

A Comparative NMR Study of the Polypeptide Backbone Dynamics of Hemoglobin in the Deoxy and Carbonmonoxy Forms[†]

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ABSTRACT: Model-free-based NMR dynamics studies have been undertaken for polypeptide backbone amide N–H bond vectors for both the deoxy and carbonmonoxy forms of chain-specific, isotopically (¹⁵N and ²H) labeled tetrameric hemoglobin (Hb) using ¹⁵N-relaxation parameters [longitudinal relaxation rate (*R*₁), transverse relaxation rate (*R*₂), and heteronuclear nuclear Overhauser effect (NOE)] measured at two temperatures (29 and 34 °C) and two magnetic field strengths (11.7 and 14.1 T). In both deoxy and carbonmonoxy forms of human normal adult hemoglobin (Hb A), the amide N–H bonds of most amino acid residues are rigid on the fast time scale (nanosecond to picosecond), except for the loop regions and certain helix–helix connections. Although rigid in deoxy-Hb A, β146His has been found to be free from restriction of its backbone motions in the CO form, presumably due to the rupture of its hydrogen bond/salt bridge network. We now have direct dynamics evidence for this structural transition of Hb in solution. While remarkably flexible in the deoxy state, α31Arg and β123Thr, neighbors in the intradimer (α₁β₁) interface, exhibit stiffening upon CO binding. These findings imply a role for α31Arg and β123Thr in the intradimer communication but contradict the results from X-ray crystallography. We have also found that there is considerable flexibility in the intradimer (α₁β₁) interface (i.e., B, G, and H helices and the GH corner) and possible involvement of several amino acid residues (e.g., α31Arg, β3Leu, β41Phe, β123Thr, and β146His) in the allosteric pathway. Several amino acid residues at the intradimer interfaces, such as β109Val, appear to be involved in possible conformational exchange processes. The dynamic picture derived from the present study provides new insights into the traditional description of the stereochemical mechanism for the cooperative oxygenation of Hb A based on X-ray crystallographic results.

Hemoglobin is an indispensable protein of vertebrates and is the classic molecule for understanding the structural basis of a complex set of cooperative, linked interactions. The oxygenation of hemoglobin is regulated by interactions between its O₂-binding sites (homotropic interactions) and interactions between individual amino acid residues in the protein molecule and various solutes (heterotropic interactions). Heterotropic effectors include hydrogen ions, chloride ions, CO₂, inorganic phosphate, inositol hexaphosphate (IHP),¹ and 2,3-bisphosphoglycerate (2,3-BPG). Human normal adult hemoglobin (Hb A) has a molecular mass of ~64.5 kDa, consisting of four subunits, namely, two identical α-chains of 141 amino acids each and two identical β-chains of 146 amino acids each. For a review on hemoglobin, see

Dickerson and Geis (1). The arrangement of the subunits (i.e., quaternary structure) of Hb depends on the ligation state of the protein. Two classical quaternary structures are the T (tense) form for the low-affinity deoxy-Hb and the R (relaxed) form for the high-affinity oxy-Hb (crystals grown in high salt conditions), first reported by Perutz (2). There are two distinct types of subunit interfaces, α₁β₁ or α₂β₂ (intradimer) and α₁β₂ or α₂β₁ (interdimer). On the basis of the crystal structures of ligated and unligated Hb A, the α₁β₂ or α₂β₁ interface undergoes a sliding motion in going from the deoxy to the oxy state, whereas the α₁β₁ or α₂β₂ interface remains unchanged during the ligation process. A new quaternary structure in the ligated state, R₂ (crystals grown in low-salt conditions), was observed by Silva et al. (3), and more recently, the R₂ structure has also been observed under high-salt conditions (4). Kavanaugh et al. (5) reported their crystallographic studies on a number of mutants with mutations in the β37Trp cluster and have identified structural transitions referred to as T-to-T_{high} quaternary transitions, i.e., between the quaternary T structure of wild-type deoxy-Hb A and an ensemble of related T-like quaternary structures that are induced by some mutations in the β37Trp cluster and/or by exposing crystals of wild-type or mutant deoxy-Hbs to O₂. Using residual dipolar coupling (RDC) measurements, we have found that the solution structure of HbCO A is a dynamic ensemble of R and R₂ crystal structures (6).

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¹ Abbreviations: Hb A, human normal adult hemoglobin; HbCO, carbonmonoxyhemoglobin; deoxy-Hb, deoxyhemoglobin; met-Hb, methemoglobin; NMR, nuclear magnetic resonance; *R*₁, longitudinal relaxation rate; *R*₂, transverse relaxation rate; *S*, generalized order parameter; RDC, residual dipolar coupling; NOE, nuclear Overhauser effect; IHP, inositol hexaphosphate; 2,3-BPG, 2,3-bisphosphoglycerate; HSQC, heteronuclear single-quantum coherence; TROSY, transverse relaxation-optimized spectroscopy.

More recently, we have assigned the polypeptide backbone resonances of deoxy-Hb A (7) and have observed spontaneous preferential orientation of deoxy-Hb A in solution at high magnetic fields, thus allowing us to obtain RDC values (8). By comparing the measured RDCs with the calculated RDCs (based on the crystal structure of deoxy-Hb A, PDB ID 1XXT), it appears that the solution structure of deoxy-Hb A may also be different from the crystal structure. The functional properties of Hb A in crystals are distinctly different from those in solution (9). This raises a very important point, namely, whether the structure of Hb in solution is not the same as that in the crystalline state, or the crystal structure may be one of many possible structures that can exist in solution. Thus, the conformational states (e.g., motion of the polypeptide chains and/or side chains) of the Hb molecule in solution and in crystals could be different. In order to better understand the Hb molecule, we need to correlate its structure, dynamics, and function in deoxy, oxy, and partially oxygenated states under physiological conditions. The CO-ligated form of Hb A is a good model for the oxy form and is considerably more stable, thus being more amenable for time-consuming NMR measurements than the oxy form that oxidizes spontaneously over time in solution becoming the nonfunctional methemoglobin (met-Hb).

