A High Resolution NMR Study of Localized Dynamic and Structural Perturbations in Human Hemoglobin Modified with Thiol Reagents*

Constantin T. Craescu‡, Joel Mispelter‡, Corinne Schaeffer‡, and Yves Beuzard‡

From the ²Institut National de la Santé et de la Recherche Médicale Unité 91 Hôpitaux Henri Mondor, 94010 Créteil, France and the 1Institut National de la Santé et de la Recherche Médicale Unité 2lB, Institut Curie, Section de Biologie, 94100 Orsay, France

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The hydrogen exchange kinetics of the NH proton in His F8 of iodoacetamide- and N-ethylmaleimide-treated human deoxyhemoglobin were studied using a NMR method. Comparison with unmodified hemoglobin shows that the reagents, covalently bound to Cys β93, significantly increase (about one order of magnitude) the exchange kinetics in β chains only. This effect was partially reversed by the strong allostERIC effector inositol hexaphosphate. Study of the high resolution 400-MHz NMR spectra of modified oxy- and deoxy-hemoglobins permitted localization of the extent of chemically induced structural perturbations. The resonances corresponding to hydrogen bonds specific to the deoxy conformation are not changed, in accord with the preserved cooperativity. Under the experimental conditions (0.1 m bis-Tris, 10 mM Cl+, pH 7.2), the salt bridge at the C terminus of the β chain in the deoxy state (His β146-Asp β94) is perturbed by both modifications. The His β146 appears to be rendered more immobilized by the reagents in the oxy conformation. From the resonances corresponding to heme pocket protons of oxyhemoglobin it is deduced that the perturbations do not extend over the distal side of the heme pocket but are limited to the FG, F, and HC segments of the β chain.

In its native conformation, human hemoglobin has two titratable cysteine residues in the F9 position of β subunits (β93). The reactivity of these sites with specific reagents as well as the crystallographic and functional properties of the modified hemoglobins have been the subject of a number of investigations (1–7). Generally the treatment with cysteine-targeted reagents significantly increased the O2 affinity and reduced the alkaline Bohr effect (3, 7, 8). For some reagents a diminished cooperativity, as measured by the Hill coefficient, has also been noted (8). The possible application of the thiol reagents as anti-sickling drugs is currently under study in our laboratory (8, 10).

A detailed molecular description of the above effects may contribute to a better understanding of the hemoglobin function and to the design of more potent hemoglobin-directed anti-sickling drugs.

The available crystallographic results on modified hemoglobins (2, 4, 5) provide some explanations for the ligand-linked reactivity of the cysteine groups and for the diminished alkaline Bohr effect. These crystallographic approaches were limited to a single crystal T structure and the resolution was in the range of 3.5 Å. A more complete picture of a functional protein must also take into account an equilibrium between dynamic states controlled by changes of the physicochemical parameters in the solution.

High resolution NMR spectroscopy is a well adapted method for such a description; its application to the study of the hemoglobin is based on a large number of intrinsic spectroscopic probes such as protons in the heme pocket and in hydrogen bonds stabilizing the tertiary or the quaternary structure or in the surface histidine residues (11). Starting from the crystallographic framework, the NMR results may be expressed in terms of structural refinements in a well-defined environment or may be used to estimate the local, dynamic fluctuations.

Recently, it was shown by a NMR method that the exchange rate of the NH proton in proximal histidines (β8) of human hemoglobin is largely subunit- and quaternary state-dependent (12). The exchange kinetic constant for these histidines measures the motional fluctuations of the amino-acid residues and/or of a whole protein segment (13) in the proximal side of the heme pocket, a region already implicated in the manifestation of cooperativity (14). We used the same method to study the influence of sulphhydryl reagents (N-ethylmaleimide and iodoacetamide) covalently bound to Cys F9 β93 of human hemoglobin on the exchange kinetics of the proximal histidine (F8) NH proton. In addition, we analyzed the 400-MHz proton NMR spectra of the modified hemoglobins and correlated the static and dynamic perturbations with the known functional effects of the reagents.

