Coupling of Tertiary and Quaternary Changes in Human Hemoglobin: A 1D and 2D NMR Study of Hemoglobin Saint Mandé (βN102Y)†

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ABSTRACT: Hemoglobin Saint Mandé (βN102Y) is a low-affinity mutant with the substitution site situated in the quaternary-sensitive $\alpha_1\beta_2$ interface. In adult hemoglobin the Asn102β contributes to the stability of the liganded (R) state, forming a hydrogen bond with Asp94α. The quaternary and tertiary perturbations subsequent to the Tyr for Asn substitution in monocarboxylated hemoglobin Saint Mandé have been investigated by one- and two-dimensional nuclear magnetic resonance (NMR) spectroscopy. Analysis of the one-dimensional NMR spectra of the liganded and unliganded samples in ^1H_2O provides evidence that both R and T quaternary structures of Hb Saint Mandé are different from the corresponding ones in HbA. In the monocarboxylated form of the mutant hemoglobin, at acid pH, we have observed the disappearance of an R-type hydrogen bond and the appearance of a new one whose proton resonates like a deoxy T marker. Using two-dimensional NMR methods and on the basis of previous results on the monocarboxylated HbA, we have obtained a significant number of resonance assignments in the spectra of monocarboxylated Hb Saint Mandé at pH 5.6 in the presence or absence of a strong allosteric effector, inositol hexaphosphate. This enabled us to characterize the tertiary conformational changes (relative to the liganded normal hemoglobin) triggered by the quaternary-state modification. The observed structural variations are confined within the heme pocket regions but concern both the α and β subunits. Most of them, localized in the C, F, G, and FG segments, could result directly from the side-chain substitution, while others, such as Leu141β, could be explained only by long-range interactions.

The aim of the major part of the recent physicochemical studies on human hemoglobin is to find the detailed structural basis of its positive cooperative binding of ligands. The first stereochemical model of the Hb[1] cooperativity (Perutz, 1970) was based on the identification and crystallographic characterization of the quaternary structures corresponding to the unliganded (T) and liganded (R) states. A successful refinement of this model requires further structural information on the pathway of heme–heme interaction and on the less stable transition states induced by the progressive saturation of the unliganded form. Much effort was concentrated on the description of these aspects of cooperativity by studying valency hybrids (Cassoly & Gibson, 1972; Ogawa & Schulman, 1972), metal hybrids (Ikeda-Saito & Yonetani, 1980), and natural mutants substituted at the interface between heterologous subunits (Greer, 1971; Andersen, 1975; Salhany et al., 1975).

Despite their limitation to some structurally localized physicochemical features (stretching modes of several chemical bonds, NMR resonances of hyperfine-shifted or hydrogen-bonded protons, electronic transitions in the heme group, etc.), the spectroscopic studies on Hb solutions give valuable information that is complementary to the crystallographic results because they can explore a large range of solution conditions close to the biological ones.

We have recently demonstrated (Schaeffer et al., 1988; Craescu & Mispelter, 1988, 1989) that a systematic application of 2D NMR techniques on isolated subunits and Hb tetramers in the liganded (CO) state permits assignment of a large number of resonances (corresponding to about 15% of the residues). The assigned resonances provide an extensive set of structural probes for the investigation of the conformational properties of the protein in solution. We have thus shown that the tertiary structure of the heme pocket in solution and that in the crystal state are highly similar (Craescu & Mispelter, 1989). A corresponding study on the natural unliganded T conformation should be significantly more difficult a priori due to the paramagnetic effects of the high-spin ferrous iron in this state. Large paramagnetic shifts and significant line broadening are expected, especially for the heme pocket resonances for which we have obtained substantial structural information in the liganded R state.

A convenient diamagnetic model of the T or an intermediate quaternary state for NMR studies would be a hemoglobin variant, which in the liganded state could be shifted in a low-affinity, T-like structure. Functional and structural studies on Hb Kansas, a low-affinity variant that has a substitution localized in a critical R-type $\alpha_1\beta_2$ contact (βN102T), have shown that at acid pH and in the presence of a strong allosteric effector, IHP, the liganded (CO) tetramer has a T-like quaternary structure (Ogawa et al., 1972; Shulman et al., 1972). Hb Saint Mandé (βN102Y), a similar natural variant discovered in our laboratory (Arous et al., 1981), also has a low oxygen affinity, reflecting a highly destabilized R state. We

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[1] Abbreviations: Hb, hemoglobin; Hb A, human adult hemoglobin; Hb SM, hemoglobin Saint Mandé (βN102Y); IHP, inositol hexaphosphate; Bis-Tris, [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)-methane; 1D, one-dimensional; 2D, two-dimensional; COSY, two-dimensional homonuclear *J*-correlated spectroscopy; NOESY, two-dimensional nuclear Overhauser effect spectroscopy; DSS, 4,4-dimethyl-4-silapentane-1-sulfonate; ppm, parts per million.

