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Solution Structure and Dynamics of Human Hemoglobin in the Carbonmonoxy Form

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Abstract

The solution structure of HbCO A was refined using stereospecifically assigned methyl groups and residual dipolar couplings based on our previous NMR structure. The tertiary structures of individual chains were found to be very similar to the X-ray structures, while the quaternary structures in solution with low-salt resembled the X-ray R structure more than the R2 structure. On the basis of chemical shift perturbation by inositol hexaphosphate (IHP) titration and docking, we identified five possible IHP binding sites in HbCO A. Amide-water proton exchange experiments demonstrated that α Thr38 located in the $\alpha 1\beta 2$ interface and several loop regions in both α - and β -chains were dynamic on the sub-second timescale. Side-chain methyl dynamics revealed that methyl groups in the $\alpha 1\beta 2$ interface were dynamic, but those in the $\alpha 1\beta 1$ interface were quite rigid on the ns-ps and ms- μ s timescales. All the data strongly suggest a dynamic $\alpha 1\beta 2$ interface that allows conformational changes among different forms (like T, R, and R2) easily in solution. Binding of IHP to HbCO A induced small structural and dynamics changes in the $\alpha 1\beta 2$ interface and the regions around the hemes, but did not increase the conformational entropy of HbCO A. The binding also caused conformational changes on the ms timescale, very likely arising from the relative motion of the $\alpha 1\beta 1$ dimer with respect to the $\alpha 2\beta 2$ dimer. Heterotropic effectors like IHP may change the oxygen affinity of Hb through modulating the relative motion of the two dimers and then further altering the structure of heme binding regions.

Hemoglobin (Hb) is a tetrameric protein consisting of two identical α -subunits and two identical β -subunits. It has been used as a model system in textbooks for understanding

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PDB accession code

Coordinates for the refined structure of HbCO A has been deposited in the PDB with accession code 2m6z.

Supporting Information Available

One figure showing the stereospecific assignment of methyl groups, one showing the IHP binding sites and chemical shift perturbations mapped onto the ribbon structure, one showing peak shifts with the increase of IHP concentration, one showing amide hydrogen-water exchange profiles, one showing $S^2_{\text{axis}} - \langle S^2_{\text{axis}} \rangle$ in the absence and presence of IHP, and one table listing the RDC Q-factors for 20 lowest energy structures. This material is available free of charge via the Internet at <http://pubs.acs.org>.

protein cooperativity, allosteric effects and structure-function relationships. In the two-structure MWC concerted model¹ and Perutz's stereochemical model,² hemoglobin has been assumed to exist in two interconvertible forms, the tense (T) deoxy-form and the relaxed (R) oxy-form. Early X-ray crystallographic studies showed that Hbs could adopt only two stable quaternary structures, one corresponding to the deoxy-Hb (T state) while the other to the ligand-bound Hb (R state, e.g., in complex with oxygen or carbon monoxide). Although the R and T quaternary structures are significantly different, the tertiary structures of each subunit are nearly identical in the two forms. These studies supported the two-structure models that explained the experimental oxygen-binding data. However, a number of new ligand-bound Hb structures known as R2,³ RR2,⁴ and R3⁴ were later discovered by altering the crystallization conditions for human adult carbonmonoxy Hb (HbCO A). Due to the significant differences in the quaternary structure among the identified Hb structural forms, the two-structure models have been challenged. To address which quaternary structure represents the true ligand-bound state in solution, we investigated the quaternary structures of Hb in the absence and presence of the allosteric effector IHP using one-bond ¹H-¹⁵N residual dipolar couplings (RDC) assuming that the structure of each $\alpha\beta$ dimer in solution was the same as that in the crystal state. From these studies, it was concluded that the solution structure of HbCO A was a dynamic intermediate between the R and the R2 forms and IHP could shift the structure towards the R form.^{5,6} Then, we determined a solution structure of HbCO A using an NOESY-based strategy⁷ and found that the NMR solution structures of each subunit and the $\alpha\beta$ dimer agreed well with those in the R2 or R form, but the tetramer structure was notably different from the X-ray structures.

Based on the early crystal structures of the Hb molecule,² it has been thought that the T-form has lower affinity for oxygen than the R-form and thus the quaternary structure of Hb controls its oxygen affinity. However, recent studies demonstrated that the quaternary T and R forms did not correlate with the oxygen affinity of Hb since the T and R forms in the presence of artificial allosteric effectors L35 and inositol hexaphosphate (IHP) had nearly the same affinity for oxygen.^{8,9} Because L35 could greatly reduce the oxygen affinity of Hb without switching the ligand-bound structure to the T form,¹⁰ a dynamic allosteric model was proposed to account for the contribution of protein dynamics to the oxygen affinity.⁸ This model was supported by a recent molecular dynamics (MD) simulation study showing that the effector 2,3-bisphosphoglycerate (2,3-BPG) increased significantly the dynamics of Hb on a nanosecond timescale.¹¹ However, NMR relaxation experiments did not detect any significant IHP-induced changes in the backbone dynamics of Hb on nanosecond to picoseconds timescales.¹² Instead, IHP greatly increased the backbone dynamics of HbCO A on millisecond to microsecond timescales, but had nearly no effect on the dynamics of the deoxy form.¹³ Here, we present the refined solution structure of HbCO A in the absence of IHP, water-amide proton exchange results, and the methyl dynamics in the absence and presence of IHP.

Materials and Methods

Human normal adult hemoglobin samples

Chain-specifically ^{13}C , ^{15}N -labeled HbCO A and ^2H , ^{15}N -labeled HbCO A were prepared as described previously.¹⁴ For stereo-specific assignments of methyl groups of valine and leucine residues, chain-specifically and 10% fractionally ^{13}C -labeled HbCO A were prepared following the procedure described previously.¹⁵ In the ^1H - ^{15}N HSQC experiments of IHP titration to HbCO A, the concentration of Hb was about 0.5 mM (in term of a tetramer) and the final IHP concentration ranged from 0 to 3 mM. In all other experiments, the concentration of Hb was about 0.6 mM, corresponding to an effective concentration of 1.2 mM per labeled chain type (α or β). It should be mentioned that in our NMR experiments, one-type of the Hb chain was isotopically labeled and the other was not labeled.¹⁴ All samples were equilibrated with a 0.1 M sodium phosphate buffer (pH 7.0) containing ~5% D_2O and saturated with CO gas.

Stereospecific assignment

2D ^1H - ^{13}C constant-time HSQC and non-constant-time HMQC spectra were collected at 30 °C on a Bruker Avance 600 MHz spectrometer using the 10% fractionally ^{13}C -labeled samples. The pro-R ($\text{C}^\gamma 1$ for Val and $\text{C}^{\delta 1}$ for Leu) and pro-S ($\text{C}^\gamma 2$ for Val and $\text{C}^{\delta 2}$ for Leu) methyl groups were assigned on the basis that pro-R and pro-S methyls give rise to doublet and singlet peaks in the HMQC spectrum, respectively. The doublet peaks in the HMQC appeared as negative singlets in the constant-time HSQC.