**MATERIALS AND METHODS**

Iodoacetamide and bis-Tris were purchased from Sigma; N-ethylmaleimide was from Calbiochem; and deuterated water (D2O) 99.9% was from the Commissariat a l'Energie Atomique, France. Adult human hemoglobin A was prepared from fresh blood obtained by venipuncture from volunteers in our laboratories.

**Sample Preparation**—Hb Malmo (β97 His→Gln) and Hb Barcelona (β94 Asp→His) were purified by ion-exchange chromatography (15, 16). The method of preparation of modified hemoglobin was previously described (8); isoelectric focusing (17) showed that a 100% fixation was achieved for both reagents. Final solutions of 20% in bis-Tris buffer (0.1 m bis-Tris, pH 7.2, 10 mM Cl+) were prepared in D2O or H2OO were obtained by ultrafiltration in CentriSart I tubes (Sartorius, France).

**Measurements of Exchange Kinetics**—The exchange kinetics of NH protons from deuterons from solvents in histidines F8 of α and β

The abbreviations used are: bis-Tris, bia(2-hydroxyethyl)iminotris(hydroxymethyl)methane; Hb, hemoglobin; Hb A, adult human hemoglobin; IAM, iodoacetamide; NEM, N-ethylmaleimide; NEM-Hb, N-ethylmaleimide-treated hemoglobin; IAM-Hb, iodoacetamide-treated hemoglobin; IHP, inositol hexaphosphate; DPG, 2,3-diphosphoglycerate.

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subunits was measured under deoxygenated conditions as in Ref. 12. Briefly, 200–300 μl of a deoxygenated hemoglobin solution (20% in 0.1 M bis-Tris, pH 7.2, 10 mM Cl" in 2H2O) were mixed at time zero with 50 μl of a deoxygenated 0.1 M dithionite solution in the same buffer and 350 μl of deoxygenated 2H2O. The NMR spectra of the proximal histidine exchangeable protons were recorded at different times after mixing and the intensities of the peaks corresponding to the NH protons in α and β subunits (18–20) were represented as a function of time. Progressive decrease of peak intensities is a measure of the exchange of NH protons with solvent deuterons. The experiments were performed at constant room temperature (20 °C).

NMR Techniques—Proton NMR measurements were performed at 400 MHz on an AM 400-WB Bruker spectrometer in 2H2O (containing 5% of 2H2O for the field/frequency lock) or in various mixtures of 2H2O/H2O. Depending on the experiments, different methods were used for the elimination of the undesirable strong solvent peak.

For the exchange kinetics of the His F8 NH protons, a spectrum was recorded every 45 min after the mixing of the Hb solution with 2H2O. Every record takes about 15 min using 6720 scans with an acquisition time of 30 ms. Prior to acquisition, a presaturation pulse of 60 ms was applied at the water resonance. Due to a strong baseline distortion and to the large broadening of the two resonances (about 1000 Hz), the transmitter frequency and the spectral width (70 kHz) were chosen to minimize the base-line correction in the region of interest. Thus, only a linear correction was necessary for this region. A line broadening of 400 Hz was applied prior to Fourier transformation.

For the observation of the exchangeable protons appearing in the range 5 to 10 ppm from 2H2O for the deoxy-Hbs, the Jump and Return method (21) for the reduction of the water resonance was found to be the most convenient one. Thus, acquisition (spectral width of 17 kHz, acquisition time of 0.5 s on 16K data points) was performed with the sequence 90° – τ – 90° – τ – (τ = 90 μs), and the transmitter frequency settled at the exact water resonance frequency.

Finally, recording of the histidine and ring current-shifted resonances was performed using a continuous saturation of the water resonance during acquisition. A Gaussian multiplication was applied (LB = –300 Hz, GB = 0.05 and an acquisition time of 0.68 s) prior to Fourier transformation in order to enhance the resolution. Comparison of such obtained spectra with those obtained without this multiplication showed that no artifactual distortions were introduced by the resolution enhancement procedure.

