Metal Complexes as Allosteric Effectors of Human Hemoglobin: An NMR Study of the Interaction of the Gadolinium(III) Bis(*m*-boroxyphenylamide)diethylenetriaminepentaacetic Acid Complex with Human Oxygenated and Deoxygenated Hemoglobin

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ABSTRACT The boronic functionalities on the outer surface of the Gd(III) bis(*m*-boroxyphenylamide)DTPA complex (Gd(III)L) enable it to bind to fructosamine residues of oxygenated glycated human adult hemoglobin. The formation of the macromolecular adduct can be assessed by NMR spectroscopy via observation of the enhancement of the solvent water proton relaxation rate. Unexpectedly, a strong binding interaction was also observed for the oxygenated unglycated human adult hemoglobin, eventually displaying a much higher relaxation enhancement. From relaxation rate measurements it was found that two Gd(III)L complexes interact with one hemoglobin tetramer ($K_D = 1.0 \times 10^{-5}$ M and 4.6×10^{-4} M, respectively), whereas no interaction has been observed with monomeric hemoproteins. A markedly higher affinity of the Gd(III)L complex has been observed for oxygenated and aquo-met human adult hemoglobin derivatives with respect to the corresponding deoxy derivative. Upon binding, a net change in the quaternary structure of hemoglobin has been assessed by monitoring the changes in the high-resolution ^{1}H-NMR spectrum of the protein as well as in the Soret absorption band. On the basis of these observations and the ^{11}B NMR results obtained with the diamagnetic La(III)L complex, we suggest that the interaction between the lanthanide complex and deoxygenated, oxygenated, and aquo-met derivatives of human adult hemoglobin takes place at the 2,3-diphosphoglycerate (DPG) binding site, through the formation of N→B coordinative bonds at His$^{143\beta}$ and His$^{2\beta}$ residues of different β-chains. The stronger binding to the oxygenated form is then responsible for a shift of the allosteric equilibrium toward the high-affinity R-state. Accordingly, Gd(III)L affinity for oxygenated human fetal hemoglobin (lacking His$^{143\beta}$) is significantly lower than that observed for the unglycated human adult tetramer.

INTRODUCTION

In recent years, gadolinium (III) complexes have received increasing attention, in view of their ability to enhance water proton relaxation rates of their aqueous solutions. This property has been widely exploited in the design of contrast agents for magnetic resonance imaging (MRI) (Lauffer, 1987; Tweedle, 1989; Koenig and Brown, 1990).

Further developments in this field are expected to be aimed at the search for an improved capability of the metal complexes to act as reporters of the molecular environment. This means that the relaxation rate of water protons in a given tissue may become dependent on the interactions that the metal complex is able to set up with other substrates. This goal may be pursued through the introduction of suitable functionalizations on the outer surface of the ligand to enable the complex to recognize specific target molecules. A simple way to introduce the desired functionality is provided by the formation of bisamide derivatives of diethylenetriaminepentaacetic acid (DTPA) according to Chart 1 (Quay, 1987; Konings et al., 1990; Aime et al., 1993a,b, 1994, 1995; Rizkalla et al., 1993; Geraldes et al., 1993, 1995).

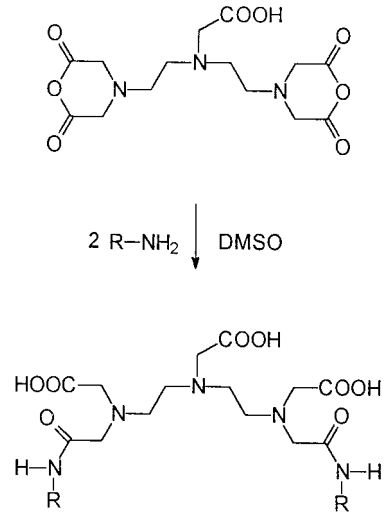

CHART 1

A bisamide DTPA ligand from DTPA anhydride and *m*-aminophenylboronic acid, namely bis(*m*-boroxyphenylamide)DTPA (hereafter L), was first reported in 1993 (Aime et al., 1993b). Its Gd(III) complex (Chart 2) shows a relatively high thermodynamic stability ($K_f = 2.0 \times 10^{16}$ M^{-1}), good relaxivity at 25.0°C ($R_{1p} = 4.6$ mM^{-1} s^{-1}) and, through the boronic functionalities, the peculiar capability to bind sugars containing the *syn*-diol moiety (Aime et al.,

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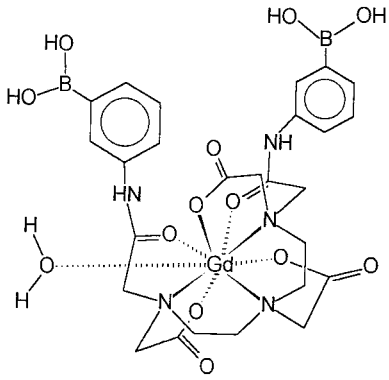


CHART 2

1993a). (Millimolar relaxivity, R_{1p}, is the paramagnetic contribution to the overall solvent water proton magnetic relaxation rate in the presence of a 1 mM concentration of the paramagnetic species. R_{1p} is given by $R_{1p} = (T_1^{obs})^{-1} - (T_1^{dia})^{-1}$, where $(T_1^{dia})^{-1}$ is the water proton relaxation rate of a solution containing an analogous diamagnetic complex, which in a dilute solution is equal to that of pure water. At 25.0°C and 20 MHz, $(T_1^{dia})^{-1} = 0.38\ s^{-1}$.) This property was exploited to determine the extent of glycation of human serum albumin, the binding of the bis(*m*-boroxyphenylamide)diethylenetriaminepentaacetic acid gadolinium(III) (Gd(III)L) complex to the macromolecule, inducing a significant increase in relaxivity as a consequence of the lengthening of the molecular reorientational time (τ_R).

A straighforward extension of this study led us to consider glycated Hb as a target for Gd(III)L for the important implications this species has in the control of sugar levels in diabetic patients.