Water-amide proton exchange

All NMR experiments for water-amide exchange and methyl dynamics were performed at 30 °C on a Bruker Avance 800 MHz spectrometer equipped with a cryogenic TXI probe. The phase-modulated CLEAN chemical exchange (CLEANEX-PM) approach with a fast-HSQC (FHSQC) detection scheme¹⁶ was employed to record ^1H - ^{15}N HSQC spectra on the chain specifically ^2H , ^{15}N -labeled samples with a series of mixing times: 5, 10, 15, and 20 ms. The effective exchange rates were calculated using the initial slope analysis.¹⁶

Methyl dynamics

Methyl ^{13}C spin-lattice relaxation rates (R_1) and cross-correlated relaxation rates (Γ) were measured at 30 °C on a Bruker 800 MHz spectrometer using our recently developed methods.^{17,18} In the R_1 measurements, data were acquired with 4 scans, a spectral width of 22 ppm in ^{13}C (t_1) dimension, a recycle delay of 1 s, a proton saturation time of 2.5 s, and a series of relaxation delays (0.01, 0.05, 0.1, 0.2, 0.3, 0.4, and 0.6 s). In the measurements of Γ values, ^1H - ^{13}C HSQC spectra were recorded with 8 scans, a spectral width of 22 ppm in t_1 dimension, a recycle delay of 2.5 s, a constant-time (T) of 27.6 ms, and a series of relaxation delays (): 0.012, 0.6, 1.2, 1.8, 2.4, 3.0, 3.6, 4.2, 4.8, 5.4, 6.0, 6.6, 7.2, and 8.0 ms. One duplicate spectrum at a relaxation delay of 1.2 ms was recorded to estimate the experimental errors in peak intensities for each sample. 90 t_1 increment points were used in all experiments. R_1 values were obtained by fitting the peak decay profiles to a mono-

exponential function. Γ values were calculated from the dependence of peak intensities on the relaxation delay () using the following equation: ¹⁷

$$I(\Delta, T) = \frac{3 \cos(3\pi J\Delta) \exp(1 - 4\Gamma T) + \{1 + 15\Gamma\tau_a^* [1 - \exp(-y\Gamma)]\} \cos(\pi J\Delta)}{3 \exp(-4\Gamma T) + \{1 + 15\Gamma\tau_a^* [1 - \exp(-y\Gamma)]\}} \quad [1]$$

where I is the peak intensity for a given methyl group, J is the one bond ^1H - ^{13}C scalar coupling constant which was determined from data fitting, τ_a is the delay used in the first INEPT period in the pulse scheme and was set to 1 ms in this study, and y is a correction factor given by $y = 0.2229 - 0.0046\tau_m + 0.000052\tau_m^2$ in which τ_m is the overall tumbling time of the protein.

The measured ^{13}C relaxation rates R_1 and Γ for a specific methyl group depend on its motional properties in a protein. Using the model-free analysis, we can extract the internal motional parameters (S^2 and τ_e) from the following equations:

$$\begin{aligned} R_1 \approx & 3 \frac{\hbar^2 \gamma_C^2 \gamma_H^2}{10r_{CH}^6} \left(\frac{\mu_0}{4\pi} \right)^2 [J(\omega_H - \omega_C) + 3J(\omega_C) + 6J(\omega_H + \omega_C)] \\ & + \frac{\hbar^2 \gamma_C^4}{10r_{CC}^6} \left(\frac{\mu_0}{4\pi} \right)^2 [J(0) + 3J(\omega_C) + 6J(2\omega_C)] \\ & + \frac{2\omega_C^2 \Delta\sigma^2}{15} J(\omega_C), \end{aligned} \quad [2]$$

$$\Gamma = \frac{\hbar^2 \gamma_H^2 \gamma_C^2}{10r_{CH}^6} \left(\frac{\mu_0}{4\pi} \right)^2 [4J_{HCH}(0) + 3J_{HCH}(\omega_C)] + \frac{\hbar^2 \gamma_H^4}{10r_{HH}^6} \left(\frac{\mu_0}{4\pi} \right)^3 [3J_{HHH}(\omega_H) + 3J(2\omega_H)], \quad [3]$$

$$J_{ijk}(\omega) = \frac{S_f^2 S_{axis}^2 \tau_m}{1 + \omega^2 \tau_m^2} + \frac{(P_2(\theta_{ij,jk}) - S_{axis}^2 S_f^2) \tau_1}{1 + \omega^2 \tau_1^2} \quad [4]$$

where γ_i and ω_i are the gyromagnetic ratio and Larmor frequency of spin i , respectively; $\hbar = h/2\pi$ and h is Planck's constant; μ_0 is the permeability of free space; r_{CH} is the C-H bond distance and is assumed as 1.09 Å; r_{CC} is the C-C bond distance assumed as 1.517 Å; $\sigma_{\parallel} - \sigma_{\perp}$, where $\sigma_{\parallel}$ and $\sigma_{\perp}$ are the principal components of an axially-symmetric ^{13}C CSA tensor along the parallel and perpendicular axes, and σ is assumed as 25 ppm; S_{axis} is the order parameter of the methyl rotation axis; $S_f^2 = 0.111$ by assuming a tetrahedral geometry for CH_3 methyl groups; $\tau_1^{-1} = \tau_m^{-1} + \tau_e^{-1}$, in which τ_e is the effective internal correlation time and $\tau_m > 10 \tau_e$; $\theta_{ij,jk}$ is the angle between bonds ij and jk . In the case where $ij = jk$, $J_{ijk}(\omega)$ becomes the auto-correlation spectral density function and is denoted as $J(\omega)$.

Methyl ^{13}C relaxation dispersion

Methyl ^{13}C relaxation dispersion data were recorded on uniformly ^{13}C -labeled samples at 30 °C on a Bruker 800 MHz spectrometer using a slightly modified $^1\text{H} \rightarrow ^{13}\text{C} \rightarrow ^1\text{H}$ CPMG scheme.¹⁹ To remove the effect of J coupling interactions between methyl ^{13}C and its neighboring ^{13}C spins, selective RE-BURP 180° pulses were applied during the CPMG and

P-element periods. To suppress one-bond ^{13}C - ^{13}C coupling during the t_1 period, a selective RE-BURP 180° pulse was inserted between the first $t_1/2$ and t_b periods. All the RE-BURP pulses had duration of 1 ms. The RE-BURP pulse used during the t_1 period was centered at 40 ppm, while others were centered at 15 ppm. Using this modified scheme, we could obtain only Ala, Met and Thr methyl relaxation dispersion profiles since the J coupling effect could not be effectively suppressed for other methyl groups in uniformly ^{13}C -labeled samples. The dispersion profiles were obtained from 10 spectra recorded with 24 scans, a recycle delay of 1.5 s, 90 increments and a spectral width of 22 ppm in t_1 dimension, a constant relaxation delay T of 33 ms and a series of ν_{CPMG} fields (60.6, 121.2, 181.8, 242.4, 303.0, 363.6, 424.2, 484.8, 545.4, 606.0 Hz). A reference spectrum was recorded to calculate the effective relaxation rate (R_2^{eff}) of a methyl ^{13}C when T was set to zero. One duplicate spectrum at a ν_{CPMG} value of 181.8 Hz was also recorded to estimate the errors of R_2^{eff} .

All data were processed with nmrPipe²⁰ and analyzed with Sparky (Goddard, T.D. & Kneller, D.G. SPARKY 3, University of California, San Francisco).