RESULTS

Study of the Exchange Kinetics of Histidine F8 Protons—The most low field resonances in the NMR spectrum of the deoxy-Hb correspond to the histidine F8 NH protons in α and β subunits (59.4 and 71.9 ppm, respectively) (18–20). The large chemical shifts of these protons are determined essentially by the hyperfine interactions with the unpaired electrons of the high spin ferrous ion in the deoxy-Hb.

Table I presents the chemical shifts of the two resonances in normal and modified human hemoglobins. It is seen that the attachment of NEM or IAM to the hemoglobin β chains induces a high field shift for the β chain peak while leaving unchanged the α chain resonance. A similar effect of a thiol reagent in isolated β chains was reported by La Mar and co-workers (22).

The exchange kinetics of NH protons of histidine F8 in human deoxyhemoglobin A is about 15 times more rapid in the α subunit than in the β subunit (Fig. 1A), in agreement with the results of Jue et al. (12).

As the modified hemoglobins have a high oxygen affinity (8), complete and rapid deoxygenation requires utilization of dithionite. This is why we used reagents whose binding to hemoglobin could not be reversed by dithionite, as is the case for NEM and IAM. Use of NEM and IAM has also the advantage that crystallographic structures of hemoglobins modified by these or other related reagents are available for comparison (2, 4, 5).

The exchange kinetics of NEM-Hb in the T conformation are represented in Fig. 1B. The comparison with the unmod-

<table>
<thead>
<tr>
<th>Homoglobin type</th>
<th>δ (NH) ppm</th>
<th>γ (× 10^9) min^-1</th>
<th>τmin</th>
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<td>71.9</td>
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<td>70.95</td>
<td>5.5</td>
</tr>
<tr>
<td>Hb</td>
<td>59.4</td>
<td>67.72</td>
<td>4.4</td>
</tr>
<tr>
<td>Hb + 5 mM IHP</td>
<td>59.5</td>
<td>70.2</td>
<td>5.5</td>
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NMR of Chemically Modified Hemoglobin

Modified hemoglobin is straightforward; the drug induces a large increase (≈15 times) in the exchange kinetics of the β subunits while leaving the α chains practically unchanged. Thus, the two chains become kinetically equivalent. The quantitative data for Hb A, NEM-Hb, and IAM-Hb are summarized in Table I. As is seen, the effect of IAM is slightly smaller than that of NEM.

The values of the kinetic parameters in modified hemoglobins (Table I) reflect large chemically induced modification of the dynamical structure in the heme pocket region of the β chains. The exchange rate constant in the modified unliganded β subunit is of the same order of magnitude as that of unmodified ligated β subunit (12). This means that the reagents induce R-like conformational dynamics in the β heme pocket.

It is known that the strongest allosteric effector, IHP significantly shifts the hemoglobin quaternary equilibrium toward the T conformation. The studied reagents and IHP should thus have antagonistic effects; in order to verify this point we measured the exchange kinetics of deoxy-NEM-Hb in the presence of a 3 times molar excess of IHP. The results (Table I) indicate that the polyphosphate does effectively decrease the exchange rate in the β subunit; an additional low field shift of the α chain peak was also observed (Table I).

Exchangeable Protons in Hydrogen Bonds—The tertiary and quaternary structures of hemoglobin are stabilized by van der Waals interactions, salt bridges, and hydrogen bonds. Some of the exchangeable protons implicated in crucial hydrogen bonds have been identified in the NMR spectrum and successfully used as indicators of tertiary or quaternary structural changes associated with ligand binding (23-25). These peaks could be observed in the region between 5 and 10 ppm from HzO. Two of them, at 8.17 and 7.49 ppm (Fig. 2), are independent of the ligation state and are thought to belong to the αβ interface (26). Two other peaks were suggested to be probes for the deoxy quaternary (9.34 ppm, Tyr α42-Asp β99 hydrogen bond) and tertiary (6.35 ppm, Tyr β145-Val β98 hydrogen bond) structures (23, 24, 27). Fig. 2 represents the spectra of Hb A, NEM-Hb, and IAM-Hb in deoxy state. All the resonances presented above are present equally in both modified and unmodified hemoglobins. Fung and Ho (23) reported an analogous result for the hemoglobin modified by the spin label N-(1-oxyl-2,2,6,6-tetramethylpiperidinyl)-iodoacetamide.

