NMR STUDY OF THE EXCHANGE RATES OF ALLOSTERICALLY RESPONSIVE LABILE PROTONS IN THE HEME POCKETS OF HEMOGLOBIN A

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ABSTRACT ^{1}H NMR spectroscopy has been used to measure the proximal histidyl labile ring proton (NH) rates of exchange with bulk solvent in the individual subunits of hemoglobin (Hb) A. These protons displayed a substantial decrease in their exchange rates in comparison with related monomeric proteins and exhibited sensitivity to the quarternary state. With the β subunit NH, the exchange behavior was similar to an allosterically responsive subset of protons, which have been identified using ^{1}H-^{3}H methods (Englander, J. J., R. Rogero, and S. W. Englander, 1983, *J. Mol. Biol.* 169:325–344). Assuming similar exchange mechanisms for the two subunits, the NMR data suggested a more flexible α than β subunit in Hb A.

INTRODUCTION

Even the highest resolution x-ray structures of hemoglobin (Hb) and myoglobin (Mb) (1, 2) suggest that the equilibrium structures do not possess channels large enough to permit ligand entry to and exit from the heme cavity. In the case of Mb, theoretical calculations (3) have shown that high frequency side chain and skeletal motions modulate the barrier to O_2 penetration and allow rationalization of the observed data. Thus the dynamics, as well as the structure of a protein, are likely to be relevant to its function (4, 5). One of the most powerful methods for studying rapid structural fluctuations in a protein is the observation of the exchange behavior of labile protons, which are buried in the protein interior (6, 7). The transient unfolding is a rapid but improbable event, and the resulting slow isotope exchange rates can be taken as a measure of the local dynamic stability (4–7).

The dominant isotope exchange work on Hb A has involved ^{1}H/^{3}H exchange of sites with exchange lifetimes from 10^2–10^5 s. The cumulative work with the hydrogen exchange characteristics of Hb A has clearly demonstrated that several subsets of residues are sensitive to the quarternary state (tense [T] or relaxed [R]) and manifest different exchange rates (6–12). Each of the five sensitive subsets, consisting of 4 to 18 protons, obeys first-order kinetics and experiences a 10 to 10^4 exchange-rate increase in oxy relative to deoxy Hb A. Some of these residues are putatively co-localized along with H helix of the protein (11). So far only one set of labile protons along the F-FG helix near the β heme cavity has been identified by hydrogen (H) exchange methods (12). Nevertheless, the data are interpreted to support the local unfolding model and to substantiate the plausibility of certain cooperativity models that detail the free-energy relationship associated with Hb's allosteric transition (7–9, 11, 12).

The ^{1}H/^{3}H exchange studies have the advantage of probing a large distribution of protons, but suffer from problems in analyzing the distribution of rates and in localizing the labile subsets of protons. Thus, only one subset of protons in one subunit of Hb A has been localized by meticulous proteolytic cleavage (12). Even though ^{1}H NMR cannot readily detect a significant number of protons in such a large molecule, it does offer the advantage of ready assignment of the labile protons that are located in the heme activity (13–16), the precise place where we would like to measure the structural fluctuations. For Hb A, the labile ring proton of the proximal histidine provides an ideal probe of the fluctuations that directly affect the heme cavity (17–20). In the deoxy state, the ^{1}H NMR spectrum shows two exchangeable resonances at 76

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and 64 parts per million (ppm) that, according to studies on models (14) and on Hb valency hybrids (15) and mutants (15, 16), originate from the proximal histidyl imidazole NH for β and α subunits, respectively. We demonstrate herein that NMR provides an effective technique for characterizing the quarternary state and subunit dependence of the local dynamics of the heme pocket.

EXPERIMENTAL PROCEDURE

We obtained packed red blood cells from the local blood bank and extracted Hb A according to procedures outlined by standard literature methods (21). Hb A was kept in the CO form until use. Optical spectroscopic measurements indicate <5% metHb present. The HbO_2 solution was concentrated to 25–30%. About 200 μl of this concentrated solution was placed in a 5-mm NMR tube with the appropriate buffer to make the final solution 0.1 M Tris and 0.2 M NaCl. For the deoxy Hb A studies, a slight molar excess of dithionite was introduced into the sample tube, which had been evacuated and flushed with N_2 several times. Immediately 250 μl of deaerated ^2H_2O was added to make the final protein solution concentration ~10%. The NMR spectra were immediately recorded and the time development followed. In the case of HbO_2, ^2H_2O was allowed to exchange for a required period of time and then dithionite was added to remove the oxygen. Even though the NMR spectra continued to monitor the NH peaks in the deoxy form, the intensity of the peaks reflected the extent of ^2H_2O exchange in the HbO_2 form.

All NMR spectra were recorded on a (NT 200 FT; Nicolet Instrument Corp., Madison, WI) spectrometer equipped with quadrature detection. The 90 degree pulse length was 7 μs; spectral window 20 kHz; and the free induction decay exponentially apodized to improve signal-to-noise ratio. Each spectrum was collected with 2,000–5,000 scans. The residual solvent resonance was reduced with a 60-ms presaturation pulse. All peaks were referenced to the residual water line that, in turn, was calibrated against 2,2-dimethyl-2-silapentane-5-sulfonate, DSS.

