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Structural Nonequivalence of the α- and β- Heme-Pockets in Human Methemoglobin

A Proton Magnetic Relaxation Study in Solution[1]

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Solvent-proton longitudinal magnetic relaxation rates as dependent on temperature were measured for human (H)/canine (C) valency hybrids of the type $\{\alpha^{H(III)}\beta^{C(II)}\}_2$ and $\{\alpha^{C(II)}\beta^{H(III)}\}_2$. The two metheme irons in the human methemoglobin chains induce quite different proton magnetic relaxation (pmr) rates reflecting a tighter β-heme-pocket compared to the α subunit. Both heme-pockets appear to be loosened in the presence of inositol hexaphosphate (IHP) although this allosteric effector binds only to the β chains, the binding assumed to be the same for canine as for human hemoglobin. The subunit nonequivalence is retained also in the T-quaternary state induced by IHP. In the species hybrids the pmr rates due to the metheme iron are sensitive to the valency (ligand) state, which was either CO or H_2O in the partner half of the hybrid. All results show very clearly the interrelationship of the tertiary (protomer) structure with the quaternary (oligomer) structure in hemoglobin.

The functional nonequivalence of the α and β hemoglobin subunits is of importance in the elucidation of the mechanism of cooperative oxygenation of hemoglobin. Asakura and Tamura (1) have brought this point to the forefront once again by presenting evidence that the two subunits behave differently because their heme-pockets are not structurally equivalent. The method they used was the electron spin resonance of heme-spin-labeled human hemoglobin. The other most convincing evidence in this respect is perhaps that obtained by Lindstrom and Ho (2) who delineated the different courses in oxygenation of α and β subunits in the native hemoglobin molecule by following the changes in its proton magnetic resonance high resolution spectra of the lines shifted by hyperfine contacts with the electron spins of the heme irons. Earlier data bearing on the α–β nonequivalence have been reviewed elsewhere (3).

In a proton magnetic relaxation (pmr) study of the interaction of inositol hexaphosphate (IHP)[4] with human methemoglo-

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[4] Abbreviations used: α^H, α^C, the α chains of human and canine hemoglobin, respectively; β^H, β^C, the β chains of human and canine hemoglobin, respectively; CM, carboxymethyl; DEAE, diethylaminoethyl; Hb, hemoglobin; IHP, inositol hexaphosphate; Mb, myoglobin.

bin (4), two questions related to the α and β hemoglobin subunits were posed: (a) Are the molar relaxation rates of α and β chains different? (b) Is the observed IHP effect in the relaxation rates shared by both types of subunits or is it confined to the β chains which only bind IHP?

Although the first question could possibly be answered positively in view of the already available evidence (1–3), it is nevertheless of such a general interest for the interpretation of other pmr data that we felt it necessary to check and quantitate this point by the same method, i.e., by pmr. The second question is of equal importance, especially in studies of allosteric effects, and the present paper deals with it, as well. Our approach was, for technical reasons, to use human/canine valency-species hybrids, though the pure human valency hybrids would be even more appropriate.

MATERIALS AND METHODS

Human and canine blood were obtained by venipuncture in Vacutainers with EDTA as anticoagulant or in tubes containing heparin. Plasma was removed by centrifugation and the red blood cells washed three times with physiologic saline. Hemolysates were prepared by mixing one volume of washed packed red cells, two volumes of distilled water and 0.4 volume of CCl_4 for 5 min. Cellular debris was removed by centrifugation at 5000 rpm for 20 min. The clear hemolysates were exposed for 10–20 min to a CO atmosphere.

The hemolysates were chromatographed on preparative columns (3.0 × 60 cm) of DEAE-Sephadex (5, 6). The isolated human HbA_0 was treated with an excess of $K_3Fe(CN)_6$ (one volume of 5% Hb solution with 0.1 volume of 2% $K_3Fe(CN)_6$) for 30 min. The excess of $K_3Fe(CN)_6$ was removed by chromatography on columns of Sephadex G-25 with a 0.005 M Tris-HCl developer, pH 8.5.