Hb glycation occurs at the N-terminal Val^1 residue of both β-chains through the formation of a Schiff base that successively undergoes the Amadori rearrangement by hydrogen transposition to saturate the N=C double bond and to form a ketonic group at the carbon adjacent to that moiety (fructosamine) (Baker et al., 1991). Our attempt to extend the relaxometric assay to human hemoglobin (Hb) has unexpectedly shown that a strong interaction with the protein also occurs in the absence of protein glycation. In this paper, the interaction of Hb with Gd(III)L has been assessed either by a ligand-binding assay based on the water proton relaxation enhancement analysis (Eisinger et al., 1961; Aime et al., 1996a) or by high-resolution NMR spectroscopy. These results allow us to recognize that this complex may act as an allosteric effector for human hemoglobin by stabilizing its high-affinity *R* state. Stabilization of the *R* state is rather uncommon. There are few other molecules that can do this through the link of α-chains together, thus yielding a constraint which is at minimum when the tetrameric protein is in the high-affinity *R* state (Abraham et al., 1995; Boyiri et al., 1995).

MATERIALS AND METHODS

Hemoglobins and chemicals

Oxygenated human adult Hb (HbA) was obtained from freshly drawn venous blood samples (Antonini and Brunori, 1971). Oxygenated human fetal Hb (HbF) was obtained from umbilical cord blood samples (Antonini and Brunori, 1971), and the HbF-to-total Hb ratio was determined by ion-exchange high-performance liquid chromatography. Oxygenated HbA and HbF concentration was determined using the extinction coefficients of 14.4 $mM^{-1}\ cm^{-1}$ at 541 nm and 14.6 $mM^{-1}\ cm^{-1}$ at 542 nm, respectively (pH 7.0) (Zijlstra et al., 1991). Although extinction coefficients are given per heme, Hb concentrations reported in the text refer to the tetramer content.

Ferric (aquo-met) HbA was prepared from oxygenated Hb by treatment with sodium nitrite (Antonini and Brunori, 1971). Ferric (aquo-met) HbA concentration was determined on the basis of the extinction coefficient 9.07 $mM^{-1}\ cm^{-1}$ at 500 nm at pH 7.3 (Zijlstra et al., 1991).

Deoxygenated HbA was obtained from oxygenated HbA by bubbling water-saturated N_2 through the sample.

Oxygenated unglycated human adult hemoglobin (HbA_0) and oxygenated glycated human adult hemoglobin (HbA_{1c}) were obtained from chromatographic separation of HbA by means of aminophenylboronic acid affinity gel chromatography (GHb-PBA), where agarose-bound boronate groups reversibly bind glucose as *syn*-diols (Wettre et al., 1993). By this procedure HbA_0 was eluted first by using 50 mM ammonia buffer at pH 9.25 as the eluent. HbA_{1c} was then eluted using 0.1 M sorbitol in the same buffer, and the protein was recovered by centrifugal ultrafiltration to wash out the excess of sorbitol with phosphate-buffered saline (PBS) (3 mM phosphate buffer, 0.150 M NaCl). HbA was obtained from blood samples freshly drawn from diabetic patients.

Ferric horse myoglobin and ferric horse heart cytochrome *c* were obtained from Sigma Chemical Co. (St. Louis, MO) and used without further purification.

Bis(*m*-boroxyphenylamide)DTPA and its La(III) and Gd(III) complexes were prepared according to a previously reported procedure (Aime et al., 1993a).

All chemicals were of analytical grade and used without further purification.

Values of P_{50} and *n* for oxygen binding to HbA (50–100 μM as tetramer), in the absence and presence of 1.0×10^{-4} M Gd(III)L, were determined at pH 7.2 in PBS and 25.0°C from absorbance changes accompanying oxy-HbA formation by the tonometric method (Antonini and Brunori, 1971).

Oxygenated and deoxygenated Hb samples contained less than 1% of ferric (aquo-met)Hb, under all of the experimental conditions.

Values of the sedimentation coefficient of the oxygenated, deoxygenated, and ferric (aquo-met) HbA and HbF, in the absence and presence of Gd(III)L (ranging between 0.1 and 1.0 mM), were determined with a SPINCO model E analytical ultracentrifuge at 52,000 rpm (Ascenzi et al., 1993).

NMR measurements

Water proton T_1 measurements of ferric (aquo-met) Hb, oxy-Hb, and deoxy-Hb, in the absence and in the presence of 1.0×10^{-4} M Gd(III)L, were obtained between pH 7.2 and pH 10.0 on a Stelar SpinMaster Spectrometer (Stelar, Mede (PV), Italy) operating at 20 MHz, by means of the Inversion-Recovery technique (16 experiments, four scans). Magnetization values were obtained by averaging the first 128 data points of the Free Induction Decay (FID). A phase cycle ($+x$, $-x$, $-x$, $+x$) was applied on the 90° observation pulse to cut off the *y*-scale receiver offset. A typical 90° pulse width was 3.5 μs. The tau values were linearly increased from a starting value corresponding to one-seventh of the estimated null point ($0.693 \cdot T_1$), so that the null point occurs at the middle of the inversion recovery curve (seventh experiment). In the 16th experiment the FID is acquired after a single 90° pulse, to get the M_∞ value (Bertini and Luchinat, 1986). The reproducibility in T_1 measurements was ±0.5%. The temper-

ature was controlled with a Stelar VTC-91 air flow heater, equipped with a copper-constantan thermocouple; the actual temperature in the probe head was measured with a Fluke 52 k/j digital thermometer, with an uncertainty of ±0.3°C.