The development of high-field multinuclear, multidimensional NMR and isotopic labeling techniques offers a realistic possibility for determining the structures of proteins of the size of Hb A and their dynamic properties in solution (6, 8, 10–20). This opens up the possibility of making a direct comparison between the crystal and solution structures of Hb A and of correlating the information derived from kinetic, thermodynamic, and spectroscopic studies with that obtained from studies of structure. Many of the controversial issues discussed above may then be resolved.

In this paper, we describe our results on the polypeptide backbone dynamics of Hb A in both deoxy and CO forms based on the Model-free analysis (21, 22) as a first step to correlate the structure, dynamics, and function relationship of this important allosteric protein. We have found some interesting results, and some of them are not predicted by crystal structures of Hb A in deoxy and CO forms. For example, we have found that there is considerable flexibility in the intradimer ($\alpha_1\beta_1$ or $\alpha_2\beta_2$) interface (i.e., B, G, and H helices and the GH corner) and possible involvement of several amino acid residues (e.g., $\alpha 31$ Arg, $\beta 3$ Leu, $\beta 41$ Phe, $\beta 123$ Thr, and $\beta 146$ His) in the allosteric pathway. Two residues, $\alpha 31$ Arg and its neighbor, $\beta 123$ Thr, exhibit interesting dynamic behavior and suggest that they play a role as cementing and communicating connectors between the two intradimer ($\alpha_1\beta_1$ and $\alpha_2\beta_2$) subunits.

MATERIALS AND METHODS

Hb A Samples. Uniformly (^2H , ^{15}N) labeled recombinant Hb A was expressed and purified as described previously (23, 24). Two types of chain-specifically labeled hemoglobin samples, namely, (α -labeled)(β -unlabeled) and (α -unlabeled)(β -labeled), each in the deoxy or CO form, were prepared by the procedures used in our laboratory (12, 25), equilibrated with 0.1 M sodium phosphate buffer at pH 7.0 in 90% water and 10% D_2O , reaching a final Hb concentration of 5%

(~ 0.8 mM). These α - and β -chain chain-specifically labeled tetrameric Hb samples were used in all of our relaxation measurements.

^{15}N Relaxation Measurements. The backbone ^{15}N R_1 , R_2 , and steady-state ^{15}N - $\{^1\text{H}\}$ NOE were measured at 11.7 and 14.1 T on Bruker DRX-500 and DRX-600 NMR spectrometers at 29 and 34 °C using 5 mm inverse detection probes equipped with triple-axis pulsed-field gradients. The pulse sequences of Farrow et al. (26, 27) were used with minor modifications. The carrier frequency for the directly detected ^1H dimension was set to the water resonance, with spectral widths of 20.0 ppm for the deoxy samples and 17 ppm for the CO samples at both fields, using digital quadrature (DQD) detection and typical time domain of 2K (1024 complex points). The water signal was suppressed using the 3-9-19 WATERGATE sequence. For the indirectly detected (^{15}N) dimension, the spectral width was typically 25 ppm for the CO samples and 20.6 or 22.8 ppm for deoxy, with the carrier at 122 ppm (CO) and at 121 or 118 ppm for deoxy. Typically, 128 time domain points (64 complex) were acquired in the States mode (with receiver phase inversion). ^{15}N R_1 relaxation rates were measured using eight data points with seven relaxation delays (repeated values marked with *): 0.11, 0.33, 0.66, 1.32*, 2.64, 4.4, and 6.6 s for both fields. ^{15}N R_2 experiments were performed with 8-relaxation delays (10 data points): 0, 16, 32*, 48, 64*, 80, 96, and 112 ms for both fields. Both R_1 and R_2 measurements were performed with a 3 s recovery delay and typically 32 (at 14.1 T) or 48 scans (at 11.7 T). Heteronuclear NOE measurements were performed using similar spectral parameters, except that for the ^{15}N dimension sometimes the time domain was 96, other times up to 140. Typically, the number of scans was 96, but up to 144 were used on occasion. The NOE experiment employed a 12 s proton saturation period, and the control (no NOE) employed a 12 s delay instead. NMR relaxation data were processed using NMRPipe (28) software on SGI or SUN workstations. The peak heights and volumes of the cross-peaks were calculated using the nonlinear least-squares fit (nlinLS) implemented in NMRPipe.

Analysis of Relaxation Data. The backbone dynamics information of Hb was obtained on the basis of the Model-free formalism, as described by Lipari, Szabo, Clore, and co-workers (21, 22, 29). The order parameter (simplified term of the square of the generalized order parameter), S^2 , describes the amplitude of the internal picosecond to nanosecond motions of a particular bond vector and ranges from $S^2 = 0$ for a bond vector rapidly and isotropically sampling multiple orientations to $S^2 = 1$ for no internal motion. The order parameter S^2 can be fitted by an “extended model” from the NMR spectral density functions at the Larmor frequencies ω for certain spins (^1H , ^{15}N , ^{13}C , etc.), $J(\omega)$, as

$$J(\omega) = \frac{2}{5} \left[\frac{S^2 \tau_m}{1 + (\omega \tau_m)^2} + \frac{(1 - S^2) \tau'_f}{1 + (\omega \tau'_f)^2} + \frac{(S^2 - S^2) \tau'_s}{1 + (\omega \tau'_s)^2} \right] \quad (1)$$

where $\tau'_f = \tau_f \tau_m / (\tau_f + \tau_m)$, $\tau'_s = \tau_s \tau_m / (\tau_s + \tau_m)$, with τ_m as the isotropic rotational correlation time of the molecule, τ_f and τ_s as the effective correlation times for internal motions on a fast time scale ($\tau_f < 100$ – 200 ps) and slow time scale