Hybridization of human with canine hemoglobin was performed following the procedure described by Itano and Robinson (7) with a slight modification described earlier (8). Equal amounts of human and canine HbA_0 were mixed and dialyzed against 0.1 M acetate buffer, pH 4.7, for 7 h at 4°C and for an additional 12 h against 0.05 M Tris buffer, pH 10.5. Separation and isolation of hybrids was achieved by column chromatography on DEAE-Sephadex with a 0.05 M Tris–HCl developer, pH 8.2, 8.0, 7.8.

Hemoglobin solutions were concentrated on small columns of CM-Sephadex equilibrated with 0.05 M Tris–maleic acid, pH 6.5. Further concentration was achieved by ultrafiltration at 4°C under reduced pressure with concurrent dialysis against distilled water. The purity of the Hb fractions was checked by starch-gel electrophoresis in Tris/EDTA/boric acid buffer, pH 9.0 (9).

These samples were transferred overnight, in ice, for final pmr measurements which were done at pH 6 in order to enable comparison with earlier data (4, 10). The final dialysis was therefore against 0.1 M NaCl, 0.05 M Tris, 5×10^{-4} M EDTA, pH 6, with concomitant pressure application to increase the concentrations when it was necessary. The actual pmr measurements were performed with solutions in the range 1.3–3.5 mM per heme.

The concentrations were determined spectrophotometrically, using the millimolar extinction coefficient $\epsilon_{1\ cm} = 4.0$ at 630 nm for methemoglobin from spectra of the valency hybrid solutions, assuming zero absorbance of the CO component at 630 nm, and $\epsilon_{1\ cm} = 11.0$ at 540 nm for the total heme concentration, obtained by the cyanmet method.

The longitudinal proton magnetic relaxation time (T_1) measurements were performed with the same apparatus and in the same manner as described elsewhere (10).

RESULTS

The "conformational probe" in our experiments is the magnetic-dipole couple between the (spins of) solvent protons and the *paramagnetic* (Fe^{III}) heme iron in either the α or the β chain of *human* hemoglobin. The *canine* half of our hybrid $\{\alpha\beta\}_2$ molecule was in the *diamagnetic*, CO-liganded state, and thus ineffective in the paramagnetic relaxation rates (but see later for the corresponding corrections). The controls were the measurements of the fully oxidized hybrid methemoglobin, $\{\alpha^H\beta^C\}_2^{(III)}$, and of the native human methemoglobin from our earlier work (4). The chromatographic separation of hybrid hemoglobins on a DEAE-Sephadex column is shown in Fig. 1.

All the data in Figs. 2–4 were normalized per *metheme* concentration, with the diamagnetic contribution to the relaxation rates (4, 10) subtracted on the basis of the *total subunit* concentration. The (logarithmic) ordinates in these figures are therefore the molar relaxation rates induced solely by the *paramagnetic metheme iron*.

Curves I/a and I/b in Fig. 2 represent the (reciprocal) temperature dependence of the relaxation rates for the $\{\alpha^{C(II)}\beta^{H(III)}\}_2$ hybrid.

The ordinates are the directly measured molar values due to the human β chain metheme, because the metheme content in both preparations, I/a and I/b, was *less than 50%*, namely 33 and 40%, respectively. Curve II in this figure was obtained with preparation I/b to which IHP was added in a 1:1 molar (per heme) ratio.

In Fig. 3 the results are due, in turn, to the metheme iron in the human α chain. As the I/a preparation contained 45% of metheme α^H, the relaxation rates were obtained from direct measurements. Preparation I/b was 77% in metheme, so that the measured relaxation rates were corrected for the β^C metheme contribution taken from Fig. 2. Curve II in this figure was obtained with the latter preparation after addition of IHP and correcting for the β^C methaem content using the values from Fig. 2, Curve II.

Finally, Fig. 4 comprises data for the control measurements of the fully oxidized hybrid $\{\alpha^H\beta^C\}_2^{III}$ in the absence and in the presence of IHP. The other curves in this figure are either replotted from earlier data, or reconstructed from Figs. 2 and 3, as explained in the Discussion.

