Published in final edited form as:

Biochemistry. 2007 June 12; 46(23): 6795–6803. doi:10.1021/bi602654u.

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

Xiang-jin Song, Yue Yuan[§], Virgil Simplaceanu, Sarata Chandra Sahu, Nancy T. Ho, and Chien Ho^{*}

Department of Biological Sciences, Carnegie Mellon University, Pittsburgh, PA 15213

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 (R₁), transverse relaxation rate (R₂), and heteronuclear nuclear Overhauser effect (NOE)] measured at two temperatures (29 and 34 °C) and two magnetic field strengths (11.7 and 14.1 Tesla). 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 (ns-ps), 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 intra-dimer (α₁β₁) interface, exhibit stiffening upon CO binding. These findings imply a role for α31Arg and β123Thr in the intra-dimer communication, but contradict the results from X-ray crystallography. We have also found that there is considerable flexibility in the intra-dimer (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 intra-dimer 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), and 2,3-bisphosphoglycerate (2,3-BPG). Human normal adult hemoglobin (Hb A) has a molecular weight 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,

[†]This work is supported by research grants from the National Institute of Health (R01HL-024525 and S10RR-017815).

^{*}Address all Correspondence to: Dr. Chien Ho, Department of Biological Sciences, Carnegie Mellon University, 4400 Fifth Avenue, Pittsburgh, PA 15213, Phone 412-268-3395; fax, 412-268-7083; Email, chienho@andrew.cmu.edu.

[§]Present address: Department of Chemistry, Simon Fraser University, Vancouver, Canada

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, $\alpha_1\beta_1$ or $\alpha_2\beta_2$ (intra-dimer) and $\alpha_1\beta_2$ or $\alpha_2\beta_1$ (inter-dimer). Based on the crystal structures of ligated and unligated Hb A, the $\alpha_1\beta_2$ or $\alpha_2\beta_1$ interface undergoes a sliding motion in going from the deoxy to the oxy state, whereas the $\alpha_1\beta_1$ or $\alpha_2\beta_2$ 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 $\beta 37\text{Trp}$ 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 $\beta 37\text{Trp}$ cluster and/or by exposing crystals of wild-type or mutant deoxy-Hbs to O_2 . 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). 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 fully 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 non-functional 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 intra-dimer ($\alpha_1\beta_1$ 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., $\alpha 31\text{Arg}$, $\beta 3\text{Leu}$, $\beta 41\text{Phe}$, $\beta 123\text{Thr}$, and $\beta 146\text{His}$) in the allosteric pathway. Two residues, $\alpha 31\text{Arg}$ and its neighbor, $\beta 123\text{Thr}$, exhibit interesting dynamic behavior and suggest that they play a role as cementing and communicating connectors between the two intra-dimer ($\alpha_1\beta_1$ 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 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). 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 ppm 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 8-data points with 7-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 nonlinear least-squares fit (nlinLS) implemented in NMRPipe.

Analysis of Relaxation Data

The backbone dynamics information of Hb was obtained based on 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 ps-ns 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

$$J(\omega) = \frac{S^2 \tau_m}{1 + (\omega \tau_m)^2} + \frac{(1 - S_f^2) \tau'_f}{1 + (\omega \tau'_f)^2} + \frac{(S_f^2 - S^2) \tau'_s}{1 + (\omega \tau'_s)^2} \quad (1)$$

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 ($\tau_f < 100$ to 200 ps) and slow time scale ($\tau_s < \tau_s < \tau_m$), 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 experimental relaxation parameters, R_1 , R_2 and NOE, by (30)

$$\begin{aligned} R_1 &= (d^2/4)[J(\omega_H - \omega_X) + 3J(\omega_X) + 6J(\omega_H + \omega_X)] + c^2 J(\omega_X) \\ R_2 &= (d^2/8)[4J(0) + J(\omega_H - \omega_X) + 3J(\omega_X) + 6J(\omega_H) + 6J(\omega_H + \omega_X)] \\ &\quad + (c^2/6)[4J(0) + 3J(\omega_X)] + R_{ex} \\ NOE &= 1 + (d^2/4R_1)(\gamma_H/\gamma_X)[6J(\omega_H + \omega_X) - J(\omega_H - \omega_X)] \end{aligned} \quad (2)$$