Heme Environments—Other NMR probes are the heme pocket protons that are largely high field shifted in liganded hemoglobins by the ring-current of aromatic porphyrin electrons. The assignment of some of these resonances, between −5.0 and −7.5 ppm from HzO, for oxy-Hb have been specified (28). In Fig. 3, spectrum a represents the region of ring-current shifted resonances of hemoglobin A in the oxygenated state. The highest field peak at −7.20 ppm corresponds to the γα methyl protons of valine E11 in α and β subunits. This peak is very sensitive to the nature of the ligand and to allosteric effectors: replacement of O₂ by CO as ligand induces a low field shift of about 0.6 ppm, while the inorganic phosphates, DPG or IHP, selectively shift the resonance corresponding to the β subunit, with a consequent splitting (29). As is seen in Fig. 3, b and c, in the modified hemoglobins the position of the highest field peak is not perturbed. In contrast, the next two peaks (−5.74 and −5.61 ppm) are absent in these last spectra. A rough estimation of the peak integrals suggests that these resonances are now superimposed on those at −5.53 and −5.44 ppm. The absence of the peak at −5.74 ppm was also observed in a high affinity Hb variant, Hb Malmo (β97 His→Gln) (Fig. 3d).

Two supplementary features are visible in the NEM-Hb spectrum. The first is manifested in the region of two resonances at 9.34 ppm, Tyr α42-Asp β99 hydrogen bond) and tertiary (6.35 ppm, Tyr β145-Val β98 hydrogen bond) structures (23, 24, 27). Fig. 2 represents the spectra of Hb A, NEM-Hb, and IAM-Hb in deoxy state. All the resonances presented above are present equally in both modified and unmodified hemoglobins. Fung and Ho (23) reported an analogous result for the hemoglobin modified by the spin label N-(1-oxyl-2,2,6,6-tetramethylpiperidinyl)-iodoacetamide.

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contribution of a new, intercalating resonance. The second is the new resonance at $-5.90$ ppm whose large linewidth suggests that the corresponding protons are more immobilized.

Similar high field-shifted resonances were equally observed in the Hb Malmo (Fig. 3d).

Very small differences were observed in the same spectral region for nonliganded modified hemoglobins. Unfortunately their interpretation is rendered difficult by the presence of the hyperfine shift induced by the paramagnetic ferrous ion and by the lack of any peak assignment.

Surface Histidine Residues—There are 38 histidine residues in the hemoglobin tetramer; 22 of them (11 per dimer) appear in the NMR spectrum, in the oxy and deoxy states, in the region between 2.0 and 5.0 ppm downfield from $^1$H$_2$O (30). Utilization of natural mutants or artificially modified hemoglobins enabled the assignment of some histidine resonance in the liganded (O$_2$ or CO) and unliganded states (30, 31). Each titrable histidine must be represented by two peaks corresponding to the C2 and C4 protons of the imidazole ring and their chemical shifts are strongly dependent on the protonation states of the N$_2$ and N, nitrogens.

Fig. 4 represents the histidine region of the Hb A, IAM-Hb, and NEM-Hb in the oxy state; the labeling of the peaks followed the notation introduced by Russu et al. (30) for CO-hemoglobin. As it was shown by Shaanan (32), the differences in the crystallographic structure of Hb CO and Hb O$_2$ are within the experimental errors. Comparison of our spectra of normal and mutant hemoglobins (Hb Malmo $\beta 97$ His$\to$Gln, Hb Barcelona $\beta 94$ Asp$\to$His, and Hb Fort de France $\alpha 45$ His$\to$Arg) in the oxy state with the corresponding spectra of

$$\text{FIG. 4. }^1\text{H NMR aromatic resonances in the oxy state of oxy-Hb A (a), IAM-Hb (b), and NEM-Hb (c) in 0.1 M bis-Tris in }^1\text{H}_2\text{O, pH 7.2, at 20 }^\circ\text{C.}$$

the CO form shows no spectral difference between the two liganded forms in this region.

