Covalent Binding of Glutathione to Hemoglobin
II. FUNCTIONAL CONSEQUENCES AND STRUCTURAL CHANGES REFLECTED IN NMR SPECTRA*

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Binding of glutathione by disulfide linkage to Cys-β93 of hemoglobin tetramers within sickle cells increases the oxygen affinity and significantly inhibits sickling at low partial oxygen pressure (Garel, M-C., Domenget, C., Caburi-Martin, J., Prehu, C., Galacteros, F., and Beuzard, Y. (1986) J. Biol. Chem. 261, 14704–14709). This article reports a characterization of the oxygen-binding properties of glutathionyl hemoglobin (G-Hb) in solution in the presence or absence of allosteric effectors. The studies reveal a nearly 6-fold increase in oxygen affinity compared to native HbA and a Hill coefficient at half-saturation (n_Hill) of 1.50 compared to n_Hill of ~2.9 for HbA. The oxygen Bohr effect measured in the alkaline pH range is reduced by 38%. Addition of 2,3-diphosphoglycerate decreases the oxygen affinity of G-Hb and HbA to a similar extent and increases the Bohr effect, indicating that the binding sites for organic phosphates are not perturbed in G-Hb. The rate of autooxidation of G-HbO2 is slower than of HbA02. Oxidation by ferricyanide of G-HbCO is also reduced and is biphasic, demonstrating a heterogeneous susceptibility of the hemes in G-Hb. Flash photolysis experiments indicate that the tetramer-dimer dissociation constant is 1 order of magnitude greater for G-HbCO than for HbA02. High resolution NMR spectra at 400 MHz show that in G-Hb: 1) the tertiary structure of the β heme pocket is significantly perturbed, particularly in the F helix and the EF corner; 2) the formation of the salt bridge between Hiss-β146 and Asp-β94, a feature of the deoxy state, is precluded; and 3) a deoxy interchain (α3β3) contact between Asp β99 and Tyr α42 is appreciably destabilized. The NMR data provide a structural basis for interpreting the high oxygen affinity, reduced cooperativity, and diminished polymerization of G-HbS.

In the preceding paper of this series (1) it has been shown that, under certain special conditions, human adult hemoglobin (HbA1) can be reacted with reduced GSH with disulfide bond formation between Cys-β93 (F9) and the cysteine of the tripeptide. This reaction can also be performed within red blood cells, where up to 25% of the hemoglobin can be modified. The treated red blood cells show increased oxygen affinity and reduced cooperativity. When hemoglobin S is modified in intact homoyzogous sickle cells an inhibition of up to 70% of the sickling at 21 mm Hg is observed. This last observation suggests a possible application of in situ hemoglobin modification in therapy of sickle cell disease.

These properties of intracellular G-Hb prompted us to study the protein in more detail in solution to establish structure-function relationships. We have studied the oxygen-binding properties, the Bohr effect, the rate of hemoglobin oxidation, and the tetramer-dimer dissociation. For the structural studies we used high resolution NMR spectroscopy, a method particularly well adapted for the solution state of normal or modified hemoglobins (2, 3). The strategy is to observe a certain number of intrinsic spectroscopic probes such as protons in the heme pocket, exchangeable protons belonging to solvent-accessible hydrogen bonds, or C2 and C4 protons of surface histidine residues (2).

We have demonstrated in a previous paper (4) that reaction of iodoacetamide or N-ethylmaleimide with Cys-β93 in HbA induces NMR-detectable structural and dynamic perturbations localized at the proximal side of the β heme pocket. In the present paper we show that similar effects can be observed in G-Hb in addition to a specific perturbation at the α3β3 subunit contact involving the Aspβ99-Tyrα42 hydrogen bond.

MATERIALS AND METHODS

Reduced glutathione and BisTris were purchased from Sigma and 2,2'-dithiodipyridine from Fluka; deuterated water (D2O) 99.98% was from CEA, France. All other chemicals were of analytical grade.

Sample Preparation—Adult human hemoglobin was prepared from fresh blood obtained by venipuncture from volunteers in our laboratories. Purification of HbA and Hbs Creteil (Ser-β96 → Asn-β96) was achieved by standard ion-exchange chromatography as previously published (5). Preparation of glutathione and thiopryidine-modified human hemoglobin is described in detail in the accompanying paper (1) briefly. HbA was first reacted with 2,2'-dithiodipyridine, and the thiopryidine hemoglobin (Hb-S) thus obtained was then reacted with reduced glutathione in large excess. The purity of the modified Hbs was tested by isoelectric focusing (6). Samples containing more than 4% methemoglobin were discarded.