R_{1p} values (see the description of millimolar relaxivity, R_{1p}, in the Introduction) have been determined by subtracting from the observed relaxation rate (R_1^{obs}) in the presence of the Gd(III)L complex the blank relaxivity value (R_1^{dia}) measured for solutions containing the same hemoprotein in the same concentration without the gadolinium complex. Although symbols with the dia superscript usually refer to diamagnetic systems, this is not the case in the present study, where both dia- and paramagnetic protein contributions have to be subtracted to determine the contribution of the Gd(III)L complex alone. The R_1^{dia} values, determined at 298 K for a 1.0 mM protein solution, are as follows: 0.61 s^{-1} for oxy-HbA_0, 0.77 s^{-1} for deoxy-HbA_0, 1.4 s^{-1} for ferric (aquo-met) HbA, 0.59 s^{-1} for oxy-HbF, and 0.54 s^{-1} for oxy-HbA_{1c}.

High-resolution ^1H NMR spectra were obtained in 90% H_2O, 10% D_2O PBS, at 400 MHz proton Larmor frequency on a Bruker AVANCE 400 spectrometer, at 29°C and pH 7.2. The intense water signal has been suppressed using the WATERGATE 3-9-19 pulse sequence (Piotto et al., 1992).

^{11}B NMR spectra were obtained in D_2O, at 25.0°C and between pD 5.5 and 11.0, on a JEOL EX-400 Fourier transform NMR spectrometer, operating at a ^{11}B Larmor frequency of 128.3 MHz, equipped with a built-in temperature controller.

The Proton Relaxation Enhancement method

The established theory (Dwek, 1973; Bertini and Luchinat, 1986; Koenig and Brown, 1990; Banci et al., 1991) describes R_1^{obs} as the sum of three terms:

$$R_1^{obs} = R_{1p}^{is} + R_{1p}^{os} + R_1^{dia} \quad (1)$$

where R_{1p}^{is} is the contribution from the exchange of water molecules in the inner coordination sphere of the paramagnetic metal ion to the bulk water; R_{1p}^{os} is the paramagnetic contribution arising from water molecules diffusing in the proximity of the metal ion; and R_1^{dia} represents the solvent relaxation rate in the absence of the paramagnetic complex.

The R_{1p}^{is} contribution is determined by the value of the relaxation time of the coordinated water molecule (T_{1M}) and by the exchange lifetime τ_M:

$$R_{1p}^{is} = [M]/55.56(T_{1M} + \tau_M) \quad (2)$$

where [M] is the molar concentration of the paramagnetic species. T_{1M} depends on the strength of the dipolar interaction (K) between the protons of the coordinated water and the unpaired electrons modulated by a function of its correlation time $f(\tau_c)$:

$$1/T_{1M} = Kf(\tau_c) \quad (3)$$

The correlation time τ_c is determined by

$$\tau_c^{-1} = \tau_R^{-1} + \tau_M^{-1} + \tau_S^{-1} \quad (4)$$

where τ_R is the molecular reorientational correlation time and τ_S is the electronic relaxation time. The latter parameter is field-dependent, and its value increases with the observation frequency; at the operating frequency (20 MHz) it is long enough to be negligible in Eq. 4. Thus the observed relaxation rate depends basically on the reorientational correlation time τ_R and consequently on the molecular size of the system containing the paramagnetic metal. The interaction of the metal chelate with the large protein will then lead to an enhancement of the relaxation rate.

The binding isotherm obtained by plotting the relaxation enhancement factor ϵ^* versus the concentration of protein may be analyzed by means of the well-established Proton Relaxation Enhancement (PRE) procedure (Eisinger et al., 1961; Mildvan and Cohn, 1963). The observed relaxation enhancement (ϵ^*) is the result of the higher relaxation rate of the solutions containing the slowly tumbling Gd(III)L-Hb adduct:

$$\epsilon^* = R_{1p}^*/R_{1p} \quad (5)$$

where the asterisk indicates the presence of the macromolecule in solution. The enhancement factor ϵ^* is then related to the molar fraction (α) of the bound paramagnetic metal complex:

$$\epsilon^* = \alpha(\epsilon_b - 1) + 1 \quad (6)$$

where ϵ_b is the enhancement factor of the all-bound Gd(III)L-Hb form. The molar fraction (α) can be expressed in terms of the dissociation equilibrium constant (K_D) by

$$\alpha = \frac{\epsilon^* - 1}{\epsilon_b - 1} = \frac{[Hb]_f}{K_D + [Hb]_f} \quad (7)$$

The subscript f indicates the free protein. More generally, the molar fraction α depends on the total concentration of Hb, Gd(III)L, the number of binding sites, and the constant K_D. In the procedure, $[Hb]_f$ has been calculated accordingly (Aime et al., 1996a).

If the protein is able to bind the paramagnetic complex at more than one site, the K_D value will be affected by the binding stoichiometry; it is then necessary to determine independently the number of equivalent sites on the protein. To this purpose, the protein is titrated with the complex and the molar ratios obtained from the PRE data are reported under the form of a Scatchard plot:

$$\frac{r}{[Gd(III)L]_f} = \frac{n}{K_D} - \frac{r}{K_D} \quad (8)$$

where r is the molar binding ratio of the protein (i.e., complex-ligated protein over total protein), and it may easily be calculated once the all-bound enhancement ϵ_b is known:

$$r = \alpha\frac{[Gd(III)L]_T}{[Hb]_T} \quad (9)$$

$$[Gd(III)L]_f = (1 - \alpha)[Gd(III)L]_T \quad (10)$$

In the same way, the free complex concentration is obtained from the total complex concentration (Eq. 10). This gives a straight line with a negative slope of K_D^{-1} and an x axis intercept equal to n.