The three spectra in Fig. 4 are largely similar. A priori, the expected perturbations induced by the reagents may concern the histidine residues in the vicinity of the $\beta$ chain cysteine F9 cavity: His FG4 ($\beta 97$) and His HC3 ($\beta 146$). The resonances of His $\beta 97$ are not visible in the oxy state. In contrast, the resonance of His $\beta 146$ (labeled C) is significantly high field-shifted and broadened. This may indicate that the reagents change the $pK$ of the imidazole cycle and/or render it more immobilized. Another significant modification is observed in the region of C4 histidine protons. In Hb A, the peak labeled O is a superposition of several resonances, in IAM-Hb and NEM-Hb one component is high field-shifted by approximately 0.1 ppm. Actually, it is difficult to assign this and other changes in this spectral region (peaks N and U) to a particular protein site.

In the deoxy state (Fig. 5) the largest effect of the thiol reagents is the upfield shift of the peaks corresponding to the C2 and C4 protons of His $\beta 146$ (peaks 3 and 12, respectively). The presence of a new resonance (arrow) between peaks 2 and 4 may well correspond to a high field shift (larger in NEM-Hb) of the resonance 3 (His $\beta 146$). Fig. 5e represents the

$$\text{FIG. 5. }^1\text{H NMR aromatic resonances in the deoxy state of Hb A (a), IAM-Hb (b), NEM-Hb (c), Hb Malmo (d), and Hb Barcelona (e). Solutions are in 0.1 M bis-Tris in }^1\text{H}_2\text{O, pH 7.10, at 20 }^\circ\text{C.}$$

3 I. M. Russu, personal communication.
spectrum of deoxy-Hb Barcelona (894 Asp→His), a variant in which the salt bridge Asp 894-His 8146 is no more possible. In this case too, the peaks 3 and 12 are shifted and comparison with spectra b and c supports the hypothesis that the C2 proton of the perturbed His β146 is now situated between peaks 2 and 4 (arrow) as it was suggested previously (33). We also noted a slight lowfield shift of the peak 1 for both modified hemoglobins. Studies on Hb Malmö (His 897→Gln) indicated that this resonance represents the His β97 (see Fig. 5d). In the region of C4 protons a new resonance was observed in the modified hemoglobin (2.30 ppm in NEM-Hb and 2.35 ppm in IAM-Hb). These peaks may correspond to the C4 proton of the modified His β146 as suggested again by the spectrum of Hb Barcelona in Fig. 5e.

**DISCUSSION**

While the crystallographic methods provide detailed information about the crystal structure of biomolecules, they cannot directly account for the dynamic aspects of molecules in solution. Starting from the x-ray structure, complementary methods like NMR or theoretical molecular dynamics may give descriptions of the molecular flexibility at atomic resolution which are more relevant for the functional explanations (34, 35).

Jue et al. (12) have recently determined the exchange kinetics of the labile N4 protons of His F8 from human hemoglobin A. This residue offers a good internal NMR probe to study the mechanism of reagent-induced perturbations in the proximal region of heme cavity.

The hydrogen exchange between a protein (usually amide protons) and the solvent has been shown to be in close relation with the structural fluctuations of the protein (13). As for the mechanism of this hydrogen exchange, two hypothesis have been proposed: the breathing (or the segmental unfolding) concept and the accessibility-penetration concept (13, 36, 37). The breathing mechanism assumes transient (more or less) local unfolding of the protein that exposes the labile protons to the solvent. The solvent penetration mechanism is based on the H-exchange catalyst (mainly OH- ions at physiological pH (37)) that diffuses through channels in the protein structure and interacts with the exchangeable proton.

In our view, the exchange mechanism of N4H of His F8 in hemoglobin may be reasonably conceived in the framework of the accessibility-penetration hypothesis. The exchange process may thus be controlled by the fluctuations of the hydrophilic leucine residues (F4, F7, and FG3), by the strength of the hydrogen bond between N4H proton of His F8 and the carbonyl oxygen of Leu F4, and by the flexibility of the propionyl groups of the heme.