Structure Re-Calculation

Using distance constraints derived from NOEs, dihedral angle restraints, hydrogen-bond constraints derived from water-amide exchange, and residual dipolar couplings (RDCs) constraints from a stretched polyacrylamide gel, we recalculated the HbCO A structures with the XPLOR-NIH program²¹ using two methods. In the first method (or conventional method), backbone torsion angles (Φ and Ψ) derived from the chemical shifts were employed as the dihedral angle restraints, and the tolerance for each torsion angle is $\pm 20^\circ$. In the second method (or non-conventional method), Φ and Ψ from the X-ray structure (RCSB accession code 1BBB, resolution 1.7 Å) were used as the restraints, and the tolerance was set to $\pm 3^\circ$. For both methods, the RDCs that are located in helices were randomly divided into two data-sets. About 80% of the selected ^{15}N - ^1H RDCs (work part) were included in the calculation. The remaining 20% of the RDCs (free part) were used for structure validation. For comparison, the structures were also calculated in the absence of RDC constraints with the two methods. Program PALES²² was used to analyze the final 20 lowest energy structures. The calculation protocols are available from the authors upon request.

Molecular Docking

AutoDock Vina²³ with AutoDockTools 1.5.4²⁴ graphical interface was used to find the potential binding sites of HbCO A for IHP. One solution structure of HbCO A derived from this study was chosen as the receptor and standard charging method within AutoDockTools was employed. The coordinates of IHP extracted from PDB bank (pdb code: 2K8R) were used directly. The gridbox with $30\text{Å} \times 30\text{Å} \times 30\text{Å}$ size was employed to search the possible binding sites around the whole surface (mainly focused on the six interfaces, i.e. $\alpha 1\alpha 2$, $\beta 1\beta 2$, $\alpha 1\beta 1$, $\alpha 1\beta 2$, $\alpha 2\beta 1$, $\alpha 2\beta 2$, which showed significant chemical shift perturbation upon IHP binding) and the central region of HbCO A. Default parameters were utilized to find the best binding models. The PyMOL (<http://www.pymol.org>) was used to analyze the data and plot the figures.

Results and Discussion

Stereospecific assignment

Using 2D ^1H - ^{13}C non-constant-time HMQC and constant-time HSQC spectra recorded on the 10% fractionally ^{13}C -labeled samples, we determined the stereo-specific assignments of all Val and Leu methyls (except for βLeu106) in the α - and β -chains of HbCO A (Supplementary Fig. S1).

Quaternary Structure

Previously, we determined a solution structure of HbCO A with dihedral angle restraints derived only from chemical shifts and NOE restraints ⁷ without stereospecific assignments. With the availability of stereospecific assignment of methyl groups, N-H residual dipolar coupling constants that were measured in a stretched polyacrylamide gel, ⁶ and hydrogen-bond constraints, we recalculated the structure of HbCO A. The tertiary structures obtained by the conventional method in the presence or absence of RDC constraints are very similar to the X-ray structures (data not shown), but the RDC Q factors for the 20 lowest energy structures that were refined with RDCs are abnormal (Table S1). The 80% RDCs used in our structure calculation displayed nearly perfect consistency between the experimental and calculated RDCs, but the remaining 20% RDCs not used in the calculation showed great discrepancy (Table S1). Moreover, the Q-factors of the unused 20% RDCs for the refined structures were even larger than the overall Q-factors for the unrefined structures. Therefore, we concluded that this method gives priority to local structural adjustments rather than to the adjustment of the relative subunit-subunit orientations during the structure calculation. In order to overcome this problem, we implemented a non-conventional method, i.e., using very tight dihedral angles restraints with small tolerance ($\pm 3^\circ$) and high weighting factor. Because the tertiary structures of individual chains determined with the conventional method are very similar to the X-ray structures, Φ and Ψ angles derived from the X-ray structure (PDB code: 1BBB) were employed as the input dihedral angles in this non-conventional protocol. The results show great improvement in term of Q factors (Table S1). The final constraints used and structural statistics obtained are listed in Table 1.

The overall quaternary structure of HbCO A in solution obtained here (Fig. S2) is more similar to the X-ray R structure of HbCO A rather than to the R2 structure. The C_2 symmetry axes of the 20 lowest energy NMR structures are close to the C_2 axis of the R structure (Fig. 1a - d). The angles between the C_2 axes of the R and R2, the R and T, and T and R2 structures are 5.5° , 7° and 11.2° , respectively. The angles between the C_2 axes of the R and the 20 solution structures are in the range of $0.4 - 4.0^\circ$ (Fig. 1b) and the average angle is 1.7° . The average angles between the C_2 axes of the R2 and 20 solution structures and the T and 20 solution structures are 5.2° and 6.7° (Fig. 1c and d), respectively. For the switch region of the $\alpha 1\beta 2$ interface, the NMR structures are quite similar to the R and R2 structures, and very different from the T structure in terms of the location of $\beta 2\text{His97}$ (Fig. 1e). These results indicate that the solution structure is closer to the R structure.

Safo et al. ⁴ reported that the R2 structure could also be crystallized under high-salt conditions (similar to conditions to obtain the classical R structure ²). Mueser et al. ²⁵

reported that the R structure of bovine HbCO was not unique, but depended on the crystallization conditions. By a comparison of the geometric coordinates of the T, R, and R2 structures, Srinivasan and Rose²⁶ concluded that in going from the deoxy to the ligated form, the quaternary structures went from T to R, and then to R2, i.e., R was the intermediate state during the ligation process. It has been pointed out that different R-type structures could coexist in equilibrium under either high salt or low salt conditions and different crystal forms may simply reflect subtle differences in crystallization conditions.^{4,25} Our present NMR structures illustrate that HbCO A can exist in multiple conformations in solution and they are more similar to the R quaternary structure. Although one can argue that the structural variations in solution result from insufficient constraints, we will show later in this paper that the interface is quite dynamic over a wide range of timescales and the dynamics can result in the multiple quaternary structures of HbCO A in solution.

Effect of IHP on the structure of HbCO A

Titration of IHP to the chain specifically ²H,¹⁵N-labeled and ¹³C,¹⁵N-labeled HbCO A showed that while several ¹H-¹⁵N and ¹H-¹³C cross peaks displayed progressive chemical shift changes (Figs. S3a and b), most ¹H-¹⁵N cross peaks were only slightly affected, and most ¹H-¹³C cross peaks did not shift at all. From the dependence of chemical shift perturbations on IHP concentration (Fig. S3c), the binding affinity should be in sub-mM although it could not be quantified since the number of the IHP binding sites is unknown. Previous ³¹P NMR study found the affinity of IHP to HbCO was about 50 μM,²⁸ consistent with our titration data. The chemical shift perturbations for different amino acid residues are shown in Figure 2. The residues with significant chemical shift changes (larger than the average value) in backbone NH or/and side chain CH₃ are mainly located in four regions. The first region is around the N-terminus (e.g., αVal1, αSer3, αLys7, αAsn9, βVal1, βLeu3) and C-terminus (αThr137, βHis143, βHis146). The second region is around the EF loop (e.g., αAsn78, αAla79, βLys82, βGly83). The third region is in the α1β2 interface around the switch region or αC helix – βFG corner (e.g., αThr38, αThr39, αThr41, βLeu96, βAsp99 and βGlu101) and around the joint region or αFG corner – βC helix (e.g., αAsp94, αVal96, αAsn97, αLeu100, βPhe41). The fourth region is around the heme group (e.g., αAla65, αLeu83, αLeu86, αLeu93, αLeu136, βVal67, βAla70, βLeu88, βLeu98 and βLeu141) (Fig. 3).