For NMR measurements, solutions of hemoglobin (~10%) in 100 mM BisTris buffer (30 mM Cl-), prepared in D2O or H2O, were obtained by ultrafiltration in Centricon I tubes (Sartorius, France).

Oxygen Equilibrium Studies—Oxygenation measurements at equilibrium of purified G-Hb and HbA were performed at 35 °C (±1) with an automatic continuous method (Hemox Analyzer, TCS, Southamp-
50 mM BisTris or Tris buffer (for pH values greater than 7.5) and 100 mM NaCl, 0.5 mM EDTA, and 20 μg/ml catalase to avoid oxidation during runs. The oxygen-binding curves were recorded in solutions containing 160–200 μM Hb (on a heme basis). A space was introduced in the optical cuvette to reduce the light path from 1.2 to 0.2 cm. The measurements performed during the 45 min duration of each experiment never exceeded 4%. Experiments were conducted under varying conditions of pH, or after addition of DPG (Na+ salt), or IHP. The recording system of the oxygen-binding experiments was interfaced with an HP 85 microcomputer programmed to store on tape up to 1000 values of absorbance and pO2. The P50 and n50 values representing the partial pressure of oxygen and the Hill coefficient at half-saturation were computed from the experimental points in the range of 40–80% saturation by linear regression analysis. The experimental oxygen-binding curves were fitted by the Adair equation using a nonlinear least squares regression program starting from rough estimates of K1 and K4 obtained graphically and the values of P50 and n50 (8). The parameters of the allosteric model L = Td/Rd = (P50 x 1/Ka) and n = Ka/KT were computed from the values of K1 and K4, where K4 and Kd are the oxygen dissociation constants for the R and T state, respectively. The number of protons released or taken up by G-Hb upon oxygenation (Bohr effect) was calculated from the linkage equation relating the changes in log P50 to pH between 6.5 and 8.5 (9),

\[ \log P_{50} = \text{constant} + \log \left( \frac{(H^+ + K^+D)}{(H^+ + K^+D)} \left( \frac{(H^+ + K^+d)}{(H^+ + K^+d)} \right) \right) \]

where K+D, K+O2, K+D, K+O2 are the ionization constants of hypothetical oxygen-linked groups involved in the alkaline Bohr effect.

The same curve-fitting procedure was used as described above, using as starting estimates of the four unknowns those given in the literature for HbA (9).

**Kinetics of G-Hb Oxidation**—The rates of oxidation of G-Hb (100 μM heme) were measured at 37°C, under pure oxygen in 50 mM BisTris buffer (pH 7.0) and 100 mM NaCl. The amount of heme oxidized was calculated from changes in absorbance at 576.5 nm (α band) and at 500 nm, a maximum of the methemoglobin spectrum. The oxidation rates were also measured in solutions of G-Hb (~100 μM heme) after equilibration with pure CO and in the presence of potassium ferricyanide (final concentration, 1 mM), as described by Lanir et al. (10).

**Measurements of the Dissociation Constant**—The tetramer-dimer dissociation constants for HbA and G-Hb were determined by the flash-photolysis method (11). The measurements were made with a Durrum-Gibson stopped flow apparatus interfaced with a Data General microcomputer (Nova 2/16 K) programmed for the acquisition and treatment of data. A dye laser phase R with rhodamine 6G was used. CO hemoglobin solutions were 5 × 10−4 M (heme) in Tris buffer (50 mM), pH 7.4.

**NMR Techniques**—Proton NMR measurements were performed at 400 MHz on an AM 400-WB Bruker spectrometer in 1H2O (containing 5% 1H2O for the field/frequency lock) or in 2H2O at 20°C. Depending on the experiments different methods were used to eliminate the undesirable strong solvent peak. For the observation of the exchangeable protons appearing in the range 5–10 ppm from 1H2O for deoxy Hb the Jump and Return method (12) for the reduction of the water resonance was performed using a continuous saturation of the water resonance during acquisition. A Gaussian multiplication (LB = −30.00 Hz, GB = 0.05, acquisition time, 0.50 s) was applied prior to Fourier transformation in order to enhance the spectrum resolution. Comparison with spectra recorded without correction showed that this procedure introduces no significant artifacts.

Chemical shifts are expressed in ppm from the residual water signal (1H2O). The pH was measured with a Radiometer pH meter with no correction for the deuterium isotope effect on the glass electrode.