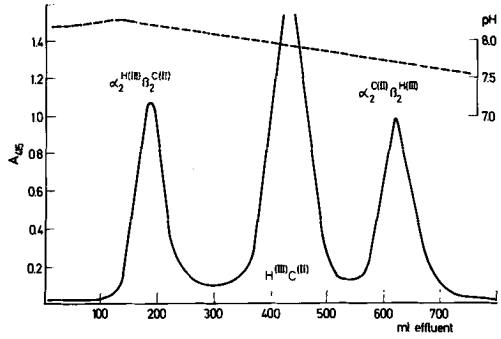

FIG. 1. Chromatographic separation of hybrid hemoglobins on a DEAE-Sephadex column.

DISCUSSION

Although not exactly identical functionally, the hybrids $\{\alpha^H\beta^C\}_2$ and $\{\alpha^C\beta^H\}_2$ were found to be quite similar, in their oxygenation behaviors, to the native human hemoglobin $\{\alpha\beta\}_2^H$ (11, 12). Figure 4, on the other hand, reveals that the data for the fully oxidized hybrid $\{\alpha^H\beta^C\}_2^{III}$ either without or in the presence of IHP (see the two sets of experimental points, circles) are quite close to the corresponding data for the native human methemoglobin from Ref. (4), replotted as dotted lines in Fig. 4. On the contrary, this temperature dependence for human methemoglobin reconstructed from the corresponding subunit data obtained

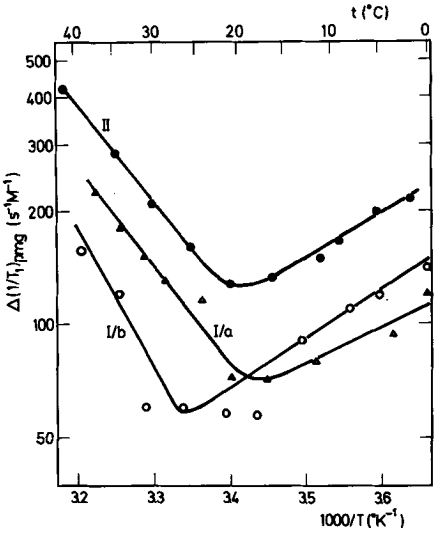

FIG. 2. The temperature dependence of the solvent-proton longitudinal magnetic relaxation rates due to the metheme iron in the human β chains of canine/human hybrid hemoglobin. I/a and I/b, original samples; II, I/b + inositolhexaphosphate.

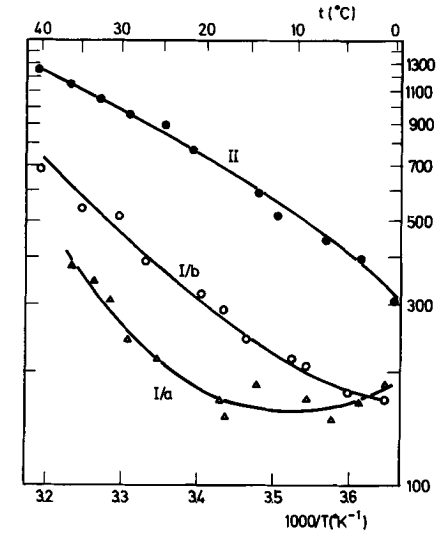

FIG. 3. The temperature dependence of the solvent-proton longitudinal magnetic relaxation rates due to the metheme iron in the human α chains of canine/human hybrid hemoglobin. I/a and I/b, original samples; II, I/b + inositolhexaphosphate.