Table 1: Isotropic, Axially Symmetric, and Fully Anisotropic Diffusion Parameters Determined for Deoxy and CO (in Parentheses) Forms of Hb Using R1R2-Diffusion(I) and Quadric_1.11(II)^a

deoxy (CO)	tensor	field (T)	$\tau_{c,eff} = (6D_{iso})^{-1}$ (ns)	$2D_z/(D_x + D_y)$	D_x/D_y
29 °C	I	isotropic	11.7	32.74 ± 0.05 (32.00 ± 0.06)	
			14.1	31.62 ± 0.05 (30.68 ± 0.06)	
		axial	11.7	32.71 ± 0.05 (32.07 ± 0.06)	1.09 ± 0.01 (1.07 ± 0.01)
			14.1	31.65 ± 0.05 (30.75 ± 0.06)	1.12 ± 0.01 (1.11 ± 0.02)
	II	isotropic	11.7	32.93 ± 0.05 (32.06 ± 0.06)	
			14.1	31.92 ± 0.05 (30.79 ± 0.06)	
		axial	11.7	32.98 ± 0.05 (32.13 ± 0.06)	1.10 ± 0.01 (1.08 ± 0.01)
			14.1	31.89 ± 0.05 (30.84 ± 0.06)	1.11 ± 0.01 (1.13 ± 0.02)
		anisotropic	11.7	32.91 ± 0.06 (32.15 ± 0.07)	1.09 ± 0.01 (1.07 ± 0.01)
			14.1	31.94 ± 0.06 (30.80 ± 0.06)	1.11 ± 0.01 (1.14 ± 0.02)
	I	isotropic	11.7	28.90 ± 0.03 (27.91 ± 0.05)	
			14.1	28.04 ± 0.03 (27.58 ± 0.04)	
34 °C	I	isotropic	11.7	28.80 ± 0.03 (28.02 ± 0.05)	1.10 ± 0.01 (1.05 ± 0.01)
			14.1	28.08 ± 0.04 (27.69 ± 0.04)	1.11 ± 0.01 (1.06 ± 0.01)
	II	isotropic	11.7	29.05 ± 0.04 (27.96 ± 0.05)	
			14.1	28.27 ± 0.04 (27.63 ± 0.04)	
		axial	11.7	28.94 ± 0.03 (27.98 ± 0.05)	1.10 ± 0.01 (1.06 ± 0.01)
			14.1	28.28 ± 0.04 (27.70 ± 0.04)	1.12 ± 0.01 (1.10 ± 0.01)
	I	isotropic	11.7	28.94 ± 0.04 (28.05 ± 0.06)	1.01 ± 0.01 (1.04 ± 0.01)
			14.1	28.27 ± 0.04 (27.71 ± 0.05)	1.01 ± 0.01 (1.01 ± 0.01)

^a Principal components only.

($\tau_f < \tau_s < \tau_m$), respectively, $S^2 = S_f^2 S_s^2$ is the square of the generalized order parameter, and S_f^2 and S_s^2 are the squares of the order parameters for the internal motions on the fast and slow time scales. The spectral density functions are computed from the experimental relaxation parameters, R_1 , R_2 , and NOE, by (30)

$$R_1 = (d^2/4)[J(\omega_H - \omega_X) + 3J(\omega_X) + 6J(\omega_H + \omega_X)] + c^2 J(\omega_X)$$

$$R_2 = (d^2/8)[4J(0) + J(\omega_H - \omega_X) + 3J(\omega_X) + 6J(\omega_H) + 6J(\omega_H + \omega_X)] + (c^2/6)[4J(0) + 3J(\omega_X)] + R_{ex}$$

$$NOE = 1 + (d^2/4R_1)(\gamma_H/\gamma_X)[6J(\omega_H + \omega_X) - J(\omega_H - \omega_X)] \quad (2)$$

where $d = \mu_0 h \gamma_X \gamma_H \langle r_{XH}^{-3} \rangle / (8\pi^2)$, $c = \omega_X \Delta\sigma / (3)^{1/2}$, μ_0 is the permeability of free space, h is Planck's constant, γ_H and γ_X are the gyromagnetic ratios of 1H and the X spin ($X = ^{13}C$ or ^{15}N), respectively, r_{XH} is the X-H bond length [$r_{NH} = 1.02 \text{ \AA}$ (31) was used in our analysis], and $\Delta\sigma = \sigma_{||} - \sigma_{\perp}$ is the chemical shift anisotropy in an axial symmetric system of the X spin [$\Delta\sigma = -172 \text{ ppm}$ (32) was used for ^{15}N in our analysis].

The Model-free program version 4.10 (33) was used in our backbone dynamics analysis. This program uses Monte Carlo simulations to estimate uncertainties in the Model-free parameters and helps to assign appropriate models for different residues according to a statistical protocol including the F -test (33, 34). Five subsets of the above extended model are provided for model selection, with reduced number of parameters under certain conditions (as in the parentheses): (1) $S^2 = S_f^2$, ($S_s^2 = 1$, $\tau_f \rightarrow 0$, $\tau_s = 0$); (2) $S^2 = S_f^2$, $\tau_e = \tau_f$, ($S_s^2 = 1$, $\tau_s = 0$); (3) $S^2 = S_f^2$, R_{ex} , ($S_s^2 = 1$, $\tau_f = 0$, $\tau_s = 0$); (4) $S^2 = S_f^2$, $\tau_e = \tau_f$, R_{ex} , ($S_s^2 = 1$, $\tau_s = 0$); (5) S_f^2 , S_s^2 , $\tau_e = \tau_s$, ($\tau_f \rightarrow 0$). In the models with R_{ex} , nonzero chemical exchange contributions to the R_2 relaxation rates are considered.