RESULTS AND DISCUSSION

Fig. 1 shows the time course of deoxy Hb A proximal histidyl ring NH exchange upon dilution with ^2H_2O to a final solution concentration of 50% ^2H_2O. The rate of intensity loss as a function of time is illustrated in Fig. 2. The same exchange rates are obtained either from the initial slope method or by a reversible first-order kinetics scheme. For the α subunit NH the half-life is $\sim 1.5 \times 10^4$ s, whereas for the β subunit NH it is $\sim 2.6 \times 10^5$ s at 25°C. When the protein undergoes a T $\rightarrow$ R transition upon ligation with O_2, the exchange rates increase significantly (Fig. 2). Although the contribution from tertiary structural changes upon ligation is not known, preliminary studies with deoxy β tetramer (not shown), which is in the R state, suggest that the quarternary structural changes play a pivotal role in influencing the NH exchange rates.

We observe that the exchange rates of the proximal histidyl ring NHs in deoxy Hb are several orders of magnitude slower than in any of the monomeric heme proteins (17–20). Such exchange-rate retardation is consistent with the additional quarternary constraint imposed by a tetrameric protein. Moreover, the β subunit's NH exchange rate is similar to that obtained (7) from recent ^1H-^3H experiments that localized an allosterically responsive set of protons in the β subunit along the F-FG helix (12). Although the pH behavior and mechanism of the

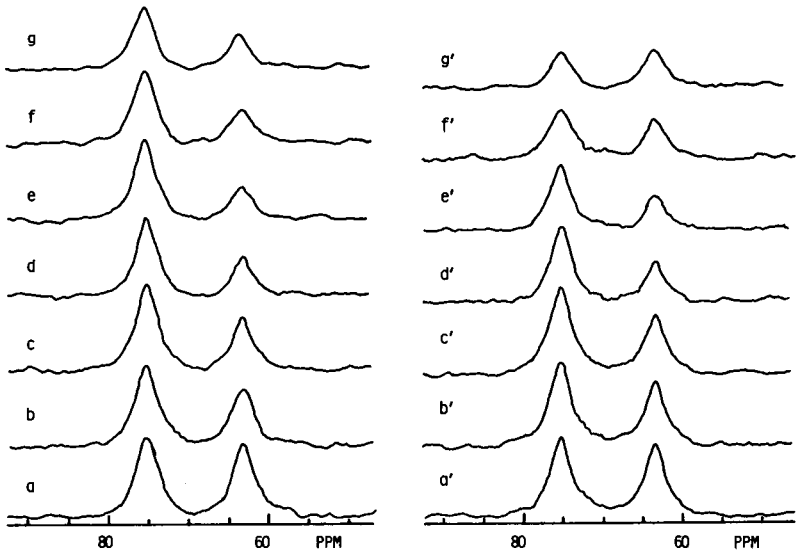

FIGURE 1 The time evolution of the ^1H-^2H exchange of the proximal histidyl NH resonances, α(64 ppm) and β(76 ppm) subunits of Hb A after mixing deoxy Hb A in H_2O with ^2H_2O to give a final ^2H_2O concentration of 50% is shown in traces *a*–*g*. Deoxy Hb A was in 0.1 M *bis*-Tris, 0.2 M NaCl, at pH 6.97, and 25°C. The traces correspond to the time after mixing with ^2H_2O: (*a*) 7 min, (*b*) 9×10^1 min, (*c*) 1.5×10^2 min, (*d*) 2.5×10^2 min, (*e*) 4.2×10^2 min, (*f*) 9.0×10^2 min, (*g*) 1.7×10^3 min. Traces *a'*–*g'* reflect analogous time evolution with HbO_2. ^2H_2O was added to HbO_2 to give a final ^2H_2O concentration of ~55%. After a variable mixing time in which ^1H-^2H exchanged in HbO_2, we introduced dithionite to remove the O_2 and monitored the extent of exchange with the intensity loss of the proximal histidyl NH resonances in the deoxy form of Hb A. HbO_2 was also in 0.1 M *bis*-Tris, 0.2 M NaCl, at pH 6.95, and 25°C. Traces *a'*–*g'* reflect the ^1H-^2H mixing time in HbO_2: (*a'*) 2×10^{-1} min, (*b'*) 6×10^{-1} min, (*c'*) 1.5 min, (*d'*) 2.5 min, (*e'*) 3×10^1 min, (*f'*) 1.2×10^2 min, (*g'*) 1.8×10^2 min.