where $d = \mu_0 h \gamma_X \gamma_H \langle r_{XH}^{-3} \rangle / (8\pi^2)$, $c = \omega_X \Delta\sigma / \sqrt{3}$, 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 ($X = ^{13}\text{C}$ 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 $\Delta\sigma = \sigma_{\parallel} - \sigma_{\perp}$ 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^2 , $\tau_e = \tau_s$, ($\tau_f \rightarrow 0$). In the models with R_{ex} , non-zero 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 5 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). 3D 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 - $\{^1\text{H}\}$ NOE were measured at 11.7 and 14.1 T, at 29 and 34 °C. The backbone dynamics information of Hb A was obtained based on the Model-free formalism. S^2 , the square of the generalized order parameter, describes the amplitude of internal ps-ns 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 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- 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, 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 intra-dimer ($\alpha_1\beta_1$ or $\alpha_2\beta_2$) interface, $\alpha 31$ Arg and $\beta 123$ Thr, 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 the residue $\beta 69$ Gly is also more restricted in HbCO A. On the contrary, residues $\beta 146$ His and $\beta 3$ Leu (not shown in Figure 1, but shown in Supporting Information) become more flexible in the CO form than in the deoxy form. $\beta 41$ Phe is among the most mobile residues in HbCO A. Unfortunately, no comparable dynamics information for $\beta 41$ Phe 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 (μ s-ms time scale) conformational exchange processes. We report the R_{ex} terms obtained for our Hb samples in Figure 2. In both deoxy and CO-ligated states, $\beta 109$ 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 inter-dimer ($\alpha_1\beta_2$ or $\alpha_2\beta_1$) interfaces, but instead at the intra-dimer ($\alpha_1\beta_1$ or $\alpha_2\beta_2$) interfaces, which were previously considered a rigidly packed region in the Hb structure.

DISCUSSION

Mobility Change of $\beta 146$ His and the Inter-dimer “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, $\beta 146$ 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 $\beta 146$ His can be explained because in the T-state of Hb $\beta 146$ 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 inter-dimer ($\alpha_1\beta_2$ or $\alpha_2\beta_1$) interfaces. As one of the important events during the allosteric transition of Hb from T to R state, a dimer-dimer rotation unlocks $\beta 146\text{His}$ from both salt-bridge and H-bond 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 Intra-dimer ($\alpha_1\beta_1$ or $\alpha_2\beta_2$) Interfaces

The intra-dimer ($\alpha_1\beta_1$ 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 intra-dimer ($\alpha_1\beta_1$ 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 intra-dimer 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.

A high-resolution crystal structure of deoxy-Hb A (PDB ID: 1A3N) (38) shows a tightly packed “hot-dog” picture of $\alpha 31\text{Arg}$ side-chain arm and a groove in 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 intra-dimer interface during the allosteric transition of Hb.

On the contrary, as an element in another “cementing unit” of the intra-dimer 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 intra-dimer 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 α 31Arg- β 123Thr 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 α 31Arg- β 123Thr site, thus initiating intra-dimer signal transduction from the distal side of the α -heme pocket. This observation provides support to the possible intra-dimer 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 α 139Lys and α 140Tyr, according to Model-free analysis (Figure 1). The α C-terminus of Hb is near the so-called “flexible joint” area at the inter-dimer 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 non-zero 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 β 109Val, and the frequent occurrence of non-zero R_{ex} terms among the amino-acid residues after 100 imply a highly likelihood of slow time scale (μ s-ms) motions in the G and H helices. Interestingly, these residues are not at the FG corner or C helices where the inter-dimer hinge areas (so-called “joint” and “switch” regions) are located, but along the intra-dimer ($\alpha_1\beta_1$ 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 inter-dimer 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 ns-ps, 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 above discussions about 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 inter-dimer interfaces. For example, $\alpha 31$ Arg and $\beta 123$ Thr located in the intra-dimer 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 intra-dimer ($\alpha_1\beta_1$ and $\alpha_2\beta_2$) interfaces. $\beta 146$ 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank Dr. Qingguo Gong and Dr. David Maillat for helpful discussions. We also want to thank Dr. Arthur G. Palmer for providing the Modelfree 4.10 program used in this work.