**RESULTS**

**Oxygen Affinity**—Hill plots of oxygen-binding curves of G-Hb compared to HbA are presented in Fig. 1. In the absence of DPG, G-Hb exhibits a more than 5-fold increase in oxygen affinity compared to native HbA and low heme-heme interactions (Fig. 2). A large shift to the left is observed at the bottom of the Hill plot for G-Hb indicative of a destabilization of its deoxy quaternary structure. Addition of 5 mM DPG reduces the oxygen affinity of G-Hb and increases slightly the cooperativity (Table I). The effect of DPG on G-Hb estimated as Δlog P50 ± DPG is comparable for G-Hb and HbA (0.55 and 0.49, respectively). This demonstrates that binding glutathione to the —SH groups of the β93 (F9) cysteinyl residues does not alter the oxygen-linked binding of DPG. A similar conclusion was reached from the Δlog P50 ± 2 mM IHP at pH 7.0 which were 1.2 and 1.05 for G-Hb and HbA, respectively. In the presence of IHP, the n50 values were similar (~2.2) for the two hemoglobins. Fig. 1 also shows that the upper asymptotes for G-Hb both in the presence or absence of DPG are shifted slightly to the right compared to HbA.

Values of the parameters of the two-state allosteric model and of the oxygen dissociation constants in the T and R states are given in Table I. In the absence of DPG the 6-fold increase in oxygen affinity compared to native HbA and low heme-heme interactions (Fig. 2). A large shift to the left is observed at the bottom of the Hill plot for G-Hb indicative of a destabilization of its deoxy quaternary structure. Addition of 5 mM DPG reduces the oxygen affinity of G-Hb and increases slightly the cooperativity (Table I). The effect of DPG on G-Hb estimated as Δlog P50 ± DPG is comparable for G-Hb and HbA (0.55 and 0.49, respectively). This demonstrates that binding glutathione to the —SH groups of the β93 (F9) cysteinyl residues does not alter the oxygen-linked binding of DPG. A similar conclusion was reached from the Δlog P50 ± 2 mM IHP at pH 7.0 which were 1.2 and 1.05 for G-Hb and HbA, respectively. In the presence of IHP, the n50 values were similar (~2.2) for the two hemoglobin, Fig. 1 also shows that the upper asymptotes for G-Hb both in the presence or absence of DPG are shifted slightly to the right compared to HbA. Values of the parameters of the two-state allosteric model and of the oxygen dissociation constants in the T and R states are given in Table I. In the absence of DPG the 6-fold increase in oxygen affinity compared to native HbA and low heme-heme interactions (Fig. 2). A large shift to the left is observed at the bottom of the Hill plot for G-Hb indicative of a destabilization of its deoxy quaternary structure. Addition of 5 mM DPG reduces the oxygen affinity of G-Hb and increases slightly the cooperativity (Table I). The effect of DPG on G-Hb estimated as Δlog P50 ± DPG is comparable for G-Hb and HbA (0.55 and 0.49, respectively). This demonstrates that binding glutathione to the —SH groups of the β93 (F9) cysteinyl residues does not alter the oxygen-linked binding of DPG. A similar conclusion was reached from the Δlog P50 ± 2 mM IHP at pH 7.0 which were 1.2 and 1.05 for G-Hb and HbA, respectively. In the presence of IHP, the n50 values were similar (~2.2) for the two hemoglobin. Fig. 1 also shows that the upper asymptotes for G-Hb both in the presence or absence of DPG are shifted slightly to the right compared to HbA.
in oxygen affinity of G-Hb and its decreased cooperativity \((n_{opt} = 1.5)\) are accounted for by the very low value of the allosteric constant \(L\) and the simultaneous increase of \(c\). This latter effect is explained by the decrease in \(K_T\) as well as a small increase in \(K_R\). Addition of DPG decreases the oxygen affinity of G-Hb by increasing \(K_T\) (4.7-fold) and \(L\) (80-fold) with only minor changes in \(n_{opt}\) and \(K_R\).