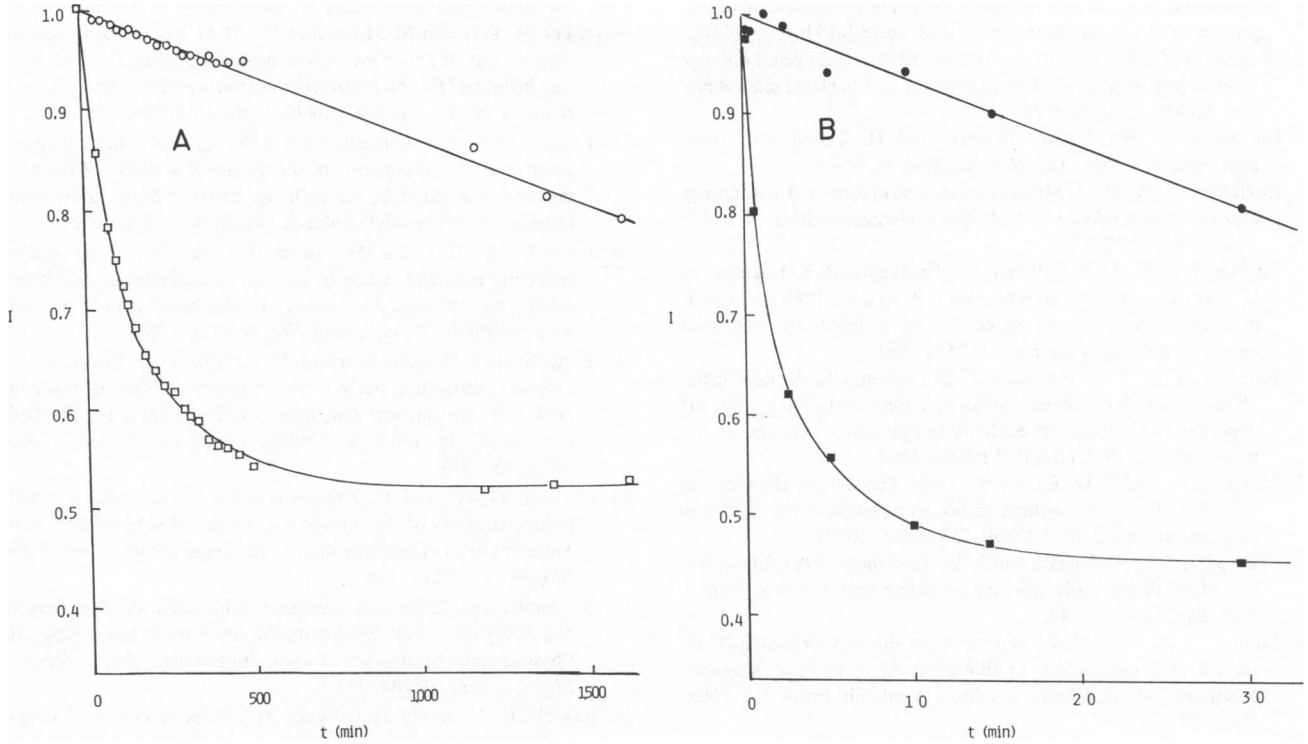

FIGURE 2 Plot of residual intensity of proximal histidyl imidazole NHs of the nonequivalent subunits of Hb A at various times after mixing Hb A in H_2O with ^2H_2O to give a final concentration ~50%. Hb A was in 0.1 M *bis*-Tris, 0.2 M NaCl, at pH 6.97, and 25°C. In *A* the top curve is for the deoxy β subunit O and the bottom curve is for the deoxy α subunit □. Analysis of the data shows the exchange half-life of the α NH to be 2.7×10^5 s and for the β NH to be 1.5×10^4 s. In *B* the plot corresponds to ^1H-^2H exchange by diluting HbO_2 with ^2H_2O to a final concentration ^2H_2O of 55%. Immediately, dithionite was added to remove oxygen. The time course of the exchange of HbO_2 for the β NH exchange is reflected on the top curve (●) and for the α NH exchange on the bottom curve, ■. When compared with deoxy Hb A NH exchange rates, the β NH exchange half-life in HbO_2 has decreased by a factor of ~40, whereas for the α NH, it has decreased by a factor of ~80.

exchange of the His F8 ring NHs have not been determined, the large increase in exchange rate in HbO_2 relative to deoxy Hb, by a factor comparable with that reported for a subset of protons by Englander et al. (9–12), indicates that not only are these protons directly in the heme cavity allosterically responsive, but the β subunit NH may belong to the same subset characterized by ^1H-^3H exchange (12).

Differences in proximal histidyl NH exchange rates have been shown to be interpretable in terms of the differential overall rigidity of the heme pocket in monomeric heme proteins (17–20). Assuming similar mechanisms of exchange for the two subunits, the relative exchange rates of the α and β subunit His F8 ring NHs may be taken in support of significantly greater flexibility on the proximal side of the heme in the α than the β subunit. This would be in contrast to ligand binding (22, 23) and reduction (24) rates that were found to be faster in the β subunits than in the α subunits.

We have demonstrated the utility of a simple and rapid ^1H NMR method for monitoring exchange rates of labile protons directly in the heme environment in Hb A, and propose that this method may have general applicability for effectively examining the dynamic processes affecting the heme pocket in other hemoproteins. Additional work in this laboratory is underway to define the mechanisms of NH exchange in Hb A and to explore the general utility of this simple NMR probe for studying potentially allosterically responsive dynamic processes in mutant and modified Hbs.

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