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thus decided to study in more detail the solution conformation of the monocarboxylated Hb Saint Mandé using 1D and 2D NMR methods. In attempting to stabilize a liganded T-like structure we chose to perform the experiments at an acid pH and in the presence of IHP, which is known to shift the quaternary equilibrium toward the T structure. Analysis of the spectroscopic probes corresponding to the intersubunit hydrogen bonds indicated that, under these physicochemical conditions, both liganded and unliganded quaternary states of the mutant are different from the standard R and T states of the Hb A. Using 2D NMR methods, we then assigned a large number of resonances in the spectrum of monocarboxylated Hb Saint Mandé with and without IHP. This enabled us to characterize the amplitude and delocalization of the tertiary perturbations (relative to the liganded R structure of Hb A) accompanying the modification in the quaternary structure. Some of the observed tertiary changes could only be explained by long-range interactions along the lines of those triggered by quaternary transitions.

Materials and Methods

Hb Saint Mandé was purified by cation-exchange chromatography on a CM52 column. Samples for NMR experiments (8 mM heme) were prepared in 100 mM phosphate buffer, pH 5.6 in ^2H_2O or in 90% ^1H_2O/10% ^2H_2O. Th pH was measured with a Radiometer pH meter, and no correction was applied for the deuterium isotope effect on the glass electrode.

Deuterated potassium was purchased from Merck; ^2H_2O (99.98%) was obtained from the Commissariat â l'Energie Atomique, France.

The 1D and 2D NMR spectra were recorded at 400 MHz on an AM 400 WB Bruker spectrometer with an Aspect 3000 computer for data processing. 1D spectra in ^1H_2O were obtained by using an efficient hard-pulse sequence for solvent suppression proposed by von Kienlin et al. (1988). Phase-sensitive COSY and NOESY spectra were accumulated and processed as previously reported (Craescu & Mispelter, 1988).

The chemical shifts were measured relative to the residual water proton signal, which is 4.63 and 4.78 ppm downfield from the proton resonance of DSS at 37 and 25 °C, respectively. The broad line width of the exchangeable protons (>50 Hz) limits the precision of the chemical shift determination to 0.1 ppm. The other chemical shifts are reported to 0.01 ppm.

Results

Spectroscopic Probes for the Quaternary State. Amino acid subsititutions in the $\alpha_1\beta_2$ and $\alpha_2\beta_1$ interfaces have the tendency to destabilize the tetrameric assembly of the Hb to various extents. Experiments performed in our laboratory (J.-P. Mahieu and C. T. Craescu, unpublished results) indicated that in the case of Hb SM(CO) the Tyr for Asn substitution increases the tetramer/dimer dissociation constant by a factor of 10. This dissociation constant predicts that less than 2% of the protein may be in a dimer form at the high protein concentration used for the NMR studies (8 mM heme). Therefore, the results reported here correspond to the tetrameric form of the Hb.

The largest conformational changes accompanying the quaternary transition in hemoblobin are localized in interface regions between heterologous subunits ($\alpha_1\beta_2$ and $\alpha_2\beta_1$). The low intrinsic mobility of the side chains in these buried regions determines broad NMR resonances for the corresponding protons, rendering their assignment more difficult. Fortunately, some of the protons implicated in hydrogen-bonding

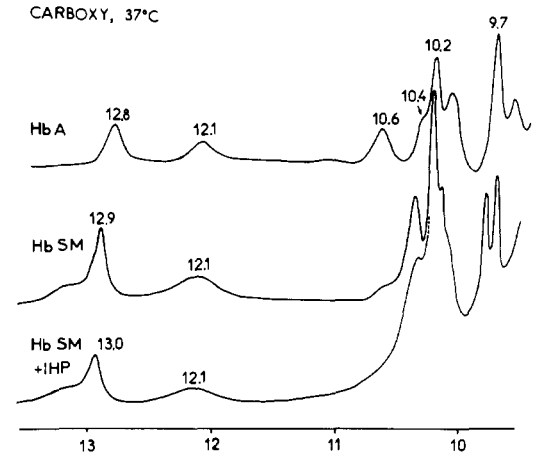

FIGURE 1: Low-field region of the NMR spectra of monocarboxylated Hb A, Hb Saint Mandé, and Hb Saint Mandé with 10 mM of inositol hexaphosphate. Hemoglobin samples (8 mM in heme) are in 100 mM phosphate buffer in ^1H_2O, pH 5.6 at 310 K. The high-field part of the spectrum of Hb SM+IHP was not shown for clarity.