We have applied the statistical approach, as described by Mandel et al. (33), to assign the best motional model for each amino acid residue. Most residues can be fitted satisfactorily. For each residue not fitted well by any of the five models, the model with the minimum sum of squared errors (SSE) between the dynamic model and the experimental data was used (35).

A complete listing of our relaxation data for deoxy- and carbonmonoxy-Hb A at 11.7 and 14.1 T and at 29 and 34 °C is given in Supporting Information.

Diffusion parameters were determined independently by two different programs, R2R1-Diffusion (36) and Quadric_diffusion 1.11 (37). Three-dimensional crystal structures of Hb A [PDB IDs 1A3N (38) and 1BBB (3)] were used for deoxy- and carbonmonoxy-Hb A, respectively. All residues with NOE < 0.65 and significantly deviating R_2/R_1 and R_1R_2 values (39) were excluded from the analysis.

RESULTS

Uniformly (2H , ^{15}N) chain-specifically labeled deoxy- and carbonmonoxy-Hb A samples were prepared as 5% solutions (~0.8 mM) in aqueous phosphate buffer. The polypeptide backbone ^{15}N R_1 , R_2 , and steady-state ^{15}N - $\{^1H\}$ NOE were measured at 11.7 and 14.1 T at 29 and 34 °C. The backbone dynamics information of Hb A was obtained on the basis of the Model-free formalism. S^2 , the square of the generalized order parameter, describes the amplitude of internal picosecond to nanosecond motions for a particular bond vector and ranges from $S^2 = 0$, for a bond vector rapidly sampling multiple orientations, to $S^2 = 1$, for no internal motion. Diffusion parameters τ_c , $2D_z/(D_x + D_y)$, and D_x/D_y were determined independently by two different programs, R2R1-Diffusion (36) and Quadric_diffusion 1.11 (37) (see Materials and Methods for details).

Diffusion Analysis. The principal components of the diffusion tensors are summarized in Table 1. The values of the relaxation parameters, $\tau_c = (6D_{iso})^{-1}$, $2D_z/(D_x + D_y)$, and D_x/D_y , obtained from different methods, are essentially identical. There is an apparent and expected temperature