Abbreviations used

Hb A	human normal adult hemoglobin
HbCO	carbonmonoxy-hemoglobin
deoxy-Hb	deoxy-hemoglobin
met-Hb	

	methemoglobin
NMR	nuclear magnetic resonance
R₁	longitudinal relaxation rate
R₂	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
and TROSY	transverse relaxation-optimized spectroscopy

References

1. Dickerson, RE.; Geis, I. Hemoglobin: structure, function, evolution, and pathology. Benjamin-Cummings; Menlo Park, CA: 1983.
2. Perutz MF. Stereochemistry of cooperative effects in haemoglobin. *Nature* 1970;228:726–739. [PubMed: 5528785]
3. Silva MM, Rogers PH, Arnone A. A third quaternary structure of human hemoglobin A at 1.7 Å resolution. *J Bio Chem* 1992;267:17248–56. [PubMed: 1512262]
4. Safo MK, Abraham DJ. The enigma of the liganded hemoglobin end state: a novel quaternary structure of human carbonmonoxy hemoglobin. *Biochemistry* 2005;44:8347–59. [PubMed: 15938624]
5. Kavanaugh JS, Rogers PH, Arnone A. Crystallographic evidence for a new ensemble of ligand-induced allosteric transitions in hemoglobin: the T-to-T_{high} quaternary transitions. *Biochemistry* 2005;44:6101–6121. [PubMed: 15835899]
6. Lukin JA, Kontaxis G, Simplaceanu V, Yuan Y, Bax A, Ho C. Quaternary structure of hemoglobin in solution. *Proc Natl Acad Sci USA* 2003;100:517–520. [PubMed: 12525687]
7. Sahu SC, Simplaceanu V, Ho NT, Giovannelli JL, Ho C. Backbone resonance assignment of human adult hemoglobin in the deoxy form. *J Biomol NMR* 2006;36:1. [PubMed: 16703421]
8. Sahu SC, Simplaceanu V, Gong Q, Ho NT, Glushka JG, Prestegard JH, Ho C. Orientation of deoxyhemoglobin at high magnetic fields: structural insights from RDCs in solution. *J Am Chem Soc* 2006;128:6290–6291. [PubMed: 16683773]
9. Eaton WA, Henry ER, Hofrichter J, Mozzarelli A. Is cooperative oxygen binding by hemoglobin really understood. *Nat Struct Biol* 1999;6:351–358. [PubMed: 10201404]
10. Wüthrich K. NMR studies of structure and function of biological macromolecules (Nobel Lecture). *Angew Chem Int Ed* 2003;42:3340–3363.