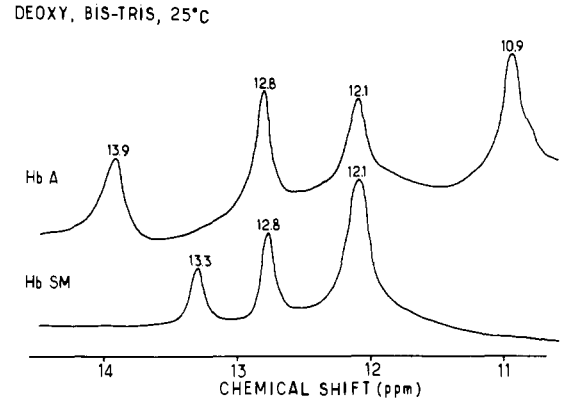

FIGURE 2: Low-field region of the NMR spectra of deoxygenated Hb A and Hb Saint Mandé. Hemoglobin samples (8 mM in heme) are in 100 mM Bis-Tris buffer in ^1H_2O, pH 7.2 at 298 K.

interactions between subunits give rise to well-defined resonances in the uncrowded, low-field region of the NMR spectrum (10–14 ppm). Simple comparison of the low-field spectral regions in tetrameric Hb and in isolated α and β chains shows that these peaks are observed only when the subunits are assembled, thus indicating their intersubunit origin (Russu et al., 1987). Figure 1 shows this low-field region of the NMR spectra in ^1H_2O of Hb A, Hb SM, and Hb SM with IHP in monocarboxylated form. The two exchangeable resonances at 12.1 and 12.8 ppm are insensitive to quaternary changes and were therefore associated with hydrogen-bond protons in the $\alpha_1\beta_1$ ($\alpha_2\beta_2$) interfaces between homologous subunits (Fung & Ho, 1975; Russu et al., 1987). While the resonance at 12.1 ppm is only slightly broadened in the spectra of Hb SM, the peak at 12.8 ppm is significantly low field shifted. Two other significant changes are observed in the spectra of Hb variant: the broadening (accentuated in the presence of IHP) of the resonance at 10.6 ppm, considered to be a R-state marker, and the appearance of a supplementary, broad peak at the low-field end of the spectral region. No changes were observed in the spectrum of Hb A(CO) when equal amounts of IHP were added, in agreement with previous observations (Fung et al., 1975).

Two of the four exchangeable proton resonances in the unliganded state of Hb A have been associated with hydrogen bonds characteristic of the quaternary T-like structure (Figure 2). Both are altered in the mutant Hb. One of them (10.9

Table I: Resonance Assignment in the Monocarboxylated Hb A (Craescu & Mispelter, 1989), Hb Saint Mandē, and Hb Saint Mandē in the Presence of 10 mM of IHP[a]