According to the chemical shift perturbations, many of the perturbed residues are not located on the protein surface (Fig. S2). This indicates that IHP binding causes slight structural changes for the regions far away from the binding sites and determination of the binding sites is very difficult from the perturbation data alone. Thus, we performed docking of IHP to the protein with AUTODOCK^{23,24} based on the NMR structure determined here. We found two potential binding sites in the β cleft which are located at the entrance of the central cavity, two in the α cleft, and one inside the central cavity (Fig. 4 and Fig. S2). Each site in the β cleft involves 6 positively charged groups: β1Val1, β1His2, β1Lys82, β2Lys144, β2His143 and β2His146 in one site, β1His143 and β1His146, β2Val1, β2His2, β2Lys82 and β1Lys144 in the other (Fig. 4a and Fig. S2b). Each site in the α cleft involves only three positively charged groups: α1Val1, α1Lys7 and α2Arg141 in one site, and α1Arg141, α2Val1 and α2Lys7 in the other (Fig. 4b and Fig. S2a). Note that the N-terminal

amino group of a polypeptide is positively charged. Overall, the docking results are consistent with the chemical shift perturbation result (Fig. 2 and Fig. 4). The methyl groups of α Val1 and β Val1 and the backbone amide groups of α Lys7, β Lys82, β His143 and β His146 displayed very large chemical shift changes upon IHP binding (note that the amides from α Arg141, β Val1 and β His2 were not detectable in the ^{15}N - ^1H HSQC in the absence of IHP on an 800 MHz NMR spectrometer). Because the protein was assumed to be rigid in the docking process, the positively charged N-terminal amino group of the α -chain did not interact with the negatively charged IHP directly (Fig. 4b). It is likely that a direct charge-charge interaction may exist by re-orientating the N-terminus of the α -chain that is very flexible in the absence of IHP. The chemical shift of the backbone amide of β Lys144 did not change significantly, implying that only the side-chain of β Lys144 is involved in the interaction with IHP while its backbone is at a distance away from IHP. Besides β Lys144, other binding residues located in the β cleft were also found previously to be involved in the binding of IHP to the deoxy T-state Hb.²⁹

A recent computational study proposed that IHP binds to the α 1 and α 2 chains via interactions with α 1Lys99, α 1Arg141, α 2Lys99 and α 2Arg141 in the ligated R-state Hb.³⁰ Our docking also identified this binding site, i.e., the binding site inside the central cavity, but α Arg141 did not participate in the interaction with IHP in our model (Fig. 4c and Fig. S2c). In most X-ray structures, α 1Lys99 (located inside the central cavity of the Hb tetramer) is more than 15 Å away from α 1Arg141 that is exposed to solvent; it seems that IHP (the largest separation between two oxygen atoms in IHP is ~10 Å) cannot be held tightly by these four charged residues without conformational changes of the protein. When the dihedral angles around α Arg141 are allowed to change a few degrees, the charged α Arg141 and α Lys99 side-chains can interact simultaneously with IHP. In solution, the C-terminal region (α Lys139- α Arg141) of the α -chain was not observable on an 800-MHz NMR, but α Arg141 gave rise to weak signals on a 500-MHz NMR spectrometer in the absence of IHP, indicating that this region is flexible on ms- μ s timescales. In the presence of IHP, the ^1H - ^{15}N correlation of α Lys99 disappeared, indicating that α Lys99 was influenced by IHP via direct interaction or allosteric effect. In addition, the residues proximal to α Lys99 and α Arg141 displayed significant chemical shift changes upon IHP binding. Thus, it is possible that α 1Arg141 and α 2Arg141 move in towards the cavity in solution and then interact with one IHP molecule together with α 1Lys99 and α 2Lys99. Nearly all of the methyl groups around the heme in HbCO A displayed significant chemical shift changes upon IHP binding. Binding of IHP to any of the binding sites discussed above would not involve direct interaction of IHP with the heme or the methyl groups situated in the vicinity of the heme. Thus, the chemical shift perturbation for these methyl groups should result from slight tertiary structural changes around the heme induced by the heterotropic effector IHP. This is consistent with the previously proposed model, i.e., heterotropic effectors alter the tertiary structure of Hb and in turn change the function or oxygen binding affinity.^{9,31} Besides the chemical shift perturbation around the hemes, many residues located in the α 1 β 2 interface and several residues in the α 1 β 1 interface displayed significant chemical shift changes upon IHP binding. These shift changes should not be caused by the direct interaction with IHP either, but induced by alteration of the quaternary structure. This agrees very well with our results obtained from the recent RDC and backbone relaxation

studies.^{6,12} Therefore, the binding of IHP to Hb alters not only the tertiary structures around the heme groups, but also the quaternary structure. It seems that the heterotropic effectors regulate the affinity of Hb to its ligands (like O₂ and CO) through modulating both the tertiary and quaternary structures.

Amide-water exchange

Table 2 lists the amide-water proton exchange results obtained from exchange profiles (Fig. S4) using the CLEANEX-PM experiment for all measurable residues in HbCO A. Residues with amide-water proton exchange rates slower than 1 s⁻¹ could not be detected by this method. Only about 10% of the amides showed relatively large amide-water proton exchange rates under our experimental condition (pH 7.0 and 30 °C). These amides are distributed mainly in the N-terminal regions, AB connection (α Ala19 – α His20, β Val18 – β Asn19), α CD/ β CE loop (α Pro44 – α Ser52, β Phe41 – β Asp52), EF loop (α His72 – α Ser81, β Leu78 – β Thr87), and GH loop (α Pro114 – α Phe117, β Phe118 – β Phe122). The exchange results show that the loops and N-termini of the α - and β -chains are dynamic on sec to ms timescales. According to the R and R2 structures of HbCO A, all amides with measurable exchange rates in the absence of IHP, with the exception of α His20 and α Ser52, do not form hydrogen bonds, demonstrating that the tertiary structure of HbCO A in solution is very similar to the crystal R and R2 structures. The amides of α His20 and α Ser52 had detectable exchange rates, indicating that they may not be involved in hydrogen bonding in solution, although they can form hydrogen bonds with the respective backbone oxygen atoms located at α Val17 and α Ser49 in the crystal structures. The difference for these two residues between the solution and crystal structures is further supported by a previous backbone dynamics study,¹² which suggested that the backbone amides of α His20 and α Ser52 are significantly more mobile than the amides in regular helices on ps-ns timescales. Interestingly, α Thr38 located in the α 1 β 2 interface displayed a very large exchange rate, indicating that the interface around this residue is accessible to water and dynamic on the sec to ms timescale.