11. Gardner KH, Kay LE. The use of ^2H , ^{13}C , ^{15}N multidimensional NMR to study the structure and dynamics of proteins. *Annu Rev Biophys Biomol Struct* 1998;27:357–406. [PubMed: 9646872]
12. Simplaceanu V, Lukin JA, Fang TY, Zou M, Ho NT, Ho C. Chain-selective isotopic labeling for NMR studies of large multimeric proteins: application to hemoglobin. *Biophys J* 2000;79:1146–1154. [PubMed: 10920044]
13. Yuan Y, Simplaceanu V, Lukin JA, Ho C. NMR investigation of the dynamics of tryptophan side-chains in hemoglobins. *J Mol Biol* 2002;321:863–878. [PubMed: 12206767]
14. Kern D, Zuiderweg ER. The role of dynamics in allosteric regulation. *Curr Opin Stru Biol* 2003;13:748–757.
15. Palmer AG III. NMR characterization of the dynamics of biomacromolecules. *Chem Rev* 2004;104:3623–3640. [PubMed: 15303831]
16. Gong Q, Simplaceanu V, Lukin JA, Giovannelli JL, Ho NT, Ho C. Quaternary structure of carbonmonoxyhemoglobins in solution: structural changes induced by the allosteric effector inositol hexaphosphate. *Biochemistry* 2006;45:5140–5148. [PubMed: 16618103]
17. Pervushin K, Riek R, Wider G, Wuthrich K. Attenuated T_2 relaxation by mutual cancellation of dipole-dipole coupling and chemical shift anisotropy indicates an avenue to NMR structures of very large biological macromolecules in solution. *Proc Natl Acad Sci USA* 1997;94:12366–12371. [PubMed: 9356455]
18. Sui X, Xu Y, Giovannelli JL, Ho NT, Ho C, Yang D. Mapping protein-protein interfaces on the basis of proton density difference. *Angew Chem Int Ed* 2005;44:5141–5144.
19. Wider G, Wüthrich K. NMR spectroscopy of large molecules and multimolecular assemblies in solution. *Curr Opin Stru Biol* 1999;9:594–601.
20. Yang DW, Zheng Y, Liu DJ, Wyss DF. Sequence-specific assignments of methyl groups in high-molecular weight proteins. *J Am Chem Soc* 2004;126:3710–3711. [PubMed: 15038713]
21. Lipari G, Szabo A. Model-free approach to the interpretation of nuclear magnetic resonance relaxation in macromolecules, 1. Theory and range of validity. *J Am Chem Soc* 1982;104:4546–4559.
22. Lipari G, Szabo A. Model-free approach to the interpretation of nuclear magnetic resonance relaxation in macromolecules, 2. Analysis of experimental results. *J Am Chem Soc* 1982;104:4559–4570.
23. Shen TJ, Ho NT, Simplaceanu V, Zou M, Green BN, Tam MF, Ho C. Production of unmodified human adult hemoglobin in *Escherichia Coli*. *Proc Natl Acad Sci USA* 1993;90:1085–1097.
24. Shen TJ, Ho NT, Zou M, Sun DP, Cottam PF, Simplaceanu V, Tam MF, Bell DAJ, Ho C. Production of human normal adult and fetal hemoglobins in *Escherichia Coli*. *Protein Eng* 1997;10:1085–1097. [PubMed: 9464574]
25. Lukin JA, Simplaceanu V, Zou M, Ho NT, Ho C. NMR reveals hydrogen bonds between oxygen and distal histidines in oxyhemoglobin. *Proc Natl Acad Sci USA* 2000;97:10354–10358. [PubMed: 10962034]
26. Farrow NA, Zhang O, Forman-Kay JD, Kay LE. Comparison of the backbone dynamics of a folded and an unfolded SH3 domain existing in equilibrium in aqueous buffer. *Biochemistry* 1995;34:868–878. [PubMed: 7827045]
27. Grzesiek S, Bax A. The importance of not saturating water in protein NMR. Application to sensitivity enhancement and NOE measurements. *J Am Chem Soc* 1993;115:12593–12594.
28. Delaglio F, Grzesiek S, Vuister G, Zhu G, Pfeiffer J, Bax A. NMRPipe: a multidimensional spectral processing system based on UNIX pipes. *J Biomol NMR* 1995;6:277–293. [PubMed: 8520220]
29. Clore GM, Szabo A, Bax A, Kay LE, Driscoll PC, Gronenborn AM. Deviation from the simple two-parameter Model-free approach to the interpretation of nitrogen-15 nuclear magnetic relaxation of proteins. *J Am Chem Soc* 1990;112:4989–4991.
30. Abragam, A. Principles of nuclear magnetism. Clarendon Press; Oxford: 1961.
31. Jeffrey, G. Accurate molecular structures, their determination and importance. Domenicano, A.; Hargittai, I., editors. Oxford Science Publication; Oxford: 1992. p. 270-298.
32. Kroenke CD, Rance M, Palmer AG III. Variability of the ^{15}N chemical shift anisotropy in *Escherichia coli* Ribonuclease H in solution. *J Am Chem Soc* 1999;121:10119–10125.