**Oxygen Bohr Effect**—Fig. 3A illustrates the pH dependence of \(\log P_{50}\) in G-Hb in the absence and presence of 5 mM DPG. These data are compared to HbA in Fig. 3B. In the absence of DPG, G-Hb exhibits a 38% reduction of the alkaline Bohr effect. Addition of 5 mM DPG increases the alkaline Bohr effect to nearly the same extent in G-Hb (+0.29 \(\mathrm{H}^+\)/heme) as in HbA (+0.23 \(\mathrm{H}^+\)/heme), demonstrating normal binding of DPG to G-Hb and a normal additional Bohr effect due to anion binding. The calculated parameters of the Bohr effect for G-Hb and HbA are given in Table II, and the Bohr curves are illustrated in Fig. 4. In the absence of DPG the Bohr curve of G-Hb is shifted toward the left and exhibits a decrease of 0.7 pH units in the \(pK^*_1\) of the hypothetical alkaline Bohr group in the deoxy conformation. Interestingly, we did not observe any change in the \(pK^*_1\) corresponding to the acid Bohr group between the oxy and deoxy states, in contrast to the behavior of this group in HbA where an increase of the \(pK^*_1\) occurs upon oxygenation. In the presence of DPG the \(pK^*_1\) (the pH at which maximum proton release occurs) is increased (4.1 \(\pm\) 0.5) \(\times\) \(10^{-6}\) M in 50 mM Tris, pH 7.4, in accord with previous results (11). For G-HbCO, in the same experimental conditions, we found a value of \((4.8 \pm 0.5) \times 10^{-6}\) M which indicates that G-Hb has a significantly greater tendency to dissociate in the CO form.

**Rate of Oxidation of G-Hb**—The rate of autoxidation of oxy G-Hb was studied at 37 °C and compared to HbA. Fig. 5A shows that the plot of \(\log (\text{HbA}_{\text{O}2})\) versus time is linear during the 3 h of measurements during which about 20% of the hemes have been oxidized. The rate of oxidation of G-Hb is 0.7 pH units in the \(pK^*_1\) of the hypothetical alkaline Bohr group in the deoxy conformation. Interestingly, we did not observe any change in the \(pK^*_1\) corresponding to the acid Bohr group between the oxy and deoxy states, in contrast to the behavior of this group in HbA where an increase of the \(pK^*_1\) occurs upon oxygenation. In the presence of DPG the \(pK^*_1\) (the pH at which maximum proton release occurs) is increased (4.1 \(\pm\) 0.5) \(\times\) \(10^{-6}\) M in 50 mM Tris, pH 7.4, in accord with previous results (11). For G-HbCO, in the same experimental conditions, we found a value of \((4.8 \pm 0.5) \times 10^{-6}\) M which indicates that G-Hb has a significantly greater tendency to dissociate in the CO form.

**Tetramer-Dimer Equilibrium in G-Hb**—Flash photolysis with HbACO showed a dissociation constant (tetramer-dimer) of \((4.1 \pm 0.5) \times 10^{-6}\) M in 50 mM Tris, pH 7.4, in accord with previous results (11). For G-HbCO, in the same experimental conditions, we found a value of \((4.8 \pm 0.5) \times 10^{-6}\) M which indicates that G-Hb has a significantly greater tendency to dissociate in the CO form.

**FIG. 2.** Hill coefficient \(n\) versus \(\log (y/1 - y)\) for solutions of G-Hb and HbA. These curves were computed as the first derivative of the Hill plots shown in Fig. 1 (curves 1 and 3) by linear regression analysis for each point between the \(i + 3\) and \(i - 3\) adjacent points. Squares are experimental points, and the lines correspond to the best fits of the experimental curves calculated by a nonlinear least-squares fitting procedure.

**FIG. 3.** The oxygen Bohr effect for G-Hb (A) and for control HbA (B). Filled circles, 100 mM NaCl; open circles, 100 mM NaCl + 5 mM DPG. Other conditions were for both sets: 150-200 \(\mu\)M heme, 50 mM BisTris buffer (or Tris buffer for pH values greater than 7.5), temperature 25 °C. Continuous lines are the best fits to the experimental data computed as indicated under "Materials and Methods." Open squares in A and B indicate the values of \(\log P_{50}\) upon addition of 2 mM IHP.

**TABLE I**

<table>
<thead>
<tr>
<th>Conditions: 150-200 (\mu)M heme, temperature, 25 °C, 50 mM BisTris buffer.</th>
<th>G-Hb</th>
<th>HbA</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 mM NaCl</td>
<td>100 mM NaCl</td>
<td>100 mM NaCl</td>
</tr>
<tr>
<td>+ 5 mM DPG</td>
<td>+ 5 mM DPG</td>
<td>+ 5 mM DPG</td>
</tr>
<tr>
<td>(\Delta H^*) max/heme</td>
<td>0.34</td>
<td>0.63</td>
</tr>
<tr>
<td>pH max</td>
<td>7.00</td>
<td>7.60</td>
</tr>
<tr>
<td>(pK^*_1) deoxy</td>
<td>7.27</td>
<td>8.34</td>
</tr>
<tr>
<td>(pK^*_1) oxy</td>
<td>6.86</td>
<td>6.78</td>
</tr>
<tr>
<td>(pK^*_1) deoxy</td>
<td>7.10</td>
<td>7.24</td>
</tr>
<tr>
<td>(pK^*_1) oxy</td>
<td>6.90</td>
<td>7.42</td>
</tr>
</tbody>
</table>

*The pH value at which maximum proton release occurs.