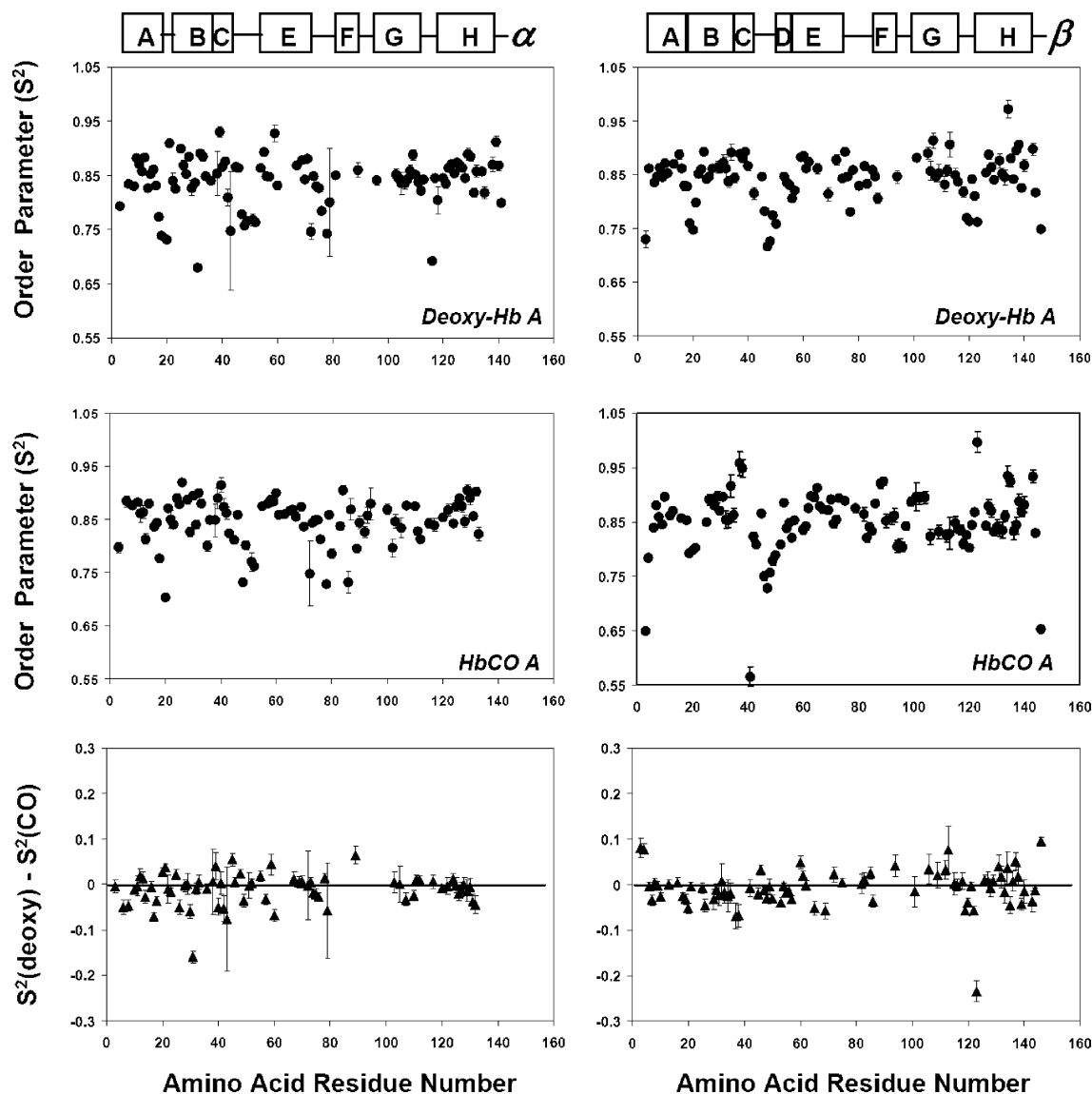


FIGURE 1: Order parameters (S^2) of amide N–H bonds in Hb at 29 °C.

effect on the tumbling time, τ_c , with shorter τ_c at 34 °C (27.5–29 ns) than that at 29 °C (31.5–33 ns). Slightly but uniformly, under the same conditions, τ_c values for HbCO A are shorter than those for deoxy-Hb A; also slightly but uniformly, for the same sample, τ_c values measured at the higher field (14.1 T) are shorter than those at the lower field (11.7 T), when other experimental conditions are identical. The anisotropy ratios, $2D_z/(D_x + D_y)$, are around 1.10, showing slight rotational anisotropy of Hb, with no significant variation for different forms and conditions.

Model-Free Analysis. As seen in Figure 1, deoxy-Hb A and HbCO A share similar backbone mobility distribution profiles, where the amide N–H bonds of most amino acid residues are rigid, except for the loop regions. The CE loop (F43–G51), EF loop (H72–A79), and GH loop (L113–F117) in the α -chain and the CD loop (E43–S49), EF loop (H77–T84), and GH loop (F118–F122) in the β -chain are apparently more mobile than the neighboring helical regions. There is no loop structure between the A and B helices. However, the residues close to the A–B connection, V17–G22 in the α -chain and V18–D21 in the β -chain, exhibit great flexibility, comparable to any of the loop regions. On

the other hand, some residues, such as T39 and G59 in the α -chain and F37, T38, V134, and A135 in the β -chain, are consistently very rigid in all cases.

Besides the common helix–loop contrast in both the deoxy and carbonmonoxy forms of Hb A, certain amino acid residues demonstrate differential rigidity with and without CO binding (Figure 1). Two neighboring residues at the intradimer ($\alpha_1\beta_1$ or $\alpha_2\beta_2$) interface, α 31Arg and β 123Thr, exhibit the most dramatic mobility change due to CO binding, with greatly increased rigidity in the carbonmonoxy form, compared to that in the deoxy form, at both 29 and 34 °C. To a lesser degree, the motion at residue β 69Gly is also more restricted in HbCO A. On the contrary, residues β 146His and β 3Leu (not shown in Figure 1 but shown in Supporting Information) become more flexible in the CO form than in the deoxy form. β 41Phe is among the most mobile residues in HbCO A. Unfortunately, no comparable dynamics information for β 41Phe is available for the deoxy form, as this residue does not give rise to an observable signal.

According to the Model-free formalism, the R_{ex} terms in models 3 and 4 are indicators of slow (microsecond to millisecond time scale) conformational exchange processes.