Upon binding of IHP, the exchange rates for most amides remained nearly unchanged, showing that the interaction of HbCO A with IHP does not alter the secondary structure significantly. However, several residues displayed significant changes in the exchange rates – α Ala5, β Phe41, and β Ser44 showed increases larger than 10 s⁻¹, while α Thr38, α Ala82, β Leu81 and β Lys82 displayed decreases of more than 10 s⁻¹ (Tab. 2). Since β Leu81 and β Lys82 are located in one possible IHP binding site, IHP binding may reduce the access of water to the amides of β Leu81 and β Lys82 and in turn causes a significant reduction of the amide-water exchange rates for these two residues. Because α Ala5 is near a possible IHP binding site in the α -cleft, the change in its NH-water exchange rate may result from IHP-induced structural changes around the binding site. According to the chemical shift perturbations (Fig. 2), the binding of IHP perturbs the structure of the α F helix. The slight structural alteration in this helix may contribute to the change of the exchange rate for α Ala82 (located at the beginning of the α F helix). α Thr38 is located in the α 1 β 2 switch region while β Phe41 and β Ser44 are in the joint region. Significant changes of the exchange rates for these residues further indicate that the α 1 β 2 interface structure (quaternary structure) is altered upon IHP binding.

Order parameters of methyl groups

Methyl ^{13}C R_1 and Γ values were determined for HbCO A in the absence and presence of IHP. Only cross peaks in the ^1H - ^{13}C HSQC without overlap were analyzed. Methyl groups involved in strong J coupling interactions with their respective adjacent ^{13}C spins were excluded in our analysis. Because the rotational motion of HbCO A in solution is nearly isotropic,¹² an isotropic overall motional model (with an overall correlation time of 31 ns determined from ^{15}N relaxation data using the previous method³²) was used in the extraction of order parameters from the R_1 and Γ values. Values of S^2_{axis} were calculated for 81 out of 94 methyl groups in the α -chain and 79 out of 95 methyl groups in the β -chain of HbCO A in the absence of IHP, while for 76 methyl groups in the α -chain and 83 methyl groups in the β -chain of HbCO A in the presence of IHP. The order parameter data are shown in Fig. 5 for the α - and β -chains. The S^2_{axis} values are distributed in wide ranges, from ~ 0.2 to ~ 1 for the α -chain (Fig. 5a and b) and from ~ 0.1 to ~ 1 for the β -chain (Fig. 5c and d). For a given methyl-containing residue, the S^2_{axis} value of the methyl group depends not only on the backbone mobility of this residue, but also on how far away the methyl group is from the backbone.³³ To correct for the positional dependence, averages of the S^2_{axis} values for each methyl type ($\langle S^2_{\text{axis}} \rangle$) were used to analyze the difference between S^2_{axis} and its corresponding $\langle S^2_{\text{axis}} \rangle$. The $\langle S^2_{\text{axis}} \rangle$ values in the absence and presence of IHP are listed in Table 3. As there are only two Met in the α -chain and one in the β -chain, respectively, their S^2_{axis} values instead of $\langle S^2_{\text{axis}} \rangle$ are shown in Table 3.

Methyl dynamics on ps-ns timescales in the absence of IHP

The S^2_{axis} values of Ala methyl groups reflect the mobility of Ala C_α - C_β bonds, i.e., representing the backbone dynamics of Ala residues. Similar to backbone S^2 values measured from ^{15}N relaxation, the S^2_{axis} values are distributed in a small range. Only αAla19 in the A-B connection had a significantly smaller S^2_{axis} value (0.67). ^{15}N relaxation data also indicate that a couple of residues in this short loop are more flexible than other loop regions.¹²

Unlike backbone order parameters, S^2_{axis} values for Thr, Val and Leu methyl groups did not correlate with the secondary structure. Many residues located in the α -helical elements had small S^2_{axis} values, while some residues located in the loop regions showed relatively large S^2_{axis} values. Nevertheless, methyl order parameters for Thr, Val, and Leu residues correlated well with the tertiary and quaternary structures as discussed below.

The methyl groups of αThr39 and αThr108 are completely buried inside the protein molecule according to the R, R2 and NMR structures; their S^2_{axis} values were 0.95 and 0.81, respectively, and were significantly larger than the average (0.70). On the other hand, the S^2_{axis} values for some partially solvent-exposed Thr methyl groups (e.g., 0.58 for αThr8 , 0.51 for βThr12 and 0.31 for βThr87) were smaller than the average. Although αThr67 , αThr118 , βThr4 , βThr50 and βThr123 are partially solvent-exposed, their S^2_{axis} values were 0.81, 0.91, 0.81, 0.73 and 0.89, respectively, significantly larger than the averages (0.70 for the α -chain and 0.65 for the β -chain) (Fig. S5a and c). Interestingly, in both the R and R2 structures the side chain oxygen atoms of αThr118 , βThr4 , βThr50 and βThr123 can form

hydrogen bonds with their proximal backbone NH hydrogen atoms of α Val121, β Glu7, β Ala53 and β Val126, respectively (hydrogen bonds are defined with the criteria: $r_{\text{H(N)O}} < 2.4$ Å and $\theta_{\text{HNO}} < 35^\circ$). The side chain of α Thr67 can form a hydrogen bond with the side chain NεHε hydrogen of α Trp14 only in the R2, but not in the R structure. If the side chain of a Thr residue is involved in hydrogen bonding, the motion of the $\text{C}_\beta\text{-C}_\gamma$ bond is greatly restricted and the S^2_{axis} value should be quite large, similar to what we observed for CH_2 groups in some Asp, Gln and Ser of which side-chains form H-bonds in drkN SH3 domain.³³ Although the accuracy of the NMR structure is not good enough for determining the presence of a hydrogen-bond between two atoms, the dynamics data obtained here indicate that the side chains of α Thr67, α Thr118, β Thr4, β Thr50 and β Thr123 are involved in hydrogen bonding in solution. α Thr38 and α Thr41 are located in the $\alpha 1\beta 2$ interface and their S^2_{axis} values were 0.47 and 0.43, respectively, significantly smaller than the average (0.70). The side chain oxygen atom of α Thr41 is predicted to form a hydrogen-bond with the side chain NH_2 hydrogen atom of β Arg40 only in the R structure, but not in the R2 structure. The dynamics result clearly indicates that there is no hydrogen bonding between α Thr41 and β Arg40 and the $\alpha 1\beta 2$ interface is quite flexible on the ns-ps timescale in solution. The high S^2_{axis} value of α Thr67 and low value of α Thr41 indicate that the local structure at these two residues in solution resembles more closely the R2 structure than the R structure although the overall solution structure is more similar to the R than the R2 structure in terms of the relative orientation of the two $\alpha\beta$ dimers. Most of the methyl groups of Leu and Val also have S^2_{axis} values larger than the averages when they are buried inside, while they have S^2_{axis} values smaller than the averages when they are solvent-exposed. However, several methyl groups do not follow this trend. The methyl groups of α Leu2, α Leu105, α Leu109, β Leu3, β Leu14, β Leu68, and β Leu75 are nearly completely buried, yet their S^2_{axis} values were significantly smaller than the averages. A similar phenomenon was observed previously for one Leu residue in the PLCC SH2-peptide complex.³⁴ α Leu2 and β Leu3 are located at the N-termini and their backbone amides were shown to be quite flexible.¹² Thus, the low S^2_{axis} values for these two residues should be caused by the high backbone mobility. α Leu105, α Leu109, β Leu14, β Leu68, and β Leu75 are located in regular helices with high backbone rigidity. The high mobility of these buried Leu methyl groups can result from local breathing motion or/and the presence of internal cavities around these residues.³⁴ On the other hand, α Leu91 and β Leu96 methyl groups are partially exposed to solvent, but the S^2_{axis} values were 0.88, 0.71, 0.83 and 0.69 for α Leu91 δ 1, α Leu91 δ 2, β Leu96 δ 1 and β Leu96 δ 2, respectively, surprisingly larger than the averages (0.55 and 0.52 for the α -chain and β -chain). Interestingly, both α Leu91 and β Leu96 methyl groups are proximal to hemes (Fig. 3) and we observed several inter-molecular NOEs between each of these methyl groups and the heme. The high S^2_{axis} values of these methyl groups can thus be attributed to their interactions with the heme.