**TABLE II**

| Parameters of the oxygen Bohr effect for G-Hb and for HbA in the absence or presence of 5 mM DPG |
|---|---|---|---|---|---|
| Conditions: 150-200 \(\mu\)M heme, temperature, 25 °C, 50 mM BisTris buffer. | G-Hb | HbA |
| 100 mM NaCl | 100 mM NaCl | 100 mM NaCl |
| + 5 mM DPG | + 5 mM DPG | + 5 mM DPG |
| \(\Delta H^*\) max/heme | 0.34 | 0.63 | 0.54 | 0.77 |
| pH max | 7.00 | 7.60 | 7.30 | 7.70 |
| \(pK^*_1\) deoxy | 7.27 | 8.34 | 7.97 | 8.52 |
| \(pK^*_1\) oxy | 6.86 | 6.78 | 6.53 | 6.55 |
| \(pK^*_1\) deoxy | 7.10 | 7.24 | 7.03 | 6.13 |
| \(pK^*_1\) oxy | 6.90 | 7.42 | 7.33 | 6.54 |

*The pH value at which maximum proton release occurs.

**TABLE III**

| Oxygen-binding parameters for solutions of G-Hb and HbA in the absence and after the addition of 5 mM DPG |
|---|---|---|---|---|
| Conditions: pH 7.20, temperature, 25 °C, 150-200 \(\mu\)M heme in 50 mM BisTris buffer. | P_{50} | K_{f} | K_{r} | L |
| 100 mM NaCl | G-Hb | HbA | G-Hb | HbA |
| 100 mM NaCl + 5 mM DPG | 1.1 | 1.50 | 0.56 | 2.2* | 12.6 |
| 100 mM NaCl | 5.8 | 2.94 | 0.34 | 35.0 | 9.74 \(\times\) 10^{-4} |
| 100 mM NaCl + 5 mM DPG | 3.9 | 1.77 | 0.68 | 10.4 | 1.0 \(\times\) 10^{-5} |
| 100 mM NaCl | 17.0 | 2.87 | 0.36 | 94.0 | 6.5 \(\times\) 10^{-6} |

*This value has been corrected for the participation of \(-8\%\) of Hb tetramers being in the R state in the absence of oxygen.
much slower and not linear indicating an inequivalence between $\alpha$ and $\beta$ chains. After 3 h at 37°C 12% met-G-Hb was formed.

To investigate these differences more precisely we studied the oxidation rate of carboxylated G-Hb by ferricyanide following the method proposed by Lanir et al. (10). In solutions equilibrated under 1 atm CO the rate of heme oxidation depends upon the dissociation rate of the ligand forming unliganded heme in the R state, which is then oxidized by the ferricyanide (10). Comparison of the oxidation rates of G-HbCO and HbACO in the presence of ferricyanide is shown in Fig. 5B. The rate of oxidation of HbA is linear over the 4 h of observation with a rate constant of 24 pmol of heme oxidized per h. In contrast, the rate of oxidation of G-HbCO is not linear. Assuming equal amounts of the two reacting species, the curve may be fitted with two rate constants: 25 pmol h$^{-1}$ and 4 pmol h$^{-1}$ (Fig. 5B).

State of Heme Pocket Residues in G-Hb—In NMR measurements some amino acid residues in the heme environment are significantly perturbed by the induced magnetic moment of the porphyrin $\pi$-electron system. The direction and intensity of this perturbation (expressed as a spectral NMR shift) depend on the position of the residue relative to the heme. This permits the use of the corresponding resonances for the characterization of the protein in the vicinity of the prosthetic group. Fig. 6 represents the NMR region of such ring-current-shifted resonances in the oxy form of HbA, G-Hb, Hb-SP, and a high affinity variant, Hb Créteil (Ser-889 $\rightarrow$ Asn-889). The resonance at $-7.20$ ppm in HbA represents the $\gamma$ CH$_3$ group of Val E11 from the distal side of the $\alpha$ and $\beta$ heme cavities (13). While in HbA, Hb-SP, and Hb Créteil the resonances corresponding to $\alpha$ and $\beta$ chains are superimposed at pH 6.85, in G-Hb a slight splitting of the $-7.20$-ppm resonance was observed indicating a slight subunit inequivalence in the heme cavity. A similar splitting of this peak was observed in normal HbA by decreasing the pH of the solution or adding certain inorganic or organic anions (Cl$^-$, SO$_4^{2-}$, IHP, DPG) (14, 15). Three other resonances noted, II, III, and VIII, are also low-field shifted in G-Hb indicating that the corresponding protons moved away from the heme center. Some analogies can be noted between the spectra of G-Hb and Hb Créteil, in which a residue of the F helix (F5) is different; however, the heme pocket of this variant and of Hb-SP appear to be significantly more perturbed than in GSH-modified hemoglobin (Fig. 6).