chemical group	Hb A(CO)	Hb Saint Mandē (CO) −IHP	Hb Saint Mandē (CO) +IHP	chemical group	Hb A(CO)	Hb Saint Mandē (CO) −IHP	Hb Saint Mandē (CO) +IHP
			Heme Substituents				
α-meso α	9.58	9.44	9.47				
α-meso β	10.05	10.15	10.17	3-CH_3 α	2.56	2.55	
β-meso α	9.34	9.32	9.32	5-CH_3 α	2.63	2.60	2.63
β-meso β	9.36	9.34	9.34	5-CH_3 β	2.79	2.93	
γ-meso α	10.22	10.22	10.22	8-CH_3 α	3.57	3.56	3.56
γ-meso β	10.22	10.22	10.22	8-CH_3 β	3.54	3.58	3.58
δ-meso α	9.72	9.72	9.72	vinyl-2α $C_\alpha H$	8.25	8.29	8.22
δ-meso β	9.72	9.78	9.78	vinyl-2α $C_\beta H_2$	5.85	5.85	5.85
1-CH_3 α	3.49	3.50	3.50	vinyl-2β $C_\alpha H$	8.03	8.19	8.19
1-CH_3 β	3.25	3.36	3.36	vinyl-2β $C_\beta H_2$	6.04	6.09	6.18
					6.09	6.17	
			Amino Acid Residues				
Trp14α η^2-CH	6.94	6.95	6.95	Val67β α-CH_3	3.57	3.58	3.61
Trp14α ζ_2-CH	7.52	7.52	7.54	Val67β NH	7.32	7.2	
Val17α γ-CH_3	0.04	0.00	0.00	Ala70β β-CH_3	2.46	2.50	2.50
	0.56	0.58	0.56	Ala70β α-CH	4.90	4.90	4.90
His20α δ-CH	8.56	8.55	8.59	His72α ε-CH	8.68	8.67	8.67
His20α ε-CH	7.31	7.31	7.33	His72α δ-CH	7.56	7.56	7.57
Tyr24α δ-CH	6.36	6.38	6.38	His77β ε-CH	8.63	8.61	8.62
Tyr24α ε-CH	6.12	6.14	6.13	His77β δ-CH	7.46	7.44	7.47
Leu28β δ-CH_3	−0.66	−0.61	−0.57	Leu83α δ-CH_3	0.75	0.85	0.87
	−0.18	0.03	−0.2		1.11	1.20	
Leu28β γ-CH	0.60	0.63	0.58	Phe85β	7.18	7.18	7.26
Leu29α δ-CH_3	−0.81	−0.78	−0.78	Leu88β δ-CH_3	0.62	0.70	
	−0.18	−0.20			1.10	1.05	
Phe33α δ-CH	7.00	6.99	6.99	Leu91α δ-CH_3	−0.64	−0.55	−0.56
Phe33α ε-CH	6.40	6.41	6.42		0.94	0.95	0.95
Phe33α ζ-CH	4.69	4.72	4.72	Val93α γ-CH_3	0.25	0.27	0.27
Tyr42α δ-CH	7.75	7.79	7.80		0.45	0.52	
Tyr42α ε-CH	7.21	7.26	7.25	Phe98α δ-CH	7.62	7.57	
Phe42β δ-CH	6.32	6.33	6.35	Phe98α ε-CH	6.23	6.32	
Phe42β ε-CH	6.00	6.03	6.03	Leu101α δ-CH_3	0.23	0.35	0.35
Phe42β ζ-CH	4.78	4.86	4.80		0.45	0.45	0.45
Phe43α δ-CH	6.22	6.24	6.25	Phe103β δ-CH	7.52	7.50	7.45
Phe43α ε-CH	5.98	6.00	6.00	Phe103β ε-CH	6.07	6.03	6.03
Phe43α ζ-CH	4.53	4.53	4.55	Phe103β ζ-CH	6.60	6.58	6.61
Phe45β δ-CH	6.92	6.93	6.92	Leu106β δ-CH_3	0.56	0.50	
Phe45β ε-CH	6.82	6.82	6.83	His116β ε-CH	8.15	8.18	8.19
Phe45β ζ-CH	6.35	6.24	6.27	His117β ε-CH	8.10	8.11	8.11
Val62α γ-CH_3	−1.69	−1.72	−1.72	Leu136α δ-CH_3	−0.42	−0.44	−0.45
	0.16	0.16	0.15		−0.05	−0.04	−0.07
Val62α α-CH	3.67	3.66	3.66	Leu136α γ-CH	1.14	1.08	1.09
Val62α β-CH	1.28	1.26	1.26	Tyr140α δ-CH	6.92	6.91	
His63β ε-CH	8.18	8.21	8.21	Tyr140α ε-CH	6.69	6.72	
Ala65α β-CH_3	2.46	2.45	2.44	Leu141β δ-CH_3	−1.06	−0.64	−0.67
Ala65α α-CH	4.90	4.91	4.90		−0.64	−0.64	−0.59
Val67β γ-CH_3	−1.83	−1.87	−1.87	Leu141β γ-CH	0.34	0.54	0.56
	0.16	0.16	0.16				

[a] The samples (8 mM in heme) are in 100 mM deuterated phosphate buffer, pH 5.6 at 310 K. The chemical shifts are given in parts per million (ppm) from the DSS. The underlined values in HbSM correspond to variations greater than 0.05 ppm relative to HbA.

ppm) is absent from its normal position, and the other is probably high field shifted from 13.9 to 13.3 ppm. The resonance at 12.1 ppm has an integral about 2 times greater than the others, suggesting that it represents two different proton groups.