33. Mandel AM, Akke M, Palmer AG III. Backbone dynamics of *Escherichia Coli* ribonuclease HI: correlations with structure and function in an active enzyme. *J Mol Biol* 1995;246:144–163. [PubMed: 7531772]
34. Palmer AG III, Rance M, Wright PE. Intramolecular motions of a zinc finger DNA-binding domain from Xfin characterized by proton-detected natural abundance ^{13}C heteronuclear NMR spectroscopy. *J Am Chem Soc* 1991;113:4371–4380.
35. Yao S, Smith DK, Hinds MG, Zhang J-g, Nicola NA, Norton RS. Backbone dynamics measurements on leukemia inhibitory factor, a rigid four-helical bundle cytokine. *Protein Science* 2000;9:671–682. [PubMed: 10794409]
36. Tjandra N, Feller SE, Pastor RW, Bax A. Rotational diffusion anisotropy of human ubiquitin from ^{15}N NMR relaxation. *J Am Chem Soc* 1995;117:12562–12566.
37. Lee LK, Rance M, Chazin WJ, Palmer AG III. Rotational diffusion anisotropy of protein from simultaneous analysis of ^{15}N and ^{13}C nuclear spin relaxation. *J Biomol NMR* 1997;9:287–298. [PubMed: 9204557]
38. Tame JRH, Vallone B. The structures of deoxy human haemoglobin and the mutant Hb Tyr α 42His at 120K. *Acta Cryst D-Biol Crystallogr* 2000;56(Pt 7):805–811. [PubMed: 10930827]
39. Kneller JM, Lu M, Bracken C. An effective method for the discrimination of motional anisotropy and chemical exchange. *J Am Chem Soc* 2002;124:1852–1853. [PubMed: 11866588]
40. Baldwin J, Chothia C. Haemoglobin: the structural changes related to ligand binding and its allosteric mechanism. *J Mol Biol* 1979;129:175–220. [PubMed: 39173]
41. Xu C, Tobi D, Bahar I. Allosteric changes in protein structure computed by a simple mechanical model: hemoglobin T $\rightleftharpoons$ R2 transition. *J Mol Biol* 2003;333:153–168. [PubMed: 14516750]
42. Ramadas N, Rifkin JM. Molecular dynamics of human methemoglobin: the transmission of conformational information between subunits in an $\alpha\beta$ dimer. *Biophys J* 1999;76:1796–1811. [PubMed: 10096879]
43. Tsai CH, Fang TY, Ho NT, Ho C. Novel recombinant hemoglobin, rHb(β N108Q), with low oxygen affinity, high cooperativity, and stability against autoxidation. *Biochemistry* 2000;39:13719–13729. [PubMed: 11076511]
44. Tsai CH, Shen TJ, Ho NT, Ho C. Effects of substitutions of lysine and aspartic acid for asparagine at β 108 and of tryptophan for valine at α 96 on the structural and functional properties of human normal adult hemoglobin: roles of $\alpha_1\beta_1$ and $\alpha_1\beta_2$ subunit interfaces in the cooperative oxygenation process. *Biochemistry* 1999;38:8751–8761. [PubMed: 10393550]
45. Mouawad L, Perahia D, Robert CH, Guilbert C. New insights into the allosteric mechanism of human hemoglobin from molecular dynamics simulations. *Biophys J* 2002;82:3224–3245. [PubMed: 12023247]
46. Balakrishnan G, Tsai CH, Wu Q, Case MA, Pevsner A, McLendon GL, Ho C, Spiro TG. Hemoglobin site-mutants reveal dynamical role of interhelical H-bonds in the allosteric pathway: time-resolved UV resonance Raman evidence for intra-dimer coupling. *J Mol Biol* 2004;340:857–868. [PubMed: 15223326]
47. Levy A, Sharma VS, Zhang L, Rifkin JM. A new mode for heme-heme interactions in hemoglobin associated with distal perturbations. *Biophys J* 1992;61:750–755. [PubMed: 1324020]
48. Fermi G, Perutz MF, Shaanan B. The crystal structure of human deoxyhaemoglobin at 1.74 Å resolution. *J Mol Biol* 1984;175:159–174. [PubMed: 6726807]
49. Srinivasan R, Rose GD. The T-to-R transformation in hemoglobin: a reevaluation. *Proc Natl Acad Sci USA* 1994;91:11113–11117. [PubMed: 7972019]
50. Mihailescu MR, Russu IM. A signature of the T $\rightarrow$ R transition in human hemoglobin. *Proc Natl Acad Sci USA* 2001;98:3773–3777. [PubMed: 11259676]
51. Chang, C-k; Simplaceanu, V.; Ho, C. Effects of amino acid substitutions at β 131 on the structure and properties of hemoglobin: evidence for communication between $\alpha_1\beta_1$ - and $\alpha_1\beta_2$ - subunit interfaces. *Biochemistry* 2002;41:5644–5655. [PubMed: 11969426]
52. Park SY, Yokoyama T, Shibayama N, Shiro Y, Tame JRH. 1.25 Å resolution crystal structures of human haemoglobin in the oxy, deoxy and carbonmonoxy forms. *J Mol Biol* 2006;360:690–701. [PubMed: 16765986]