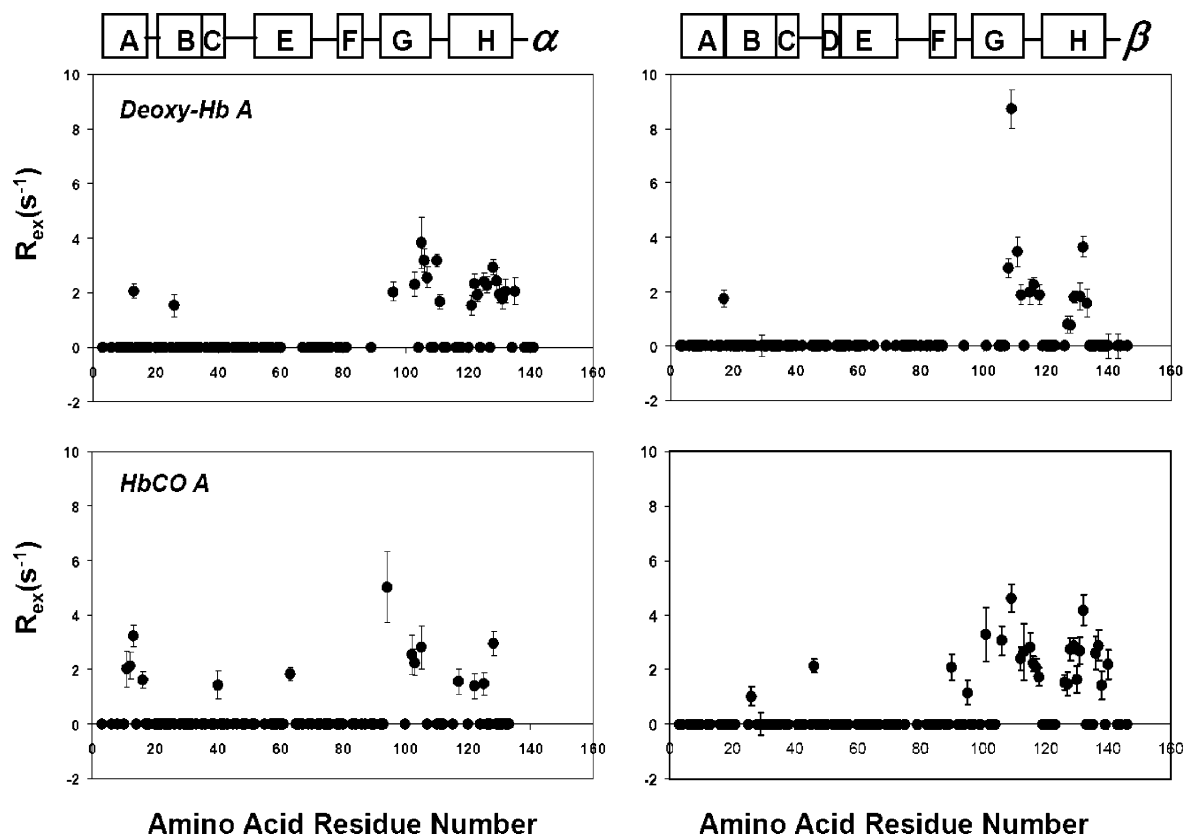


FIGURE 2: R_{ex} terms from Model-free analysis for deoxy-Hb A and HbCO A at 34 °C. Possible chemical exchange processes are implied at amino acid residues after 100, including the most dramatic $\beta 109\text{Val}$.

We report the R_{ex} terms obtained for our Hb samples in Figure 2. In both deoxy- and CO-ligated states, $\beta 109\text{Val}$ has very high R_{ex} values at both 29 and 34 °C, but the magnitudes of R_{ex} terms for this residue are significantly modified after CO binding. The amino acid residues after 100, close to the C-terminus, apparently need R_{ex} terms in fitting more than do other parts of the protein molecule, implying possible slow time scale dynamic events at the GH corners and H helices of both chains. These regions are not at the interdimer ($\alpha_1\beta_2$ or $\alpha_2\beta_1$) interfaces but instead at the intradimer ($\alpha_1\beta_1$ or $\alpha_2\beta_2$) interfaces, which were previously considered a rigidly packed region in the Hb structure.

DISCUSSION

Mobility Change of $\beta 146\text{His}$ and the Interdimer “Switch” Region. As discussed above, the backbone order parameter S^2 reflects the degree of mobility of the amide N–H bond. For example, as the C-terminal residue, $\beta 146\text{His}$ displays high rigidity in deoxy-Hb A, with order parameter values comparable to the helical parts of the protein (Figure 1). The restricted mobility of $\beta 146\text{His}$ can be explained because in the T state of Hb, $\beta 146\text{His}$ is involved in two strong interactions with neighboring residues, e.g., a salt bridge to $\alpha 40\text{Lys}$ and an H-bond to $\beta 94\text{Asp}$ (1, 40). $\beta 146\text{His}$ is a key residue in the so-called switch region at the interdimer ($\alpha_1\beta_2$ or $\alpha_2\beta_1$) interfaces. As one of the important events during the allosteric transition of Hb from the T to R state, a dimer–dimer rotation unlocks $\beta 146\text{His}$ from both salt bridge and H-bond interactions with its neighbors (1). As a consequence, the order parameter (S^2) of $\beta 146\text{His}$ amide N–H becomes as low as that seen for a loop region. This is the first dynamic evidence to support the removal of interaction at the

C-terminus ($\beta 145\text{Tyr}$, $\beta 146\text{His}$, etc.) of the β -chain during the cooperative oxygenation process of Hb A.

CO-Induced Stiffening of $\alpha 31\text{Arg}$ and $\beta 123\text{Thr}$ at the Intradimer ($\alpha_1\beta_1$ or $\alpha_2\beta_2$) Interfaces. The intradimer ($\alpha_1\beta_1$ or $\alpha_2\beta_2$) interfaces can no longer be considered merely passive parts of the allosteric machinery of Hb, a result of recent experimental and computational evidence as to their roles in the allosteric signal transduction (41–47). Although the molecular dynamics simulation by Ramadas and Rifkin (42) implied that a frozen intradimer ($\alpha_1\beta_1$ or $\alpha_2\beta_2$) interface can block the heme–heme communication within the $\alpha\beta$ dimer, X-ray crystallography results indicate no flexibility in this so-called “subunit packing” region. Specifically, the “cementing” residue, $\alpha 31\text{Arg}$, according to X-ray crystallography is involved in a very stable interaction network with neighboring $\beta 122\text{Phe}$ and $\beta 127\text{Gln}$. The triangle formed by these three residues, along with other subunit-crossing interactions involving $\beta 30\text{Arg}$, $\alpha 117\text{Phe}$, and $\alpha 122\text{His}$, contributes to the tight G–H–B packing of the $\alpha_1\beta_1$ or $\alpha_2\beta_2$ dimer (1, 41, 46, 48, 49). In our present study, the order parameter values for the amide N–H bonds of $\alpha 31\text{Arg}$ and one of its neighboring residues at the beginning of the β H helix, $\beta 123\text{Thr}$, are consistently low in deoxy-Hb A at both 29 and 34 °C, showing great flexibility for these two residues. This observation implies that one of the two “clenching bolts” at the intradimer interfaces is loosened in the deoxy (T) state in solution. Interestingly, this finding contradicts the X-ray crystallography results for deoxy-Hb A. Then, according to the NMR dynamics data (Figure 1), this “loosened bolt” in the deoxy state is tightened after CO binding, as it demonstrates high rigidity in the ligated state.

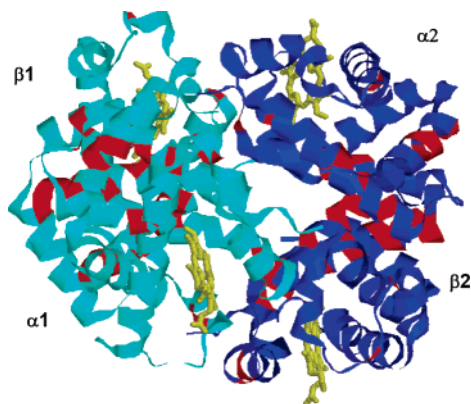


FIGURE 3: HbCO A at 34 °C with the amino acid residues with significant R_{ex} values colored red. Amino acid residues in the G and H helices of both α - and β -chains are possibly involved in chemical exchange processes.