Resonance Assignment. The strategy used for resonance assignment in the monocarboxylated Hb SM spectrum is the same we used for the Hb A(CO) (Craescu & Mispelter, 1989). Briefly, we recorded a NOESY and a COSY spectrum for the mutant Hb (CO) sample in the presence and absence of IHP. For similar concentrations and acquisition parameters, the signal-to-noise ratio in the 2D spectra is slightly lower than for Hb A(CO), mainly due to larger line widths. The presence of small proportions of slightly different conformations in low exchange may be a cause of this line broadening.

Resonance identification and assignment were easier for the protons of the heme pocket side chains, largely shifted by the heme ring current. Figure 3 shows a comparison of the high-field region of the COSY spectra of monocarboxylated β_4 chains, Hb A, Hb SM, and Hb SM+IHP. The cross-peaks were well-defined, enabling the partial identification of several side-chain spin systems. In particular, the significant changes of the Leu141β resonances among different proteins are readily visible in the figure while the symmetry-related leucine in a α subunit (Leu136α) is conserved. Analysis of the 2D spectra in comparison with the results obtained on the normal hemoglobin, under the same physicochemical conditions, enabled us to assign a significant number of resonances. The major part of the heme substituent resonances and the partial assignment for a large number of amino acids in HbA, Hb SM and Hb SM+IHP are compared in Table I. The differences in chemical shift larger than 0.05 ppm are underlined. Several trends can be pointed out from the analysis of this table:

(a) With the exception of Leu141β, there is no significant difference between the identified resonances in Hb Saint Mandē with and without IHP,

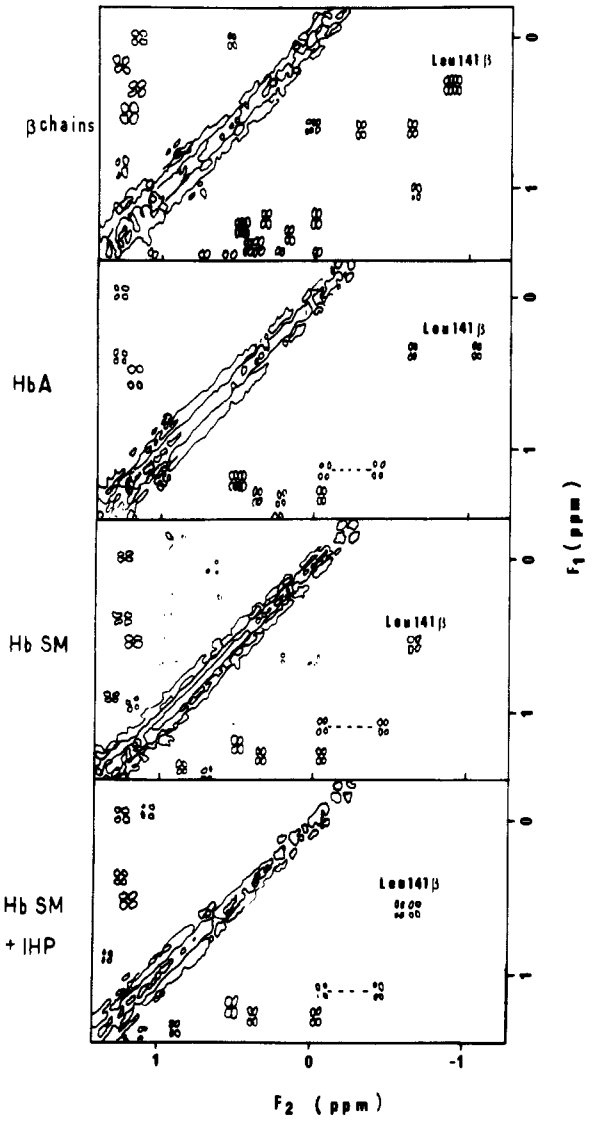

FIGURE 3: High-field region of the COSY spectra of the monocarboxylated β_4 chains, Hb A, Hb Saint Mandé, and Hb Saint Mandé with 10 mM inositol hexaphosphate. The conditions are the same as in Figure 1. The partial spin system of Leu136α is indicated by interrupted lines.

(b) Among the assigned resonances corresponding to the heme substituents, only one in the α subunits (α-meso) versus five in the β subunits is modified in the mutant hemoglobin,

(c) About two-thirds of the assigned side-chain resonances are conserved in the mutant hemoglobin irrespective of the presence of IHP,

(d) Among the resonances of the side chains, the shifted resonances correspond to the heme pockets of both subunits but are generally larger in β subunits.