53. Lukin JA, Kontaxis G, Simplaceanu V, Yuan Y, Bax A, Ho C. Backbone resonance assignments of human adult hemoglobin in the carbonmonoxy form. *J Biomol NMR* 2004;28:203–204. [PubMed: 14755170]
54. Mueser TC, Rogers PH, Arnone A. Interface sliding as illustrated by the multiple quaternary structures of liganded hemoglobin. *Biochemistry* 2000;39:15353–15364. [PubMed: 11112521]
55. Yang D, Kay LE. Contributions to conformational entropy arising from bond vector fluctuations measured from NMR-derived order parameters: application to protein folding. *J Mol Biol* 1996;263:369–82. [PubMed: 8913313]
56. Akke M, Bruschweiler R, Palmer AG III. NMR order parameters and free-energy - an analytical approach and its application to cooperative Ca^{2+} binding by calbindin-D(9k). *J Am Chem Soc* 1993;115:9832–9833.
57. Lee AL, Kinnear SA, Joshua WA. Redistribution and loss of side chain entropy upon formation of a calmodulin-peptide complex. *Nat Struc Biol* 2000;7:72–77.
58. Akke M, Skelton NJ, Kordel J, Palmer AG III, Chazin WJ. Effects of ion binding on the backbone dynamics of calbindin D9k determined by ^{15}N NMR relaxation. *Biochemistry* 1993;32:9832–9844. [PubMed: 8373781]
59. Bracken C, Carr PA, Cavanagh J, Palmer AG III. Temperature dependence of intramolecular dynamics of the basic leucine zipper of GCN4: implications for the entropy of association. *J Mol Biol* 1999;285:2133–2146. [PubMed: 9925790]
60. Fayos R, Melacini G, Newlon MG, Burns L, Scott JD, Jennings PA. Induction of flexibility through protein-protein interactions. *J Biol Chem* 2003;278:18581–18587. [PubMed: 12604595]
61. Arumugam S, Gao G, Patton BL, Semchenko V, Brew K, Van Doren SR. Increased backbone mobility in β -barrel enhances entropy gain driving binding of N-TIMP-1 to MMP-3. *J Mol Biol* 2003;327:719–734. [PubMed: 12634064]
62. Zidek L, Novotny MV, Stone MJ. Increased protein backbone conformational entropy upon hydrophobic ligand binding. *Nat Struc Biol* 1999;6:1118–1121.
63. Loh AP, Pawley N, Nicholson LK, Oswald RE. An increase in side chain entropy facilitates effector binding: NMR characterization of the side chain methyl group dynamics in Cdc42Hs. *Biochemistry* 2001;40:4590–4600. [PubMed: 11294626]
64. Song, X-j; Flynn, PF.; Sharp, KA.; Wand, AJ. Temperature dependence of fast dynamics in proteins. *Biophys J* 2007;92:L46–L48. [PubMed: 17237208]

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

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 $\beta 109Val$.

Figure 3. HbCO A at 34 °C with the amino acid residues with significant R_{ex} values painted 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 intra-dimer ($\alpha_1\beta_1$ 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.

Isotropic, axially symmetric and fully anisotropic diffusion parameters determined for deoxy and CO (in parentheses) forms of Hb using R1R2_Diffusion(I) and Quadric_1.1(I)(II). (Principal components only)

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