Fig. 7 shows the spectra in the high-field region of deoxy HbA and G-Hb; the observed differences (arrows) indicate that structural perturbations also exist in the unliganded state, but the absence of any assignment makes their interpretation difficult.

Surface Histidine Residues—It has been shown that 22 of the 38 hemoglobin histidine residues have observable resonances in the NMR spectrum between 2.0 and 5.0 ppm downfield from $^2$H$_2$O (16). The chemical shift of the histidine

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**Fig. 4.** Bohr curves calculated from the experimental data shown in Fig. 3. These curves were obtained using Equation 1 given under "Materials and Methods." Conditions are those given in the legend of Fig. 3.

**Fig. 5.** Kinetics of oxidation for G-Hb and for control HbA illustrated by the disappearance of the fully liganded species versus time. The ordinate is a logarithmic scale. The two upper curves were obtained by measuring the changes in the ratio of absorbances at 578.5 and 500 nm for the oxygenated Hb solutions. The two lower curves were obtained for solutions of Hb fully saturated with CO and in the presence of 1 mM K$_3$Fe(CN)$_6$. Changes in absorbances were measured at 568.5 and 500 nm as a function of time. Conditions were: pH 7.0, 100 $\mu$M heme, 50 mM BisTris buffer at 37 and 25°C for oxygen and CO-saturated solutions, respectively. The dashed line was drawn by extrapolation to the ordinate of the estimated rate constant of the last experimental points. Crosses indicate the rate of oxidation of G-Hb after subtraction of the second rate constant given by the slope of the dashed line.
Structure-Activity Relationships in Glutathionyl Hemoglobin

Hb $\text{A}$

I $\text{XI}$

40.0

3.0

.O

2.0

PPM FROM $^{1}\text{H}_{2}\text{O}$

FIG. 8. The aromatic region of the 400-MHz NMR spectra of deoxy HbA and G-Hb under the same conditions as in Fig. 6. Titrable histidine resonances are labeled as in Ref. 2.

Hb A

FIG. 7. High field region of the 400-MHz NMR spectra of HbA and G-Hb in the deoxygenated state; conditions as in Fig. 6.

peaks expresses the tautomeric state of the imidazole ring which is determined in a complex manner by the solution pH, the net electronic charge of the neighboring residues, and the strength of the hydrogen bond in which the histidine may be implicated. Monitoring of the aromatic spectrum may thus give information about secondary or tertiary changes in the protein; their localization is limited by the assignment of the specific probes.

In the liganded state we observed no spectral difference in the histidine region between HbA and G-Hb (data not shown). In contrast, some significant modifications were observed in the spectrum of unliganded G-Hb; a low-field shift of resonance 1, the absence of resonance 3, and a perturbation of resonance 4' (Fig. 8). These resonances were previously assigned as follows: 1 = His-$\beta$97 (3, 4), 3 = His-$\beta$146 (16), 4' = His-$\beta$77 (17). It is known that the resolution enhancement procedure may modify the peak intensities (18); our experience showed, that when applied with the same parameters in a series of slightly different hemoglobin variants, it does not perturb significantly the relative intensities of peaks within the aromatic spectral region. Thus, analysis of the two spectra
in Fig. 8 suggests that a new resonance has appeared between peaks 2 and 4 + 5 in G-Hb. By analogy with previous studies (4) on N-ethylmaleimide- and iodoacetamide-treated HbA and Hb Barcelona (p94 Asp-His), we tentatively conclude that it corresponds to the high field-shifted resonance of His-p146.