RESULTS

Fig. 1 reports the PRE factors ϵ^* of solutions containing 0.10 mM Gd(III)L and variable amounts of HbA_{1c} at pH 7.2

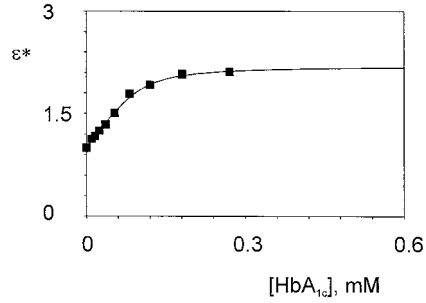


FIGURE 1 Water proton relaxation enhancement of a 0.10 mM Gd(III)L solution at different oxygenated HbA_{1c} concentration, at pH 7.2 and 25.0°C. Analysis of the data, according to the Proton Relaxation Enhancement (PRE) procedure (see Eqs. 5–7), yields a K_D value of 1.3 ± 0.1 × 10^{-5} M (ϵ_b = 2.2 ± 0.1) for Gd(III)L binding to HbA_{1c}.

in PBS and 25.0°C. The analysis of the PRE curve, according to the above theory, shows that the paramagnetic boronic probe Gd(III)L interacts with the *syn*-diol groups present in oxygenated HbA_{1c} with a K_D value of $1.3 \pm 0.1 \times 10^{-5}$ M (by assuming a 1:1 stoichiometry). The enhancement factor for this adduct (ϵ_b) is 2.2 ± 0.1, which is slightly higher than the value found for the Gd(III)L-human serum albumin adduct ($\epsilon_b = 1.5$) at the same pH and temperature (Aime et al., 1993a). It is likely that the binding mode for Gd(III)L binding to glycated human serum albumin allows larger internal motions of the paramagnetic moiety (glycation in albumin occurs on Lys side chains), which results in a decrease in the effective dipolar correlation time and, in turn, in the observed relaxivity (Lipari and Szabo, 1982).

To determine the degree of glycation in an actual HbA specimen, we needed to know the contribution to the water relaxation rate arising from the interaction between the Gd(III)L complex and oxygenated unglycated human hemoglobin (HbA_0), if any. Surprisingly, we found that Gd(III)L binds to oxygenated HbA_0 ($K_D = 1.4 \pm 0.1 \times 10^{-5}$ M for an apparent 1:1 interaction; Fig. 2) as strongly as to oxygenated HbA_{1c}. Moreover, the resulting Gd(III)L-HbA_0 adduct displays a much higher enhancement factor ($\epsilon_b = 7.1 \pm 0.2$ at 25°C). The measurements of water proton relaxation rates of solutions of Gd(III)L in the presence of different ratios of oxygenated HbA_0/HbA_{1c} showed that the interaction with sugar residues precludes the interaction with the site on the protein, suggesting that the binding site of Gd(III)L on oxygenated HbA_0 should be in the region where the glycation takes place. Indeed, the presence of the glycidic residues results in a lower enhancement of the solvent proton relaxation rate with respect to solutions containing oxygenated HbA_0 only. No relaxation enhancement takes place in the presence of monomeric hemoproteins such as horse myoglobin and horse heart cytochrome *c*. This observation supports the view that the interaction of Gd(III)L involves a site present in the tetramer only.

The addition of sorbitol to a solution of Gd(III)L and oxygenated HbA_0 causes a marked decrease in the observed relaxation rate. For a solution containing 1 mM HbA_0 and 0.1 mM Gd(III)L, R_1^{obs} decreases from 3.3 s^{-1} to 0.6 s^{-1} upon the addition of 20 mM sorbitol. This behavior may be accounted for in terms of the competition between the protein and sorbitol for the boronic functionalities on the Gd(III)L complex. Moreover, in the presence of an even larger excess of sorbitol, the measured relaxation rate is further decreased to become similar to that of the free complex ($R_1^{obs} = 0.46$ s^{-1}) indicating unambiguously that the integrity of the Gd(III)L complex is maintained.

In a related experiment it has been found that the relaxation rate of a solution of Gd(III)L and oxy-HbA_0 undergoes a drastic change between pH 8.0 and 9.5 to reach, at the basic limb, the same relaxation rate observed in the absence of oxy-HbA_0 (Fig. 3). Boronic esters are indeed known to undergo hydrolysis at basic pH values; thus the observed behavior simply reflects the release of the complex from the adduct with the protein.

The assessment of the stoichiometry of the adduct was gained by measuring the relaxation rate of solutions containing a fixed concentration of HbO_2 (0.2 mM as a tetramer) with different Gd(III)L concentrations (up to 2 equivalents). Data are conveniently analyzed in the form of a Scatchard plot according to Eq. 8. Fig. 4 clearly indicates a 2:1 stoichiometry with HbO_2; however, the slope of the straight line affords a value of K_D that does not match the one calculated from the binding isotherm, even assuming a 2:1 stoichiometry. Data may be explained by considering a two-site interaction, i.e., a high-affinity site that determines the relaxation enhancement observed in Fig. 2, and a low-affinity site whose K_D value is calculated from Fig. 4. Proton relaxation enhancement data have then been fitted using the two-site model, affording refined values for K_D of $1.0 \pm 0.1 \times 10^{-5}$ M and $4.8 \pm 0.4 \times 10^{-4}$ M, respectively. Measurements performed at a low binding ratio ($r = 0.05$–0.15) with a higher protein concentration (0.4 mM) fit to the straight line drawn using the K_D value of 1.0×10^{-5} M, as shown in the upper part of Fig. 4.

In the presence of inositol hexakisphosphate (IHP), which is known to set up a strong heterotropic interaction with the cavity where the natural allosteric effector 2,3-

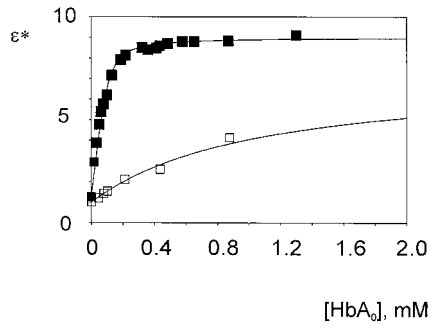


FIGURE 2 Water proton relaxation enhancement of a 0.10 mM Gd(III)L solution at different oxy-HbA_0 (■) and deoxy-HbA_0 (□) concentrations, at pH 7.2 and 25.0°C.

