The resonances in the region 25-40 ppm for Hbs Boston and Iwate and 25-70 ppm for Hb Milwaukee must arise from the mutated α and β met chains, respectively. The shift range for the met β chains in Hbs Iwate and Boston is considerably different from those observed in either metHb A (14) or even met myoglobin (15). Moreover, the non-exchangeable single proton resonances in the region 80-120 ppm have no precedent in the nmr spectra of any other ferric hemoprotein (1,2) and must be associated with the unique tyrosine coordination present in the α chain of both mutants (6,7). Based on some preliminary studies on model compounds (La Mar, G.N., Eguchi, K., to be published), we tentatively assign the furthest downfield non-exchangeable single proton resonances at 122 and 91 ppm at 35°C for Hb Iwate and at 105

ppm at 35°C for Hb Boston to the coordinated tyrosines. More extensive studies aimed at clarifying these latter assignments in the mutated chains are in progress.

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