Probes of Subunit Interfaces—Fig. 9 shows the spectral region between 6.0 and 10.00 ppm downfield from 1H2O of deoxy HbA, G-Hb, and Hb Crêteil. The resonances observed in this region represent hydrogen bond protons in slow exchange (on the NMR time scale) with the solvent. Two of them, at 8.20 and 7.50 ppm, are independent of the ligation state of the Hb and are thought to belong to the αβ接触 interface (19, 20). The other two peaks were demonstrated to be probes for the deoxy structure; in particular, the 9.30-ppm peak is associated with the specific deoxy quaternary structure hydrogen bond between Tyr-p442 and Asp-p99 (αpβ contact) (20). The principal differences between HbA and G-Hb in Fig. 9 are the significant broadening of this last resonance in the modified hemoglobin and splitting of the resonance at 7.50 ppm. A similar broadening of the low field peak was observed in the high affinity variant Hb Crêteil (Fig. 9); the peak is simultaneously high field-shifted.

Discussion

Although GSH is found at high concentration in the red cells (2-3 mmol/liter of cells) (21), only a negligible fraction appears to be associated with oxy Hb (22). In the first paper of this series (1) we have shown that under certain conditions GSH can be bound to Cys-p93 of the Hb to form a mixed disulfide.

Initial studies of partially purified G-Hb obtained by a prolonged incubation of HbA with oxidized GSH have shown an increased oxygen affinity, a decreased cooperativity, and a decreased Bohr effect (23).

Oxygen-binding measurements of the present study confirm and extend these results showing that G-Hb has a nearly 6-fold increase in oxygen affinity compared to HbA and low heme-heme interactions. The alkaline Bohr effect is reduced by ∼40%, but the allosteric effectors DPG and IHP have comparable effects on G-Hb and HbA in decreasing the oxygen affinity and restoring the Bohr effect to normal. These results are characteristic of several Hbs chemically modified at p93 (F9) cysteine such as NES-Hb or AA-Hb (24). To our knowledge GSH is the most powerful reagent in increasing the oxygen affinity while preserving normal interaction with organic phosphates.

In the two-state allosteric model (25) the abnormal function of G-Hb in terms of P0.5 and a0 is accounted for by the very low value of the allosteric constant L and the large decrease of Kσ, the dissociation constant of oxygen in the T quaternary state. The slightly increased value of Kσ contributes also, to a smaller extent, to the low L value for G-Hb. Similar observations have been made in other high oxygen affinity variants (26) and NES-Hb (24).

The low value of Kσ for G-Hb is indicative of a destabilized T deoxy quaternary structure. The mechanism for this destabilization in G-Hb is probably not unique. As is indicated in Table I, both allosteric parameters L and c are greatly modified in G-Hb, suggesting that the mechanism for affinity increase induced by glutathione is presumably a combination of quaternary and tertiary perturbations.

The results of autoxidation and ferricyanide oxidation studies indicate that in G-Hb the individual α and β chains behave as heterogeneous species. Modifications of the tertiary heme pocket structure may be invoked to explain the decreased heme oxidation rates in modified Hb.

The NMR results on the heme pocket residues in liganded G-Hb indicate that at least two types of tertiary perturbations are induced by the bound glutathione. The first is represented by the splitting of resonance l (Fig. 6) corresponding to the Cys-CH3 groups of Val-p11 (α + β) at the distal side of the cavity (13) at pH 6.85. The most probable interpretation of this splitting is that Val-p11 in β chains is slightly shifted toward the heme center, the corresponding resonance being thus high field shifted. The second kind of tertiary perturbation in the heme region affects the peaks labeled II and III. It is interesting to note that similar low field shifts in this spectral region also occur in another modified Hb (Hb-SP where 2-thiopyridine is bound to Cys-p93) and in a high affinity Hb variant, Hb Crêteil (Fig. 6). The common feature of these hemoglobins, a modified β chain, led us to envisage that the modified resonances correspond to protons in the β heme cavity. In a previous paper, where similar observations were reported for NES-Hb and AA-Hb (4), we advanced the hypothesis that at least one of the low shifted resonances belongs to Leu-p96 (FG3). With such an assignment the above spectral observations mean that in the G-Hb, part (or all) of the F helix and FG corner in the β chains have moved away from the heme center. From the spectra in the high field region of deoxy Hbs it may be deduced that spectral differences exist also in the unliganded state (Fig. 7). Unfortunately, their interpretation is not possible since no assignments have yet been made. It is reasonable to postulate that the F and FG segments show the same tendency for displacement in the deoxy state of G-Hb as in the oxy state.