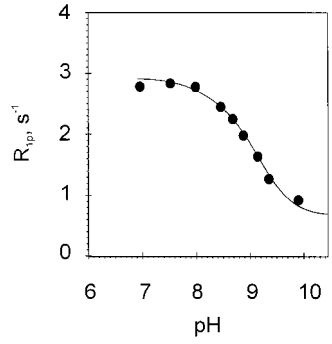


FIGURE 3 Effect of the pH on the longitudinal relaxation rates (R_{1p}) of a solution containing 1.0 mM oxy-HbA_0 and 0.1 mM Gd(III)L. Data were obtained at 20 MHz and 25.0°C.

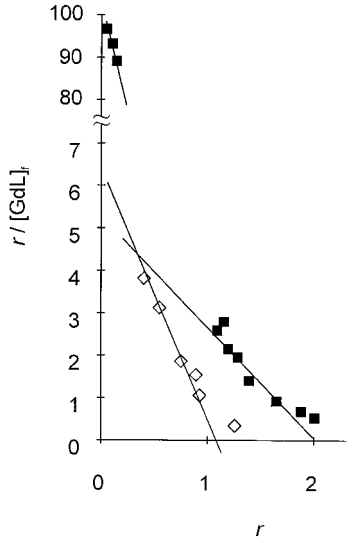


FIGURE 4 Scatchard plots for the binding interaction of Gd(III)L to oxy-HbA_0 (■) and deoxy-HbA_0 (◇). Protein concentration is 0.4 mM for the low binding ratio measurements on oxy-HbA_0 (*top of the graph*) and 0.2 mM for all other measurements.

diphosphoglycerate (DPG) binds to the tetramer, PRE data are markedly affected. Fig. 5 shows a comparison between Scatchard plots in the absence and in the presence of a threefold excess of IHP. In the latter case, the slope indicates a large K_D over the whole r range, and the x axis intercept cannot be determined accurately.

A titration of the Gd(III)L solution with the deoxygenated HbA_0 derivative (Fig. 2) was carried out at 20 MHz and 25.0°C. The analysis of the binding isotherm, using the same asymptotic enhancement factor found for the Gd(III)L-HbA_0 adduct and assuming a 1:1 stoichiometry, gives a K_D value of $2.1 \pm 0.2 \times 10^{-4}$ M for the interaction of the paramagnetic adduct with deoxy-HbA_0. The stoichiometry is confirmed by the Scatchard plot reported in Fig. 4. The observed behavior indicates, therefore, that Gd(III)L promotes a marked stabilization of the high-affinity versus the low-affinity state of the tetramer. The stabilization of the high-affinity form is further confirmed by the measurement of R_{1p} upon the addition of ferric (aquo-met) HbA to the Gd(III)L solution. This Hb derivative shows an *R*-like conformation (Fermi and Perutz, 1981), and the measured relaxation enhancement curve (not shown) is similar to that of oxy-HbA_0 ($K_D = 4.3 \pm 0.4 \times 10^{-5}$ M, assuming $n = 1$, $\epsilon_b = 7.1 \pm 0.4$).

According to the linked functions (Wyman, 1964; Antonini and Brunori, 1971), the oxygen affinity for HbA_0, in the absence of Gd(III)L ($P_{50} = 8 \pm 1$ mm Hg; $n = 2.8 \pm 0.2$), is lower than that observed for the tetramer in the presence of 1.0×10^{-4} M Gd(III)L ($P_{50} = 1.0 \pm 0.3$ mm Hg; $n = 1.6 \pm 0.2$) at pH 7.2 (PBS) and 25.0°C (Fig. 6).

The effect of Gd(III)L on the stabilization of the R state of human hemoglobin is also supported by the measurement of a 9% enhancement of the absorption spectra, in the Soret region, of deoxy HbA_o in the absence and presence of Gd(III)L, at pH 7.2 (Fig. 7). The maximum at 430 nm (133 mM^{-1} cm^{-1}) has been associated with the low-affinity T state of the unligated tetramer (Antonini and Brunori, 1971). The addition of Gd(III)L to deoxy HbA_o in the absence of atmospheric oxygen induces the transition to a species characterized by a higher molar absorbance (145 mM^{-1} cm^{-1}). Such behavior has been attributed to the shift of the conformational equilibrium toward the R state with a consequent strengthening of the proximal HisF8-Fe bond (Antonini and Brunori, 1971).

Another proof of the switch of the quaternary conformation of the tetramer from the low-affinity to the high-affinity form (i.e., from the T to the R state) comes from the observation of the exchangeable ^1H resonances of amino acid residues located at the subunit interfaces of the Hb tetramer (Russu et al., 1987). These residues are involved in the hydrogen-bonding interactions that stabilize the quaternary structure, and their resonances are absent in the spectra of isolated chains. On going from deoxy- to oxy-Hb, some of these signals show significant variation in their chemical shift, and two resonances almost disappear. These reso-

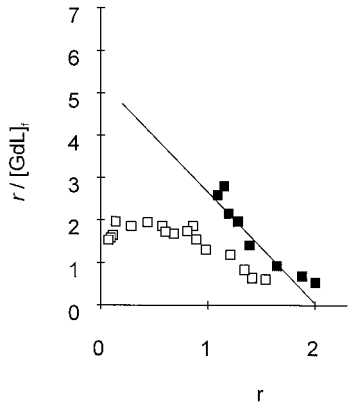


FIGURE 5 Scatchard plots for the binding interaction of Gd(III)L to 0.2 mM oxy-HbA_0 in the absence (■) and in the presence (□) of 0.6 mM inositol hexakisphosphate.

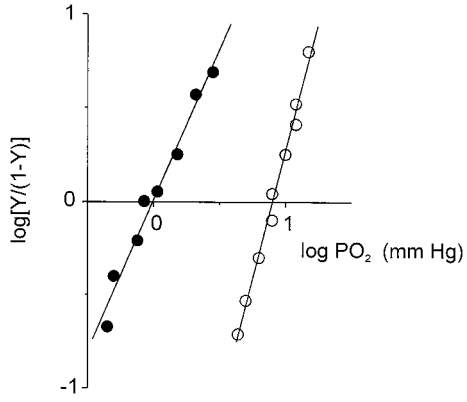


FIGURE 6 Hill plot for oxygen binding to HbA_0 in the absence (○) and in the presence (●) of 1.0×10^{-4} M Gd(III)L at pH 7.2 and 25.0°C. Values of P_{50} and n are given in the text.