Discussion

Quaternary Changes. X-ray diffraction and spectroscopic structural studies together with equilibrium and kinetic functional measurements on Hb Kansas have consistently indicated that, at acid pH and in the presence of IHP, the liganded mutant hemoglobin switched to a T-like quaternary structure. Hb SM has the same substitution site and similar functional properties as Hb Kansas (Arous et al., 1981). This leads us to predict similar quaternary features. The study of the exchangeable proton resonances in the present work gives some experimental information about the liganded and unliganded quaternary structures.

The peak at 10.6 ppm in the spectrum of Hb A(CO) (Figure 1) is an R-state marker (Fung et al., 1975), and its pertubation in Hb SM reflects the modification of an $\alpha_1\beta_2$ hydrogen bond characteristic of the liganded quaternary structure. The other significant observation, illustrated in Figure 1, is the appearance of a broad peak at 13.2 ppm, suggesting the formation of a new hydrogen bond. In an earlier NMR study of liganded Hb Kansas (βN102T) the authors have observed (Ogawa et al., 1972) that addition of IHP induces the appearance of a new, large peak at an even lower field (14.0 ppm). Similar observations have recently been made in biliganded metal-substituted hybrids (Shibayama et al., 1987; Ishimori & Morishima, 1988) which could be shifted into a T-like structure. Thus, the spectral changes observed for the exchangeable resonances in the carboxylated Hb SM are a strong indication for modification and creation of hydrogen bonds in structural regions of low solvent accessibility, situated at the subunit interface. Therefore, the relative position of the tetramer subunits and the energetics of their interactions in the liganded mutant might be different from that observed in the standard R structure.

On the other hand, the observation of the exchangeable proton resonances in the absence of ligand (Figure 2) strongly indicates that the unliganded state of Hb SM is also different from the typical T state of the deoxy Hb A. From X-ray diffraction studies Andersen also found that the liganded T state of Hb Kansas is similar to but not identical with the deoxy T state of Hb A (Andersen, 1975). The two markers for the T structure are perturbed: one peak (10.9 ppm) is absent from its position in deoxy Hb A (the doubling of the 12.1 ppm resonance intensity may result from the shift of this peak) and the other (13.9 ppm) is probably high field shifted.

The increased tetramer/dimer dissociation constant, the decreased affinity for the fourth ligand (C. Poyart, personal communication), and the perturbed intersubunit contacts described above lead us to conclude that the substitution Tyr102β for Asn102β induces a new liganded quaternary state, different from the standard R or T state described for Hb A. IHP, a strong allosteric effector that stabilizes the T state, has no significant effect on this new state. Comparison of functional and structural data consistently indicates that the changes in the liganded quaternary structure, induced by the substitution of Asn102β, are different in Hb Kansas (β102T) and Hb SM (βN102Y), pointing to the determining role played by the type of substituent side chain. The accompanying tertiary changes, resulting from analysis of the assigned resonances in the variant spectra, will be discussed in the next section.

Tertiary Changes. The interpretation of the chemical shift variations in terms of structural modifications is limited by the number and distribution (over the molecule structure) of the assigned resonances. Thus, a large part (over 75%) of the identified peaks correspond to the side chains forming the heme cavity in the two subunits. Therefore, the tertiary structure changes in the heme pocket will be better characterized. All the assigned amino acids lying at large distance from the heme pocket appear to conserve their conformation and environment in the mutant hemoglobin, suggesting that the global tertiary structure of the subunits is largely conserved. These markers cover the A, B, EF, and HC segments in the α chains and the EF and G segments in the β chains (see Table I). In contrast, the majority of the heme pocket resonances are significantly perturbed in the modified hemoglobin.

Characterization of the actual nature of the structural changes induced by the amino acid substitution requires a substantial set of proton–proton distances. For relatively small proteins (<15 kDa) these distances may by evaluated from the NOESY cross-peak intensitites (Wüthrich, 1986). The high molecular weight and the peak superposition in crowded regions of our spectra preclude, at the moment, this kind of approach. Nevertheless, the comparative analysis of the chemical shift values given in Table I provides useful information on the extent and intensity of the structural perturbations in the Hb variant. In the case of heme pocket protons the chemical shifts are largely dominated by the ring-current effect, which is very sensitive to the geometrical relationship between the heme structure and the neighboring protons. Small displacements of the protons toward the heme center may generate large high-field chemical shift changes of the corresponding resonances (Perkins & Wüthrich, 1979).