The configuration of the H bond implicating the N4H proton is qualitatively the same in both subunits and in both quaternary state, and its presence is a stabilizing factor for the α helix formed by the residues F9 to FG4 (4, 38).

Inspection of the crystallographic distances shows that the distance N4· · · O=C is inversely related to the exchange kinetics in the two chains (39). This means that the kinetic differences cannot be simply explained by considering only the hydrogen bonds involving the labile protons.

Another possible controlling factor is the presence of the negatively charged groups of the heme propionyl III and IV at the site of access to the proximal cavity. In the α subunit, the propionyl III is hydrogen-bonded to the His CD3 (α45) in both R and T conformation and the propionyl IV is hydrogen bonded to the Lys E10 (α61) only in the liganded state (40). No hydrogen bonding of these groups is noted for the β subunit in either state. As the catalyst is a charged species (largely OH- at physiological pH (37, 41), immobilization by a hydrogen bond of the propionyl group at the "gate" of the pathway may favor the exchange kinetics. This may thus explain partially the difference in exchange kinetics between subunits.

The conformational transition following ligand binding to Hb is accompanied by a displacement of the FG corner away from the heme plane (40) which is larger in the α subunit. This may render more accessible the histidine F8 to solvent and amplify the fluctuations of the hydrophobic residues of this packed region. Indeed, in liganded Hb the exchange kinetics increases 40 times in the β chain and 80 times in the α chain (12).

In the context of the above discussion we can try to explain the effect of the two thiol reagents which have been studied. First, the reagents induce an upfield chemical shift only for the N4H peak of the β subunit; the same asymmetric effect is noted for the exchange kinetics (Table I). The decreased N4H contact shift in the β subunit could be induced by an increase in proton donation of the imidazole (22) and therefore in the strength of the hydrogen bond with the peptide carbonyl of Leu F4. But the fact that, simultaneously, the exchange kinetics of the N4H proton increases renders the hypothesis improbable. A more probable mechanism could be the lengthening of Fe-N, bond via perturbation of the imidazole environment (22). This is in accord with a displacement of the FG corner away from the heme induced by the reagents in oxy state (see later).

The effect of IAM on the exchange kinetics is significantly lower than that of NEM (Table I). As the IAM has also a lower effect in increasing the Hb O2 affinity, we think that a correlation exists between the degree of destabilization of the deoxy T state (reflected in an increased exchange of N4H proton of His F8) and the O2 affinity of the hemoglobin tetramer.

Inspections of other regions of the NMR spectra of modified hemoglobins may give supplementary information necessary to understand more fully the mechanism of dynamic perturbations previously described.

In the unliganded state four exchangeable protons implicated in hydrogen bonds are observed in the NMR spectrum between 6 and 10 ppm (Fig. 2). The resonance at 6.34 ppm was tentatively assigned to the intrasubunit hydrogen bond Tyr β145-Val β98 (24). The assignment was based on studies on hemoglobin mutants at the C-terminal site of the β chain (Hb Osler β145 Tyr→Asp, and Hb Mc Kees Rocks β145 Tyr→Term) where the peak at 6.34 is absent. Recently, refined crystallographic studies (32) have shown that the hydrogen bond Tyr β145-Val β98 exists also in the oxy-Hb structure and thus the previous assignment is no longer supported. This means that the NMR observations made on Tyr β145 mutants may be rationalized in terms of mutation-induced perturbations extending over a larger region.

Whatever the assignment of 6.34 ppm peak, it rests a deoxy-like probe and our data (Fig. 2) indicate that no dramatic perturbations of interchain contacts are introduced by NEM and IAM. This is in accord with the functional studies which indicate only a slight decrease in cooperatively (Hill n) in modified hemoglobins (7, 8).