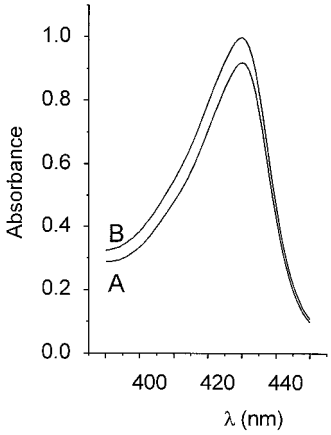


FIGURE 7 Absorption spectra, in the Soret region, of deoxygenated HbA_0 in the absence (*trace A*) and in the presence (*trace B*) of 4.0×10^{-3} M Gd(III)L. Spectra were recorded at pH 7.2 and 25°C, using a 1-cm path length cuvette fitted with a two-hole screw cap for nitrogen flushing. HbA_0 concentration was 1.73×10^{-6} M (expressed as tetramer).

nances are due to the intersubunit hydrogen bond between $Tyr^{42}(\alpha_1)$ and $Asp^{99}(\beta_2)$ (14.1 ppm) and to the intrachain hydrogen bond between $Tyr^{145}(\beta)$ and the $Val^{98}(\beta)$ carbonyl (11.1 ppm), an important feature in the deoxy tertiary structure.

Fig. 8 shows the ^{1}H-NMR spectra of deoxy-HbA_0 in the absence (*trace A*) and in the presence (*trace B*) of the diamagnetic La(III)L. Trace C reports the spectral changes upon the addition of La(III)L and IHP in a 1:1 ratio. The disappearance of the signal at 14.1 ppm in trace B is clear-cut evidence of the effect of La(III)L on the allosteric equilibrium, resulting in the cleavage of the interdimer hydrogen bond responsible for the stabilization of the T state. This does not mean that an R state is reached; rather the interchain interactions that keep the oxygen affinity low are weakened, yielding a conformational state that resembles the R state in the quaternary structure, conserving, however, the features of a T state in the tertiary structures of the globin chains (the shoulder at 11.1 ppm in all three traces indicates that the globin conformation is that of the unligated, deoxy form). Eventually, the simultaneous presence of La(III)L and IHP cancels this effect (*trace C*) and restores the spectral pattern shown in trace A.

Under all of the experimental conditions, the possible Ln(III)L-induced dissociation of HbA_0 (even at the highest Gd(III)L concentration used, 1.0×10^{-3} M) has been ruled out on the basis of the values found for the sedimentation coefficient (4.1 ± 0.1) for the oxygenated, deoxygenated, and ferric (aquo-met) derivatives of Hb, obtained in the absence and in the presence of the metal complex. Values of the sedimentation coefficient are indeed typical of the tetrameric state (Antonini and Brunori, 1971).

To better understand which amino acid residues could be involved in this heterotropic interaction, we considered the high-resolution ^{11}B NMR spectra of the corresponding diamagnetic La(III)L complex (^{11}B natural abundance = 81.17%, $I = 3/2$). La(III)L at pH 7.4 shows a ^{11}B resonance at 30.7 ppm ($W_{1/2} \approx 970$ Hz), which moves upfield to $\delta = 7$ ppm ($W_{1/2} \approx 2300$ Hz) in the presence of a fivefold excess of oxy-HbA_0. Upon recording the ^{11}B-NMR spectrum of La(III)L in the presence of amino acids like Arg, His, Lys, or Ser, all potentially capable of forming a dative bond to boron, we noted that, at pH 7.4, a marked upfield shift is observed only in the presence of His (Fig. 9). The resulting chemical shift ($\delta = 12$ ppm when the His-to-La(III)L ratio is 30:1) is close to the corresponding value found for the adduct with oxy-HbA_0. On the other hand, in the presence of Arg, Lys, and Ser, the ^{11}B chemical shift approaches the value of the La(III)L-oxy-HbA_0 adduct only at pH > 9.

To gain more insight into the possible involvement of histidines in the Gd(III)L binding to hemoglobin, we investigated the interaction of Gd(III)L with fetal hemoglobin (HbF). In the quaternary structure of HbF, β-chains are replaced by γ-chains, where Ser is substituted for $His^{143\beta}$ (Antonini and Brunori, 1971). The binding isotherms for

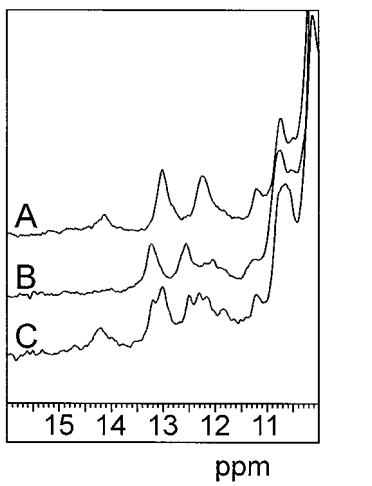


FIGURE 8 ^{1}H-NMR (400 MHz) spectra of 0.5 mM deoxygenated HbA_0 in 10%-deuterated PBS. *Trace A*: Resonances of exchangeable protons at the intersubunit interface (assignments are given in the text). *Trace B*: After the addition of 10 mM La(III)L. *Trace C:* After the addition of 10 mM La(III)L and 10 mM IHP.