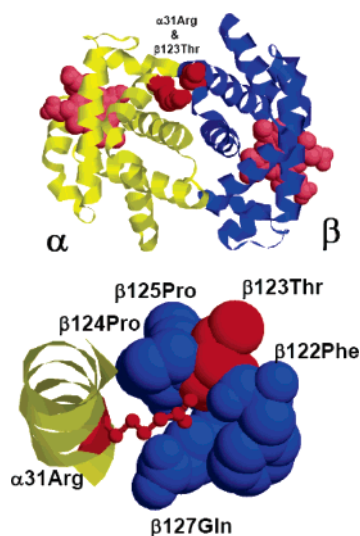


FIGURE 4: Residues $\alpha 31\text{Arg}$ and $\beta 123\text{Thr}$ are shown at the intradimer ($\alpha_1\beta_1$ or $\alpha_2\beta_2$) interface of Hb A. The amide N–H bonds of these two amino acid residues exhibit unusual flexibility in deoxy-Hb A, but both become rigid in HbCO A.

A high-resolution crystal structure of deoxy-Hb A (PDB ID 1A3N) (38) shows a tightly packed “hot-dog” picture of the $\alpha 31\text{Arg}$ side-chain arm and a groove in the β -subunit formed by $\beta 122\text{Phe}$, $\beta 123\text{Thr}$, $\beta 124\text{Pro}$, $\beta 125\text{Pro}$, and $\beta 127\text{Gln}$ (Figure 4). There is an H-bond between $\alpha 31\text{Arg}$ and $\beta 122\text{Phe}$ and an H-bond between $\alpha 31\text{Arg}$ and $\beta 127\text{Gln}$, according to X-ray crystallography. From our result, the unusual mobility of this region occurs at where $\alpha 31\text{Arg}$ contacts $\beta 123\text{Thr}$ and two prolines ($\beta 124\text{Pro}$ and $\beta 125\text{Pro}$). The flexibility of $\alpha 31\text{Arg}$ and $\beta 123\text{Thr}$ is possibly due to the motion of the β -H helix at its beginning end, where the proline twins are located. An alternative explanation is the absence of H-bonds involving $\alpha 31\text{Arg}$ in the solution, as it is possible that the solid packing in the crystal form sometimes can generate extra close contacts between amino acid residues, simulating an H-bond. No matter what actually contributes to the mobility contrast in this region before and after ligand (CO) binding, this observation implies that certain signal transduction or energy distribution crosses the intradimer interface during the allosteric transition of Hb.

On the contrary, as an element in another “cementing unit” of the intradimer assembly, $\beta 30\text{Arg}$ shows consistently high backbone rigidity in both deoxy and CO forms. However,

$\alpha 122\text{His}$, a member of this second intradimer triangle locking region, along with $\alpha 103\text{His}$, has been found to be structurally more stabilized in the R state than the T state by solvent exchange methods, implying a dynamic variation of certain degree, though less significant than in the $\alpha 31\text{Arg}$ – $\beta 123\text{Thr}$ case (50, 51). For this second triangle network, as for the first one involving $\alpha 31\text{Arg}$ and $\beta 123\text{Thr}$, X-ray crystallography gives no evidence of the removal of surrounding interactions (H-bond or salt bridges) of $\alpha 122\text{His}$ in the T state (48). $\alpha 31\text{Arg}$ and $\beta 30\text{Arg}$ are at the far ends of the distal heme pockets of the α -chain and β -chain, respectively. As previously mentioned, the distal pockets of α - and β -chains are found to be not identical in many aspects, including their ligand accessibility, cavity size in the T state, and the strengths of their H-bonds to the ligands in the R states (2, 25, 45, 52). The loose–rigid switch of the $\alpha 31\text{Arg}$ – $\beta 123\text{Thr}$ region during the T–R transition suggests that the ligand binding at the α -subunit might induce a tightening lock of the same dimer α - and β -subunits at the $\alpha 31\text{Arg}$ – $\beta 123\text{Thr}$ site, thus initiating intradimer signal transduction from the distal side of the α -heme pocket. This observation provides support to the intradimer sequential cooperativity during Hb allostery.

Comparison of S^2 Values with Crystallographic B-Factors. In the present study, we have observed no apparent correlation between the amide N–H order parameters and the B-factors for backbone nitrogen atoms, except that a minor inverse correlation is seen in the N- and C-terminal residues as well as some of the loops connecting helices. As has been suggested (33), the varying degrees of inverse correlation between S^2 values and B-factors arise mainly from the insensitivity of the order parameters to translational displacements. In fact, since the frequency of Larmor precession of nuclei matches only the frequencies of rotational motions, the faster processes including bond vibrations do not affect the order parameter. A plot comparing the crystallographic B-factors of Hb A and the NMR order parameters obtained at 29 °C can be found in Figure 3S (Supporting Information).

Mobility Change at the α -C Terminus upon CO Binding. Amino acid residues after 138 at the C-terminus of the α -chain in HbCO A are not visible in the HSQC and TROSY spectra (53). These α -C-terminal residues, however, have normal peak intensities in the spectra of deoxy-Hb A and demonstrate high rigidity for $\alpha 139\text{Lys}$ and $\alpha 140\text{Tyr}$, according to Model-free analysis (Figure 1). The α -C terminus of Hb is near the so-called “flexible joint” area at the interdimer interface and is surrounded by several H-bonds and salt bridges in deoxy-Hb A (1). The disappearance of the NMR signals of these residues in the CO form might be due to their flexible state, a consequence of the breaking of the H-bonds and salt bridges during the T \rightarrow R allosteric transition (1). Such an interpretation is supported by the B-factor plot, as shown in Supporting Information, depicting a sharp increase of the B-factor for residues past 138 (Figure 3S).