Excluding the alanines, the methyl-containing residues located in the $\alpha 1\beta 1$ interface include α Leu34, α Val107, α Thr118, β Val33, β Val34, β Met55, β Val111 and β Thr123. With the exception of α Leu34, all other residues had S^2_{axis} values significantly higher than the averages. Only one Met residue exists in the β -chain and its S^2_{axis} value (0.62) is larger than the average S^2_{axis} value of Leu methyl groups although Met methyl is one bond further away from the backbone than Leu methyl. The results indicate that the $\alpha 1\beta 1$ interface of

HbCO A is rigid on this timescale, consistent with the backbone dynamics results and the X-ray structures. α Leu34 is located at the end of helix B and one of its methyl groups is largely exposed to solvent, explaining its low S^2_{axis} values (~ 0.34). Only three methyl-containing residues, α Thr38, α Thr41, and α Val96 are located in the $\alpha 1\beta 2$ interface. Both α Thr38 and α Thr41 have flexible methyl side chains as shown above. Moreover, the S^2_{axis} values of the two methyl groups in α Val96 are 0.48 and 0.47, respectively, also significantly lower than the average (0.71). Taken together, the results demonstrate that the $\alpha 1\beta 2$ interface is flexible while the $\alpha 1\beta 1$ interface is quite rigid on ps-ns timescales in solution. Solvent hydrogen exchange experiments,³⁵ though, showed that the $\alpha 1\beta 1$ interface is dynamic as well, albeit on a much slower timescale of ms-s, faster in deoxy-Hb and slower in the ligated forms of Hb A. This dynamic behavior is revealed by the solvent exchange rates of the histidyl side-chain NeH of α His103 and α His122 and these rates increase in the presence of allosteric effectors such as IHP and inorganic phosphates or chloride ions.

Methyl dynamics on ms – μ s timescale

Except for α Thr38 and α Thr41, the methyl groups of Ala, Met and other Thr residues in HbCO A do not display relaxation dispersion in the absence of IHP. For α Thr38 and α Thr41, small relaxation dispersions can be observed (Fig. 6), indicating that the switch region in the $\alpha 1\beta 2$ interface of HbCO A is dynamic on the ms- μ s timescale, which is also dynamic on the ns-ps timescales as shown above. α Thr38 methyl had a much larger effective relaxation rate than other methyl groups, but its relaxation dispersion is small over the range of CPMG field strengths from 60 to 600 Hz. This result indicates that the conformational exchange for the α Thr38 methyl occurs on a timescale significantly faster than millisecond. The conformational exchange observed here is consistent with the result obtained from a recent backbone relaxation study – α Lys40 of HbCO A (in the $\alpha 1\beta 2$ interface) is mobile on the ms- μ s timescale.¹³ The mobility of this switch region was detected only for HbCO A, but not for the deoxy form.¹³ In the deoxy form, the positively charged side chain of α Lys40 forms strong interactions with the negatively charged C-terminus of the β -chain (β His146) according to the crystal structure, greatly reducing the mobility of the switch region. However, in the liganded form, these two residues are too far away to interact with each other in our NMR structure. In consequence, the interactions in the $\alpha 1\beta 2$ interface become weaker and the interface becomes more dynamic upon Hb ligation. This conformational exchange in the $\alpha 1\beta 2$ interface should result from local dynamics instead of reorientation of the $\alpha 1\beta 1$ dimer relative to the $\alpha 2\beta 2$ dimer. Otherwise, more residues would display relaxation dispersion. Localized conformational exchange in the $\alpha 1\beta 2$ interface was also detected early for the indole NH of β Trp37 in HbCO A in the presence of IHP.³⁶

Effects of IHP binding on methyl dynamics

As shown in Table 3, Fig. 5 and Fig. S5, upon IHP binding, some methyl groups of HbCO A became more flexible, while others became more rigid, but the average order parameters, $\langle S^2_{\text{axis}} \rangle$, showed nearly no change ($\langle S^2_{\text{axis}} \rangle(\text{free}) - \langle S^2_{\text{axis}} \rangle(\text{IHP}) = -0.004$). The changes of S^2_{axis} values [$S^2_{\text{axis}}(\text{free}) - S^2_{\text{axis}}(\text{IHP})$] were within a quite small range and the maximal change was about 0.19 (Fig. S5b and d). The residues with significant changes in S^2_{axis} ($|S^2_{\text{axis}}(\text{free}) - S^2_{\text{axis}}(\text{IHP})| > 0.05$) are distributed mainly in four regions: (i) proximal to the

IHP binding sites in the α -cleft (α Val1) and β -cleft (β Val1, β Leu3 and β Leu81); (ii) proximal to the hemes in the α -chain (α Met32, α Ala65, α Leu86, α Leu105) and β -chain (β Leu31, β Val98, β Leu106 and β Leu141); (iii) α 1 β 2 interface (α Thr38); and (iv) the α 1 β 1 interface (α Leu34, α Val107, α Thr118, β Val34 and β Ala115). Several residues in the α G helix (α Leu101, α Leu106, α Thr108 and α Leu109), β G helix (β Leu114), and β H helix (β Val134) also displayed significant changes in S^2_{axis} values upon the interaction of IHP with HbCO A. Although α Val1 and β Val1 are close to the IHP binding sites in the α - and β -clefts, their methyl groups may not interact directly with IHP and the increase in their methyl mobility may arise from structural change around the binding sites. Consistent with IHP-induced structural changes (or chemical shift changes), many methyl-containing residues in the proximity of the hemes also displayed changes in dynamics on the ps-ns timescales. These changes should be caused by the allosteric effect instead of direct interactions with IHP. Similarly, the changes in the dynamics for the residues located in α 1 β 1 and α 1 β 2 interfaces and G and H helices should originate from the allosteric effect too.