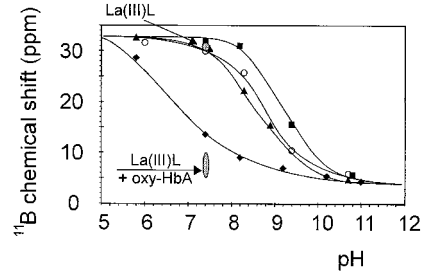


FIGURE 9 Effect of pH on the ^{11}B chemical shift (measured at 128 MHz and 25.0°C) of 10.0 mM La(III)L interacting with Lewis-basic amino acids (0.1 M) (♦, His; ○, Ser; ▲, Lys; ■, Arg). Shaded areas indicate the chemical shifts of La(III)L complex in neat water and of the oxy-HbA_0-La(III)L adduct.

Gd(III)L binding to oxy- and deoxy-HbF are reported in Fig. 10. It is noticeable that the mutation $His^{143}Ser$ in the DPG cavity implies a decrease in the asymptotic value observed for oxy-HbF ($\epsilon_b = 5.1 \pm 0.3$ versus 7.1 ± 0.2 for oxy-HbA_0). A Scatchard plot has been drawn (not shown) to determine the stoichiometry of the binding and the interaction strength of the second molecule of Gd(III) binding to the Hb tetramer ($K_D = 5.4 \pm 0.7 \times 10^{-4}$ M). By the use of the two-site model described above, binding parameters for the first molecule have been determined ($K_D = 1.3 \pm 0.2 \times 10^{-4}$ M, $\epsilon_b = 5.1 \pm 0.3$). Gd(III)L binds to deoxy HbF with an even higher dissociation constant (Fig. 10; $K_D = 1.3 \pm 0.5 \times 10^{-3}$ M, $\epsilon_b = 5 \pm 1$).

DISCUSSION

The complex Gd(III)L binds with very similar affinity to human adult glycated and unglycated hemoglobins; however, the binding to HbA_0 causes a sevenfold enhancement of the solvent water proton relaxation rate, whereas in the presence of HbA_{1c} the enhancement is equal to 2.2. Thus there are two different binding modes with very similar strengths. Results obtained for mixtures of HbA_0 and HbA_{1c} show that the glycation of Val^1 residues of β-chains precludes the kind of interaction that is observed when such N-terminal residues are unligated. This observation suggests that sugar residues in HbA_{1c} act as gates over the interaction site, thus providing an insight into the mode of interaction of Gd(III)L on HbA_0. In fact, the ends of β-chains where glycation takes place mark the boundaries of the site of interaction for DPG, the well-known allosteric effector that shifts the equilibrium toward the T state by binding at the central cavity between the two β-chains on the twofold symmetry axis of the tetramer (Fermi and Perutz; 1981). IHP binds much more strongly than DPG to Hb at the same cleft (Fermi and Perutz, 1981).

The body of experimental results—values of the dissociation constants, the measurement of P_{50} in the presence of Gd(III)L (Fig. 6), changes in the Soret band (Fig. 7) and in the ^{1}H-NMR spectra (Fig. 8)—clearly show that the complex acts as an allosteric effector by stabilizing the high-affinity form of HbA. The linked functions analysis (Eq. 11) yields a P_{50} value in good agreement with that found experimentally (Scheme 1):

$$\log P_{50} = \log P_{50}^{o} + 0.25 \log \frac{1 + K_{Hb}[L]}{1 + K_{HbO_2}[L]} \qquad (11)$$

These complexes have then to be included in the class of allosteric effectors that increase the oxygen affinity of hemoglobin (Abraham et al., 1995; Boyiri et al., 1995), although their mode of action should be rather different from that reported for bifunctional molecules able to link α-chains in a manner that constrains the tetramer in the R state. One can envisage that IHP shifts the allosteric equilibrium to the form that binds Gd(III)L less strongly, thus acting in the same way as oxygen removal. Scatchard plots reported here clearly show that IHP really competes with Gd(III)L. Indeed, T-state (deoxy) HbA possess one site with a K_D value of $2.1 \pm 0.2 \times 10^{-4}$ M, whereas IHP-saturated HbA displays a much larger dissociation of the Gd(III)L-HbA_0 adduct. Thus Gd(III)L binding occurs in the same region of DPG and IHP, although a quite different bonding mode has to be adopted. The binding of Gd(III)L to HbA_0 may be envisaged to occur through the coordination of donor atoms of the protein to the Lewis acid boron centers of the complex. The cleavage of the adduct occurs either by the addition of sorbitol or by increasing pH. On the basis of the results obtained by means of ^{11}B-NMR spectroscopy (Fig. 9), the anchoring sites for Gd(III)L on HbA_0 deal with histidine residues. (A number of attempts to prepare crystals of the Ln(III)L-HbA_0 adducts failed. Indeed, crystals of oxygenated HbA_0 cracked when they were soaked in a Gd(III)L solution.) Actually, four His residues (His^2 and His^{143} of both β-chains), together with Lys^{82} of both β-chains, define the binding site of DPG.

On going from the low-affinity to the high-affinity state of Hb, the DPG cavity varies between an "open" and a "closed" conformation (Fermi and Perutz, 1981). Clearly, the stronger interaction shown by Ln(III)L complexes with HbA_0 in the R state suggests that it matches the "closed" form better. In this conformation, $His^{2\beta}$ and $His^{143\beta}$ residues are no longer orientated toward the inside of the cavity, as they are in the T conformation (Perutz et al., 1986). As the HbA_0 tetramer undergoes a rotation and translation of the $\alpha_1\beta_1$ dimer relative to the $\alpha_2\beta_2$ dimer upon ligand binding (Fermi and Perutz, 1981), $His^{2\beta}$ and $His^{143\beta}$ resi-