Conformational Exchange at G and H Helices Implied by R_{ex} Terms. A nonzero R_{ex} term obtained from Model-free analysis is not solid evidence for the conformational exchange process. However, the significantly large R_{ex} values of certain residues, such as $\beta 109\text{Val}$, and the frequent occurrence of nonzero R_{ex} terms among the amino acid residues after 100 imply a high likelihood of slow time scale

(microsecond to millisecond) motions in the G and H helices. Interestingly, these residues are not at the FG corner or C helices where the interdimer hinge areas (so-called “joint” and “switch” regions) are located, but are along the intradimer ($\alpha_1\beta_1$ and $\alpha_2\beta_2$) interfaces (Figures 2 and 3). This observation is not sufficient to exclude the existence of certain types of conformational flexibility at the joint and switch regions, but obviously these motions are invisible to the R_{ex} indicators. Questions thus arise about whether the mobility of the amino acid residues at the interdimer hinge regions could be affected by the sliding movement between the two dimers. This sliding movement was considered as the major conformational change during the T \rightarrow R \rightarrow R2 quaternary transition (54). The time scale of this dimer–dimer sliding and the relative populations of the three or more sliding intermediate states are the two key factors for the feasibility of using NMR methods to detect this dynamic event.

Rigidity Fluctuations between Deoxy- and Carbonmonoxy-Hb A. Our results show that the order parameters (S^2) of the backbone N–H bonds of Hb A do not have a uniform trend of variation upon CO binding but fluctuate slightly within a range of 0.1, different from residue to residue (Figure 1). Since the work of Lipari and Szabo (22), the amplitude of order parameters has been correlated to the thermodynamic properties, entropy in particular, of proteins (55–57). However, the trend of the variation of backbone or side-chain rigidity, in response to ligand binding, could be either increasing (57–59) or decreasing (15, 60–62). The backbone disordering discussed above is not the sole contribution to the entropy of a protein system. Mobility of protein side chains (57, 63), motions, and conformational variations on time scales other than nanosecond to picosecond, as well as solvent factors, could also affect the general entropy level. Adding to this complexity, the paramagnetic environment at the heme sites could also alter the relaxation properties of the spin systems, leading to extra errors in the entropy estimation.

Finally, we need to emphasize that in the above discussions about the mobility of Hb amino acid residues, we mean the motion of the protein backbone, as characterized by the amide N–H bonds. This is, in fact, a convenient simplification. The side chains of the amino acid residues can act very differently in their dynamic behaviors, with much lower barriers for their motions, compared to structurally more constrained polypeptide backbones (64). Thus, biological processes that do not affect the rigidity of protein backbone scaffold may still significantly perturb the side-chain motions (57). To achieve a complete correlation between dynamics and Hb functions, a thorough investigation of side-chain dynamics of Hb is necessary.

In conclusion, our present NMR studies of the polypeptide backbone dynamics of Hb A in both deoxy and carbonmonoxy forms have provided new information regarding the structural and dynamic properties of this protein not available from the traditional crystal structures. There are clear differences in the backbone dynamics between the deoxy and carbonmonoxy forms in several regions of the Hb molecule, especially in the intra- and interdimer interfaces. For example, $\alpha 31\text{Arg}$ and $\beta 123\text{Thr}$ located in the intradimer interface are flexible in the deoxy state, but exhibit stiffening upon ligation of the Hb molecule. These two residues exhibit

interesting dynamic behavior and may suggest that they can play a role as cementing and communicating connectors between the two intradimer ($\alpha_1\beta_1$ and $\alpha_2\beta_2$) interfaces. $\beta 146\text{His}$, a key residue in the allostery of the Hb molecule, becomes flexible upon ligation. Thus, further structural and dynamic studies of Hb A in solution in conjunction with appropriate functional studies will help elucidate the structural, dynamic, and functional basis for the allostery of this interesting protein molecule at atomic resolution.

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SUPPORTING INFORMATION AVAILABLE

Figure 1S, order parameters (S^2) of amide N–H bonds in Hb at 34 °C; Figure 2S, R_{ex} terms derived from Model-free analysis for deoxy-Hb A and HbCO A at 29 °C; Figure 3S, X-ray crystallographic B -factors (filled circles) and NMR order parameters (open circles, obtained at 29 °C) [top, deoxy-Hb A, with B -factors from 1A3N (PDB ID); bottom, HbCO A, with B -factors from 1BBB (PDB ID)]; Figure 4S, NMR relaxation parameters of backbone amide ^{15}N in Hb A at 29 °C (filled circles) and 34 °C (open circles) [conditions: (1) deoxy-Hb A at 11.7 T; (2) deoxy-Hb A at 14.1 T; (3) HbCO A at 11.7 T; (4) HbCO A at 14.1 T]; Figure 5S, R_1 and R_2 relaxation decay curves for amide ^{15}N in two amino acid residues ($\alpha 31\text{Arg}$ and $\beta 146\text{His}$) at 29 °C (repeated data points are indicated by arrows); Table 1S, NMR relaxation parameters of backbone amide ^{15}N in deoxy-Hb A; Table 2S, NMR relaxation parameters of backbone amide ^{15}N in HbCO A; Table 3S, amino acid residues in Hb A selected for correlation time determination. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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