A recent MD simulation study showed that Hb became more flexible or had higher entropy upon either de-oxygenation or/and binding of 2,3-BPG. The estimated timescale and maximal amplitude of the fluctuation of helices E and F were of the order of ~ 2 ns and ~ 3 Å, respectively.¹¹ On the basis of this simulation, it was proposed that the concerted fluctuations of the EF helical region could modulate the position of the distal and proximal His residues relative to the heme Fe and, in turn, could modulate the oxygen affinity of Hb.⁸ It was also suggested that the heterotropic allosteric effects of Hb were caused by conformational entropy changes and had not much to do with quaternary structural changes.⁸ According to our experimental results, IHP binding induces limited changes in the dynamics of some residues on the ps-ns timescales. However, there were no obvious overall changes in mobility on the ns-ps timescales for the entire protein or E/F helices upon IHP binding (Fig. 5 and Fig. S5). Using the order parameters for the methyl groups in the IHP free and bound forms, we estimated the conformational entropy change ($S(\text{IHP}) - S(\text{free})$) to be about $-70 \text{ J mol}^{-1} \text{ K}^{-1}$ for HbCO A using the simple diffusion-in-a-cone model.³⁷ It is noteworthy that the entropy was calculated by considering conformational changes of only methyl groups on ns-ps timescales and neglecting the contributions from other timescales, other groups in methyl-containing residues and other residues. A more reliable empirical calibration method has recently been proposed to estimate conformational entropy change upon ligand binding from only methyl order parameters.³⁸ Using a scaling factor of $0.037 \text{ kJ mol}^{-1} \text{ K}^{-1} \text{ residue}^{-1}$ established previously³⁸, the conformational entropy change was found to be $-85 \text{ J mol}^{-1} \text{ K}^{-1}$ for the Hb tetramer, consistent with result derived from the simple model. Because the scaling factor is uncertain and may be different for different proteins,³⁹ the entropy obtained here is just an estimation. Nevertheless, our result indicates that the conformational entropy of HbCO A should decrease or remain nearly no change rather than increase significantly upon IHP binding as proposed previously.^{8,11} Therefore, the heterotropic allosteric effects should result mainly from both tertiary and quaternary structural changes, and might be influenced slightly by changes in the dynamics on the ns-ps timescales.

Upon the binding of IHP to HbCO A, six residues among all the Ala, Met, and Thr residues show obvious relaxation dispersion (Fig. 6). One of them could not be assigned unambiguously because the methyl correlation peak of this residue is in an overlapped region in the ^{13}C - ^1H HSQC spectrum of the α -chain in the absence of IHP. The other five residues (αThr38 , αThr41 , βAla70 , βAla138 , and βAla142) are distributed in three regions. αThr38 and αThr41 are located in the $\alpha1\beta2$ interface, βAla70 is in close contact with the heme (Fig. 3), βAla138 and βAla142 are near the heme, and βAla142 is adjacent to a residue (βHis143) directly involved in IHP binding (Fig. 4a). According to previous measurements of R_{ex} values for backbone amides,¹³ IHP-induced conformational exchange was also observable in these three regions: the $\alpha1\beta2$ interface, heme binding sites, and IHP binding sites. The results indicate that HbCO A exists in two or more conformations in the presence of IHP, at least for these three regions. Early ^{31}P NMR studies⁴⁰ suggested that IHP binds sequentially to hemoglobin at multiple sites in fast exchange between them with exchange rates larger than 10^4 s^{-1} . The conformational exchange observed here should not correspond to those among the fully IHP-bound, partially IHP-bound and IHP-free forms since many methyl groups displayed significant chemical shift perturbations by IHP, but did not show relaxation dispersion upon IHP binding. Instead, there should exist more than one protein conformations that undergo exchange in the presence of IHP. Assuming a two-state exchange model,⁴¹ we estimated the exchange rates to be in the range of $400 - 800 \text{ s}^{-1}$ (i.e., on the ms timescale), significantly smaller than that estimated from k_{on} and k_{D} for the binding of IHP to HbCO A.⁴⁰ Most likely, the conformational exchange observed here arises from the relative motion of the $\alpha1\beta1$ dimer with respect to the $\alpha2\beta2$ dimer. The relative motion alters the local structures (chemical shifts) around the $\alpha1\beta2$ interface and also the heme binding sites, modulating the oxygen affinity of Hb.

Concluding Remarks

The structural and dynamic results presented in this paper show that (i) the classical two-structure allosteric models for hemoglobin cannot describe the mechanisms as well as the structural and dynamic aspects of the cooperative oxygen binding to Hb A and (ii) hemoglobin is a flexible molecule. This flexibility is essential for the physiological function of Hb A as an oxygen carrier. In solution, the structure of HbCO A is a dynamic ensemble of various structures. A recent wide-angle x-ray solution scattering (WAXS) investigation shows that the observed WAXS pattern for HbCO A in solution are different from those calculated from the atomic coordinate sets, suggesting the structure of this protein in solution is different from the known crystal structures,⁴² consistent with our NMR structural and dynamic results.

The binding of the heterotropic allosteric effector, IHP, affects both tertiary and quaternary (inter-subunit interface) structures of hemoglobin as well as the relative motion of the $\alpha1\beta1$ dimer with respect to the $\alpha2\beta2$ dimer, but does not increase the conformational entropy. This result is significant, as the role of protein dynamics in the allosteric effect has gained increased recognition over the recent years.⁴³⁻⁴⁵ It appears thus that an intricate web of multiple and highly specific interactions involving both types of the inter-subunit interfaces is responsible for regulating hemoglobin function. An important implication of our results is

that allosteric interactions in other regulatory proteins and enzymes will likely require multiple pathways for signal communication, with the dynamics playing a crucial role.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

RDC	residual dipolar couplings
NOESY	nuclear Overhauser effect spectroscopy
IHP	inositol hexaphosphate
HbCO A	human adult carbonmonoxy Hb
2,3-BPG	2,3-bisphosphoglycerate
HSQC	heteronuclear single-quantum coherence
HMQC	heteronuclear multiple-quantum coherence
CSA	chemical shift anisotropy
CPMG	Carr-Purcell-Meiboom-Gill sequence

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Figure 1.

Comparison of the symmetric axis orientations (a-d) and the switch region in $\alpha 1\beta 2$ interface (e) of HbCO A in the T, R, R2 and solution conformations. (a). Distribution of the C₂ axes of different structures in a 3D frame. The angles shown in b, c and d are not drawn to scale and are enlarged for better visualization. The C₂ axes of the T, R, R2 and 20 solution conformations are in blue, red, magenta and black lines. The angles between the C₂ axes of the T structure and the 20 lowest energy NMR structures (b), between the C₂ axes of the R structure and the 20 lowest energy NMR structures (c), and between the C₂ axes of the R2 structure and the 20 lowest energy NMR structures (d). The average direction of the C₂ axes of the solution structures is denoted by a green arrow in b, c and d. The switch region in the T, R, R2 and one representative solution conformation are shown in blue, red, magenta and yellow respectively in (e). The backbone atoms of residues 38-44 in the $\alpha 1$ subunit are superimposed in order to illustrate the relative orientation of $\beta 2$ His97.

Figure 2.

Chemical shift perturbations of backbone amides and side chain methyl groups by the binding of IHP to HbCO A. The chemical shift perturbations were defined as $\delta_{av} = [(\delta_{HN}^2 + \delta_N^2/25)/2]^{0.5}$ for amide NH²⁷ and $\delta_{av} = [(\delta_{HC}^2 + \delta_C^2/4)/2]^{0.5}$ for methyl groups, where δ_{HN} , δ_N , δ_{HC} and δ_C are the chemical shift differences of amide ¹H, amide ¹⁵N, methyl ¹H, and methyl ¹³C between the samples in the presence of 3 mM IHP and in the absence of IHP. The empty regions represent no information available for residues in those regions because the NH and CH HSQC peaks of those residues were invisible or unassigned. The residues containing no methyl groups are located in the empty regions too. For Leu and Val, the data for γ_1 and δ_1 methyl groups are shown in blue bars while the data for γ_2 and δ_2 methyl groups in red bars. Other methyl groups are displayed in red bars. The dash lines represent the mean values over all residues with perturbation data.