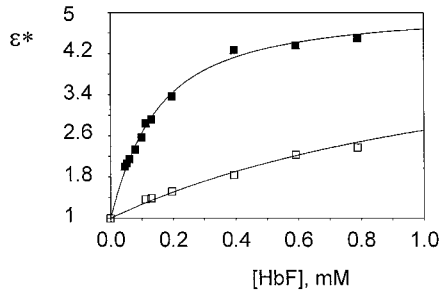
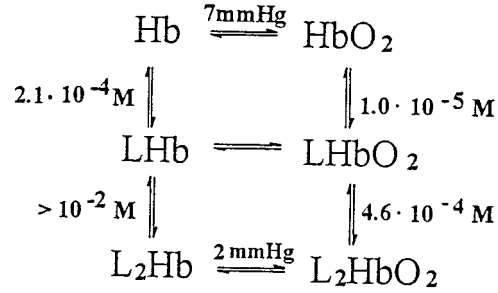
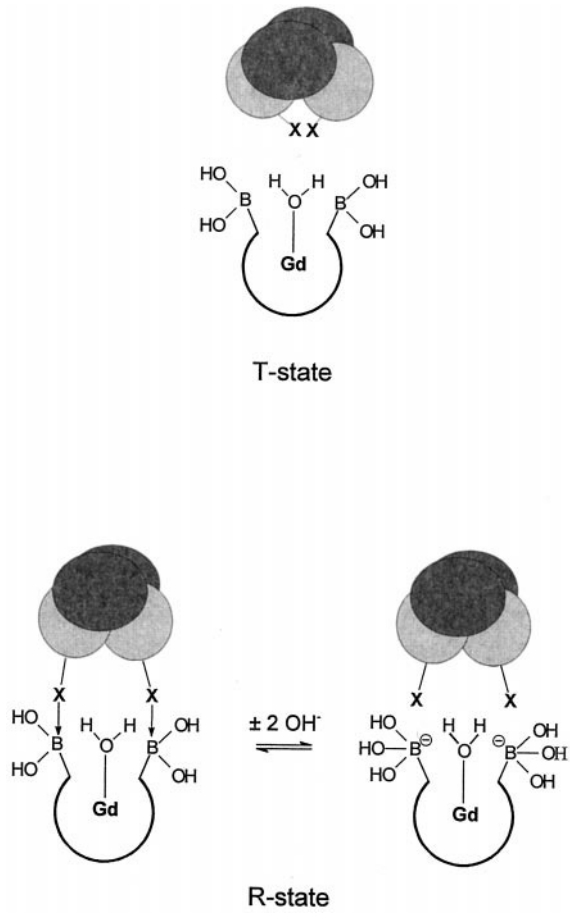


FIGURE 10 Water proton relaxation enhancement of a 0.10 mM Gd(III)L solution at different oxy-HbF (■) and deoxy-HbF (□) concentrations, at pH 7.2 and 25.0°C.

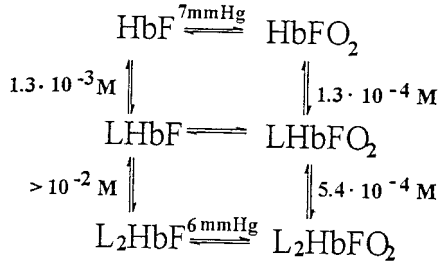


SCHEME 1

dues move outward, becoming more suitable for binding to the acceptor boron atoms provided by the Ln(III)L complex. From the x-ray crystal structure of the oxygenated HbA (Shaanan, 1983; PDB code = 1HHO), it is possible to determine the relative N-N distances inside the four pairs of donor atoms formed by $His^{2}(\beta_1)$, $His^{2}(\beta_2)$, $His^{143}(\beta_1)$, and $His^{143}(\beta_2)$ residues in the DPG pocket. An interchain linking is necessary to justify the higher affinity for the R state, as reported for the other class of effectors (Abraham et al., 1995; Boyiri et al., 1995). Taking into account the steric demand of Ln(III)L complexes and the different isomeric structures they can adopt in water solutions (Aime et al., 1995a), a potential binding site may be recognized in the $His^{2}(\beta_1)$-$His^{143}(\beta_2)$ pair (and in the symmetry-equivalent $His^{2}(\beta_2)$-$His^{143}(\beta_1)$ pair) (Scheme 2). Indeed, in each of these pairs the interresidue N-N distance may become as short as 10 Å, which fits quite well the boron-boron distance (~10 Å) evaluated with a model of Gd(III)L obtained by using the structural data from the x-ray diffraction study of the closely related bis(benzylamide)DTPA Gd(III) complex (Aime et al., 1997). On this basis one may conclude that the first molecule of Gd(III)L links the globin chains in the R state by binding to one of the two (equivalent) His pairs at the $\beta\beta$ interface. The second molecule is still able to recognize the linking site at the DPG cavity rim, but with a lower affinity. Preliminary results on a related monoboronic complex show a negligible interaction with HbA_0, thus underlining the structural requirement of this bifunctional coordination compound.



SCHEME 2



SCHEME 3

On comparing the interaction of Gd(III)L with HbA_0 and HbF, the lack of $His^{143\beta}$ appears to be responsible for both a weaker interaction and a smaller relaxation enhancement. Asymptotic values (i.e., ϵ_b in Eq. 6) are determined by a number of factors, which are, in turn, dependent on the structural determinants of the binding mode itself. The observation of a smaller ϵ_b in the case of the adduct with fetal hemoglobin reflects a different structural arrangement of the complex on the protein surface. The presence of $Ser^{143\beta}$ might, of course, provide an alternative mode for boronic acid coordination, as reported in the case of serine enzymes, where Ser residues do form boronate esters (Aime et al., 1996b).

Analysis of the dissociation constants of Gd(III)L with HbF according to Eq. 11 provides an estimation of a decrease in the P_{50} of ~1 mmHg (Scheme 3).

As a general remark, the results reported in this paper show that metal complexes may provide a novel class of allosteric effectors, thanks to their peculiar stereochemical properties. Moreover, the spectroscopic properties of metal complexes may be exploited to probe conformational variations of the protein structure and in turn act as reporters of the physicochemical determinants of the protein conformations. A further peculiar feature of this class of compounds is the fact that the binding isotherms are more easily obtained by looking directly at the spectroscopic properties of the effector itself rather than at the spectroscopic or functional changes in the protein induced by the allosteric effector.

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