As was earlier shown by crystallographic studies of the T → R transition of the Hb tetramer, Cys-p93 shifts from an external position toward Tyr-p145 (27, 28). This shift is part of a more general movement of the F and FG segments mentioned above. Arnone et al. (5) have shown that in crystals of deoxy Hb Crêteil Cys-p93 retains its internal position even in the T state, and this is one of the reasons for the highly increased affinity of this variant. The similarities in the spectral region corresponding to the oxy heme pocket in G-Hb and Hb Crêteil (Fig. 6) suggest similar conformations for the perturbed β chains, particularly that attachment of the glutathionyl residues determines an "oxy-like" position of the reacted cysteine.

Interesting information concerning the tertiary structure of the unliganded state was obtained by studying the aromatic region of the NMR spectra (Fig. 8). The low field region of these spectra represents C2H protons of the imidazole rings in tryptophan and histidines (2). Using the previous peak assignments (4, 16, 17) the present results indicate that three β chain histidines are modified in G-Hb: His-p146 (peak 3), His-p97 (peak 1), and His-p77 (peak 4'). The high field shift of resonance 3, whose new position is probably between peaks 2 and 4 + 5, reflects a decreased pK of the corresponding histidine. This indicates that the salt bridge between the carboxyl terminal end of the β chain (His-p146) and Asp-p94 is not present in G-Hb. Two consequences follow: the first is the destabilization of the T state for which the above salt bridge is an important tertiary feature (27). The second concerns the Bohr effect; in fact, it was found that His-p146 is responsible for about 40% of the alkaline Bohr effect (29) and any perturbation precluding terminal salt bridge formation reduces by about half the number of protons liberated upon oxygenation (5, 30).

The terminal carboxyl of the β chain is also salt bridged to the ε amino group of Lys-p40 (27), this interaction being one of the important αpβ2 contacts necessary for the full
expression of cooperativity. Displacement of His-β146 in the unliganded state of G-Hb makes improbable this contact, but our NMR data cannot determine its status directly.

The second histidine perturbation observed in the deoxy G-Hb spectrum (Fig. 8) is the low field shift of resonance 1 corresponding to His-β97 (3, 4). The pK of its imidazole ring which is already the highest in deoxy HbA (2) seems to be further increased. This perturbation suggests that the contribution of His-β97 to the αββ2 contact (Pro-α44...His-β97) of the T tetramer may be weakened in deoxy G-Hb.

The perturbation induced by the reagent binding at the βF position extends to the E helix, as is indicated by the perturbed His-β77 resonance (Fig. 8) and by the splitting of the peak corresponding to Val-Ell β67 (Fig. 6).

The final NMR observation in the region of exchangeable hydrogen bond protons (Fig. 9) also concerns an αββ2 interaction. Resonances in Fig. 9 represent protons in exchange with the solvent at the slow exchange limit on the NMR time scale νex ≈ νex, where νex is the exchange frequency, and νex and νH2O are the spectral frequencies of the exchanging proton and water, respectively (31). Under these conditions, the line width is determined predominantly by the exchange reaction, so we have Δνex ~ νex. A rough estimation of the line width of the resonance at 9.30 ppm in HbA and G-Hb suggests that the proton in the hydrogen bond involving Asp-β99-Tyr-α42 exchanges about two times more rapidly in G-Hb. This may be explained either by a general destabilization of the αββ2 interface or by a localized perturbation of the neighboring region. The fact that the other probe for the T state of globins (35) in a more recent paper (36) Russu and Ho presents small differences in pK of some surface histidines, altered in G-Hb renders the first hypothesis improbable. Our unliganded hemoglobin (the resonance at 6.30 ppm) is not perturbed. The 10-fold increase in the tetramer-dimer dissociation constant in the deoxy state. Many of the above suggestions are further supported by the observation that the other probe for the T state of G-Hb spectrum (Fig. 8) is the low field shift of resonance 1 in the unliganded state of G-Hb makes improbable this contact, but our NMR data cannot determine its status directly.

Throughout this paper we presented functional and structural results obtained on modified HbA, but in all probability the consequences may be directly applied to G-HbS. In fact, functional tests (33) and crystallographic studies (34) failed to reveal any significant difference between HbA and HbS. Early comparative NMR experiments have shown that the tertiary structure of the heme environment, as well as the αββ2 and αββ2 contacts are indistinguishable in the two hemoglobins (35). In a more recent paper (36) Russu and Ho present small differences in pK of some surface histidines, both in the CO and deoxy forms. As we report here, histidine perturbations in HbA induced by GSH are significantly greater than these differences, and therefore, we postulate that they are also relevant for HbS. Consequently, some of the observations presented in this paper are used in the preceding article of the series (1) to discuss the possible mechanisms of the direct antisickling effect of GSH.

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