Five of the heme substituent resonances in β subunits are low field shifted to various extents (from 0.06 to 0.16 ppm), while only one resonance coming from the α heme is significantly shifted (α-meso α). This clearly indicates that the β heme, which is closer to the substitution area, is more perturbed than the heme of the other subunit. The chemical shifts of the heme substituent protons are determined mainly by the deshielding effect of the heme ring current but also by the ring current of the surrounding aromatic side chains. Therefore, the modification of the resonances coming from the heme substituents may originate from a displacement of the heme itself relative to the globin, a heme distortion, or conformational changes of the neighboring aromatic side chains. One observation resulting from the present results enables us to favor the last mechanism. This is the fact that the resonances of the γ-CH_3 groups of Val67β, which are highly sensitive to the heme ring current, are not significantly modified in Hb SM. Other ring-current shifted resonances from Ala70β, Phe85β, or Phe103β are equally insensitive to the amino acid substitution, suggesting that the heme group does not move relative to the polypeptide backbone. This would mean that all the observed changes in the chemical shift of the β heme substituents and of the surrounding side chains correspond to a rearrangement of the spatial structure of the heme pocket in the β subunits.

Some of the structural changes in the β subunits may be induced by short-range interactions with the new Tyr substituent in position 102. This may be the case for 1-CH_3, α-meso, vinyl-2, Phe41, Phe42, and Phe45. Note that Phe41β is not given in Table I; the low signal-to-noise ratio in the COSY spectra of Hb SM precludes the precise determination of its spin system, but comparison of 1D spectra strongly suggests that the resonances are significantly low field shifted. The substitution site is also not too far from the Val98β which was suggested to play a critical role in the heme–heme interaction at the $\alpha_1\beta_2$ interface (Baldwin & Chothia, 1979). Unfortunately, we were not able to identify the resonances coming from this valine.

An interesting aspect of our results is the absence of large perturbations on the distal valines (E11) in the two subunits. The X-ray studies have shown that during deoxygenation these valines come closer to the heme, in the empty place generated by the departed ligand (Baldwin & Chothia, 1979; Luisi & Shibayama, 1989). In the case of Hb SM, which has deoxy-like functional and quaternary properties, we noted only a small tendency for such a movement in both α and β subunits; the presence of the ligand, probably in the perpendicular position, is sterically unfavorable to a larger displacement. The movement of distal valines toward the heme center seems to correspond to an energetically favored conformation. For instance, when the CO ligand is replaced by O_2, which has a bent structure, Val E11 residues in both subunits come closer to the heme plane as reflected in a large high-field shift (0.6 ppm) in the NMR spectra (Dalvit & Ho, 1985; Craescu et al., 1988).

Conformational changes in the β subunits were also observed far away from the mutation site. The largest one is that of Leu141, situated at the end of the H helix, close to the C terminus of the β chain. Part of its spin system in the different proteins studied is given in Figure 3. The conformational modulation of this residue should be explained by long-range interactions triggered by the substitution, probably via the hydrogen bond Tyr145β···Val98β linking the FG turn to the C terminus. It was shown (Shaanan, 1983) that the contact region between the two fragments in the liganded Hb A is quite mobile; in particular, the Tyr145β can be found in two different sites, one of them being typical for the unliganded state. Several other NMR studies in solution give additional evidence for the high sensitivity of the Leu141β conformation to small structural perturbations in the proximal or distal site of the heme pocket, including change of the ligand ($O_2 \rightarrow$ CO), point mutations in the β F helix (Craescu et al., 1988), binding of thiol reagents to Cys93β (Craescu et al., 1985), or deletion of the two C-terminal amino acids in the β subunits (C. T. Craescu, unpublished results). As is seen in Figure 3 the conformation of this leucine is also different in the β chain tetramers in which the subunit interactions do not participate in a cooperative transition. The position of the Leu141β seems therefore to be closely related to the tertiary and quaternary changes modulating the Hb affinity.

The modifications observed in the α subunits indicate that the perturbation induced by the substitution in the β subunits propagates also to the neighboring subunit. Here again, the conformational changes are localized in the heme pocket region adjacent to the heterologous interface: helices C, F, and G and the FG turn. Significantly, the ϵ-CH resonance of Tyr42α is significantly shifted in Hb SM. The Tyr42α is a sensitive probe for quaternary changes in the $\alpha_1\beta_2$ interface. It forms a hydrogen bond with the Asp99β in the unliganded but not in the liganded state of the Hb A. The perturbation observed in Hb SM(CO) is therefore in agreement with the above conclusion on the modification of the liganded quaternary state.