Additional information about the reagent-induced perturbations of the deoxy-Hb structure is obtained by analysis of the histidine region (Fig. 5). Our data show that the two resonances corresponding to the β146 His are high field-shifted from their normal position in deoxy-Hb A, in agreement with an earlier crystallographic study of Perutz et al. (2) predicting that the salt bridge between His β146 and Asp β94...
is broken by the NEM and IAM. The induced upfield shift of the His $\beta$146 resonance may be the composed effect of the broken hydrogen bond and of a decreased pK of the imidazole ring. The proximity of the positively charged group, Lys $\beta$144, in the new position of the histidine may explain such a pK change. If the assignment of the new peak between 2 and 4$^\prime$ (Fig. 5) to the His $\beta$146 in modified Hbs is right, this means that again the perturbation induced by NEM is larger than that induced by IAM.

The low field shift of the resonance corresponding to His $\beta$97 (peak 7, Fig. 5) in the modified hemoglobin may express a direct interaction between the negatively polarized site of the drugs (the carbonyl group) and the imidazole cycle. The absence of the salt bridge His $\beta$146$\Rightarrow$Asp $\beta$94 in Hb Barcelona (16, 33) (Fig 5e) produced, in contrast, a high field shift of the peak 1; this supports the idea that the abolished salt bridge linking the C-terminal residue to Asp $\beta$94 cannot simply explain the perturbation of the His $\beta$97 in NEM-Hb and IAM-Hb.

The spectral region of heme pocket protons is only slightly modified by the reagents in the absence of ligands and the interpretation is not straightforward. Therefore, only the perturbed C-terminal salt bridge can be presently invoked as an NMR argument for the known destabilization of the T state and increased affinity of modified hemoglobin.

Kinetic and equilibrium study of the N$\mathbf{H}$ resonance of the proximal histidines in the deligated state has shown (Table 1) that only the $\beta$ chains (to which the reagents are bound) are influenced. The spectra of heme pocket protons in oxy-Hb are helpful to further circumvent the perturbed area. Fig. 3 shows that the Val E11 residue does not change its position which is in the distal side over the heme region between pyrrole I and IV. The analogies between the spectrum of modified Hb (especially of NEM-Hb, see Fig. 3) and that of Hb Malmö in which the mutation affects the $\beta$FG corner suggest that the structural perturbations in drug-treated hemoglobins also concern this site. A significant low field shift of the resonances at $-5.74$ and $-5.61$ ppm was observed in modified hemoglobins. The assignment of the $-5.74$ ppm peak to CH$_3$ Val E11 in $\beta$ subunit was recently shown to be inappropriate (28) but it is accepted that it is a resonance corresponding to the $\beta$ chain (43). Considering the three-dimensional structure of the heme pocket, we find that a possible candidate for the $-5.74$ peak is the Leu $\beta$ 96 $\epsilon_1$ methyl group which lies over the pyrrole IV of the heme (44). The low field shift of the resonance is determined by the increase in the distance of this group to the heme plane which would correspond to a displacement of the FG corner away from the heme (as in the T$\Rightarrow$R transition (40)) in the $\beta$ subunit. Extrapolated to the deoxy state, such a scheme may equally contribute to the explanation of the increased accessibility of the N$\mathbf{H}$ in deoxy $\beta$ chains of modified hemoglobins.

In the histidine spectrum of the oxy configuration, only the resonance of His $\beta$146 is modified by the reagents in a manner that is compatible with a decreased pK of the imidazole and an immobilization of the C-terminal residue. In this case, the displacement of the imidazole ring toward the Lys $\beta$144 residue may be the dominant contribution to the decrease in the pK.

The above considerations support the idea that the localization of the reagent-induced perturbations is limited to the FG, F, and HC segments of the $\beta$ chain. Further, the intensity of spectral changes induced by the drugs seems to correlate with the efficacy in increasing the $O_2$ affinity. We may thus rationalize the reagent-induced increased $O_2$ affinity of hemoglobin in terms of local tertiary (and not quaternary) perturbations; this may be the case for all chemical modifications or hemoglobin variants which manifest by an altered affinity but a preserved cooperativity. The NMR study of some hemoglobin variants with point mutations in the FG, F, and HC areas, which are now in progress in our laboratories, will enable us to get new information about the functional and structural role played by the corresponding residues.

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