Figure 3.

Heme and its proximal methyl-containing residues in the α -chain and β -chain. Methyl carbon atoms displaying significant and insignificant chemical shift perturbations are shown in red and green, respectively.

Figure 4.

IHP binding sites in the β -cleft (a), α -cleft (b) and center cavity (c). IHP molecule is shown in blue stick.

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Figure 5.

Order parameters S^2_{axis} , describing the degree of spatial restriction of the C_3 symmetric axis for methyl groups in the α -chain (a) and β -chain (c) in the absence of IHP and in the α -chain (b) and β -chain (d) in the presence of IHP. The uncertainties of S^2_{axis} for most methyl groups were about 0.01 and the maximum uncertainty was about 0.02. The open and filled circles represent pro-R and pro-S methyl groups for Val and Leu, respectively. Ala, Leu, Met, Thr and Val methyls are shown in magenta, red, green, light blue and black.

Figure 6.

Relaxation dispersion profiles of the methyl groups with intrinsic conformational exchange in the absence and presence of IHP. The data collected in the samples with and without IHP are presented by ‘o’ and ‘*’, respectively. The solid lines are fitting curves assuming the two-state exchange model. The identity of each residue is indicated inside each panel.

Table 1Structural statistics for the final 20 conformers of HbCO A^a

Distance restraints	
Intra-residue ($i-j = 0$)	2740
Sequential ($ i-j = 1$)	1732
Medium range ($2 \leq i-j \leq 4$)	1356
Long range ($ i-j \geq 5$)	1114
Heme-subunit	218
Inter-subunit	152
Hydrogen bond	472
Total	7754
Dihedral angle restraints	
ϕ	520
ψ	520
Residual Dipolar coupling restraints	
N-H (work/free)	298 (242/56)
Dipolar coupling Q factor (%) ^b	
Work (used)	6.9
Free (non-used)	25.3
Average rmsd to the mean structure (Å) ^c	
Global (tetramer)	2.36 ± 1.31 (2.65 ± 1.19) ^d
α -chain	0.69 ± 0.19 (1.34 ± 0.18)
β -chain	0.64 ± 0.18 (1.26 ± 0.19)
$\alpha\beta$ dimer	0.93 ± 0.34 (1.46 ± 0.31)
ϕ/ψ space ^e	
Most favored region (%)	83.5
Additionally allowed region (%)	12.9
Generously allowed region (%)	3.0
Disallowed region (%)	0.6
rmsd from covalent geometry	
Bonds (Å)	0.0028 ± 0.00004
Angles (deg.)	0.4233 ± 0.0078
Impropers (deg.)	0.4712 ± 0.0114
rmsd from experimental restraints	
NOEs (Å)	0.03 ± 0.016
Dihedral angles (deg.)	0.58 ± 0.018

^a Selected from 100 calculated conformers according to overall energy. The restraints are for the 4 chains (i.e., a tetramer).^b Q factor calculated with the same method as that used in Table S1.^c Calculated with MOLMOL.⁴⁶ Residues in all alpha-helix regions were used (i.e. Helix A, B, C, E, F, G and H for α -chain, helix A, B, C, D, E, F, G and H for β -chain).

^d Averages are over heavy backbone atoms (all heavy atoms).

^e Calculated with PROCHECK-NMR. ⁴⁷

Table 2

Water-amide proton exchange rates for HbCO A in the absence and presence of IHP

Res.	k_{ex} (s^{-1}) without IHP	k_{ex} (s^{-1}) with IHP	Intrinsic $k_{\text{ex}}(\text{s}^{-1})^b$
α -Ala5	15.7 $\pm$ 0.1	34.2 $\pm$ 1.0	15.0
α -His20	2.6 $\pm$ 0.2	2.5 $\pm$ 0.1	43.7
α -Gly22	34.7 $\pm$ 0.2	35.3 $\pm$ 0.6	48.6
α -Thr38	38.3 $\pm$ 0.7	19.3 $\pm$ 0.8	12.8
α -His45	5.4 $\pm$ 0.1	4.6 $\pm$ 0.4	25.1
α -His50	60.3 $\pm$ 0.3	52.0 $\pm$ 0.8	87.2
α -Gly51	43.7 $\pm$ 0.4	39.9 $\pm$ 0.6	119.0
α -Ser52	2.3 $\pm$ 0.1	2.4 $\pm$ 0.1	90.5
α -Ala53	63.1 $\pm$ 0.9	66.1 $\pm$ 0.5	52.1
α -Gln54	6.1 $\pm$ 0.1	6.8 $\pm$ 0.2	30.0
α -Asp74	3.7 $\pm$ 0.1	3.7 $\pm$ 0.1	9.5
α -Ala82	35.6 $\pm$ 0.1	19.5 $\pm$ 0.2	52.1
α -Ala115	11.1 $\pm$ 0.2	11.5 $\pm$ 0.3	15.0
β -Leu3	4.3 $\pm$ 0.3	1.5 $\pm$ 0.1	16.8
β -Glu6	3.7 $\pm$ 0.1	5.5 $\pm$ 0.4	4.7
β -Asn19	4.8 $\pm$ 0.2	5.6 $\pm$ 0.1	58.4
β -Phe41	$_a$	40.4 $\pm$ 2.0	24.9
β -Ser44	28.8 $\pm$ 0.5	48.7 $\pm$ 0.7	43.5
β -Asp47	4.2 $\pm$ 0.1	7.5 $\pm$ 0.2	17.3
β -Asp52	1.3 $\pm$ 0.1	4.0 $\pm$ 0.2	7.6
β -Leu78	$_a$	1.2 $\pm$ 0.1	16.8
β -Asp79	6.4 $\pm$ 0.2	5.7 $\pm$ 0.1	10.2
β -Leu81	59.6 $\pm$ 0.8	19.4 $\pm$ 0.8	14.3
β -Lys82	14.8 $\pm$ 0.3	$_a$	14.7
β -Thr87	16.9 $\pm$ 0.4	17.1 $\pm$ 0.3	22.2
β -Lys120	5.2 $\pm$ 0.2	7.2 $\pm$ 0.4	35.2

^a exchange rates smaller than 1 s^{-1} , which are undetectable.^b intrinsic exchange rates derived using SPHERE (<http://www.fccc.edu/research/labs/roder/sphere/>).

Table 3

Average S^2_{axis} values for Ala, Thr, Val and Leu residues and S^2_{axis} values for Met residues in the absence and presence of IHP

$\langle S^2_{\text{axis}} \rangle$	Ala	Thr	Val	Leu	Met
α -chain without IHP	0.87±0.07	0.70± 0.20	0.71±0.17	0.55±0.18	M32, 0.90 M76, 0.21
α -chain with IHP	0.87±0.07	0.68 ± 0.17	0.72± 0.20	0.57±0.19	M32, 0.94 M76, 0.21
β -chain without IHP	0.86± 0.05	0.65± 0.22	0.73 ± 0.18	0.52 ± 0.22	M55, 0.62
β -chain with IHP	0.87±0.06	0.67 ± 0.22	0.73± 0.19	0.54 ± 0.22	M55, 0.63