Conclusions

The present results demonstrate that the combined utilization of 1D and 2D NMR methods may give valuable information on the coupled quaternary and tertiary conformational changes in a large, allosteric protein under physicochemical conditions similar to the physiological ones. These data are complementary to the results of X-ray crystallography and are very helpful for the interpretation of the functional data. We showed that a simple substitution of an amino acid in Hb SM induces a changes in the quaternary structure, thus triggering the propagation of structural perturbations over large ares of the two subunits. Besides its sensitivity to small tertiary changes, NMR spectroscopy provides information on the intensity and dynamics of the intersubunit hydrogen bonds which is very useful for the understanding of the energetic aspects of the Hb cooperativity.

Further increases in the resolution and sensitivity of our 2D NMR experiments will enable us to extend the present observations to other structural regions of the protein. In addition, experiments are now in progress in our laboratories for resonance assignment in the unliganded form of the hemo-

globin, which will provide a basis for structural analysis of the deoxy quaternary conformation in solution.

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REFERENCES

Andersen, L. (1975) *J. Mol. Biol. 94*, 33–49.
Arous, N., Braconnier, F., Thillet, J., Blouquit, Y., Galactêros, F., Chevrier, M., Bordahandy, C., & Rosa, J. (1981) *FEBS Lett. 126*, 114–116.
Baldwin, J. & Chothia, C. (1979) *J. Mol. Biol. 129*, 175–220.
Cassoly, R., & Gibson, Q. H. (1972) *J. Biol. Chem. 247*, 7332–7341.
Craescu, C. T., & Mispelter, J. (1988) *Eur. J. Biochem. 176*, 171–178.
Craescu, C. T., & Mispelter, J. (1989) *Eur. J. Biochem. 181*, 87–96.
Craescu, C. T., Mispelter, J., Schaeffer, C., & Beuzard, Y. (1985) *J. Biol. Chem. 260*, 15616–15622.
Craescu, C. T., Schaeffer, C., Blouquit, Y., & Rosa, J. (1988) *J. Biol. Chem. 263*, 3250–3255.
Dalvit, C., & Ho, C. (1985) *Biochemistry 24*, 3398–3407.
Fermi, G., & Perutz, M. F. (1981) in *Haemoglobin and myoglobin. Atlas of molecular structures in biology*, Vol. 2, p 54, Clarendon Press, Oxford, U.K.
Fung, L. W.-M., & Ho, C. (1975) *Biochemistry 14*, 2526–2535.
Greer, J. (1971) *J. Mol. Biol. 59*, 99–105.
Ikeda-Saito, M., & Yonetani, T. (1980) *J. Mol. Biol. 138*, 845–858.
Ishimori, K. & I. (1988) *Biochemistry 27*, 4060–4066.
Luisi, B., & Shibayama, N. (1989) *J. Mol. Biol. 206*, 723–736.
Ogawa, S., & Schulman, R. G. (1972) *J. Mol. Biol. 70*, 315–336.
Ogawa, S., Mayer, A., & Schulman, R. G. (1972) *Biochem. Biophys. Res. Commun. 49*, 1485–1491.
Perkins, S. J., & Wüthrich, K. (1979) *Biochim. Biophys. Acta 576*, 409–423.
Perutz, M. F. (1970) *Nature 228*, 726–734.
Russu, I. M., Ho, N. T., & Ho, C. (1978) *Biochim. Biophys. Acta 914*, 40–48.
Salhany, J. M., Ogawa, S., & Schulman, R. G. (1975) *Biochemistry 14*, 2180–2190.
Schaeffer, C., Craescu, C. T., Mispelter, J., Garel, M.-C., Rosa, J., & Lhoste, J. M. (1988) *Eur. J. Biochem. 173*, 317–325.
Shaanan, B. (1983) *J. Mol. Biol. 171*, 31–59.
Shibayama, N., Inubushi, T., Morimoto, H., & Yonetani, T. (1987) *Biochemistry 26*, 2194–2201.
Shulman, R. G., Hopfield, J. J., & Ogawa, S. (1975) *Q. Rev. Biophys. 8*, 325–420.
von Kienlin, M., Decorps, M., Albrand, J. P., Foray, M. F., & Blondet, P. (1988) *J. Magn. Reson. 76*, 169–173.
Wüthrich, K. (1986) *NMR of Proteins and Nucleic Acids*, p 117, Wiley, New York.