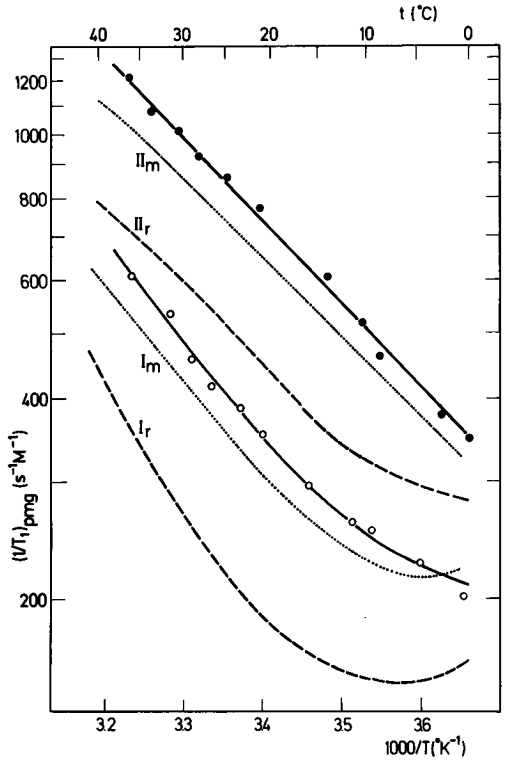

FIG. 4. The temperature dependence of the solvent-proton longitudinal magnetic relaxation rates due to the metheme iron in the fully oxidized species hybrid hemoglobins or in the native human methemoglobin: (I), (○——○), $\{\alpha^{H}\beta^{C}\}_2^{III}$-measured; (II) (●——●), $\{\alpha^{H}\beta^{C}\}_2^{III}$ + inositolhexaphosphate (IHP)-measured. (····), I_m, native human methemoglobin, and II_m, I_m + IHP. (-----), Reconstructed $\{\alpha^{H}\beta^{C}\}_2^{III}$ from Figs. 2 and 3; I_r, without IHP; II_r, with IHP.

with the valency-species hybrids (Figs. 2 and 3), plotted as dashed lines in Fig. 3, differ from both the original methemoglobin and from the fully oxidized hybrid. Hence, we come to the conclusion that it is not the species characteristics of the protein subunits that causes this difference in pmr rates but, rather, the valence state of the iron ion in the partner subunits of the hybrid tetramer. In other words, the tertiary structure of one valence pair influences the structure of the second through the quaternary level of the oligomer.

This implies differences in the metheme-environment conformation depending on whether the other half of the hybrid is in the CO-liganded (diamagnetic, divalent) state or in the oxidized one. Judging by the results in Fig. 4, the CO-liganded state of one-half of the hybrid tends to induce a more closed metheme-environment in its partner subunits. This analysis shows that it is reasonable to draw conclusions about the posed questions, from these hybrid measurements, in order to relate them cautiously to the native human hemoglobin.

The structural specificity in the heme environment of the two (α and β) chains of human methemoglobin is evident from the comparison of Curves I/a,b in Fig. 2 with I/a,b in Fig. 3. Owing to the different met/CO composition in preparations a and b and in the light of the preceding analysis on the structural influence of the valence states of each partner upon the other, one would not expect an exact identity of the data within each I/a,b-pair. At the same time it should be pointed out that *the type* of the temperature dependence and *the range* of the pmr rates in Fig. 2 differ significantly from those in Fig. 3.

For instance, all the points for the $\{\alpha^{C(II)}\beta^{H(III)}\}_2$ hybrid (Fig. 2, I/a,b) at higher temperatures lie below some 200 s^{-1} M^{-1}. At these temperatures the pmr rates reflect the rate of exchange of (water) protons between the heme-pocket and bulk solvent (10). The energies of activation for this molecular mechanism, as derived from Figs. 2 and 3, are 13 kcal M^{-1} for the $\beta^{H(III)}$ chain, and 8 kcal M^{-1} for the $\alpha^{H(III)}$ chain. This difference is obvious, too, from the temperatures at which the exchange mechanism becomes significant: ~25°C for β chains and 0°C for α chains. The ratios of these rates, R_α/R_β, calculated at 30 and 40°C, are roughly 10 and 4 respectively. A lower exchange rate with a larger E_a for $\beta^{H(III)}$ implies a "tighter" heme-pocket, assessed by the size of the fluctuating H_2O molecule(s), than that of the $\alpha^{H(III)}$ chain, in agreement with the X-ray crystal structure analysis of Perutz and co-workers (13). This is yet another independent line of evidence on the similarity between the protein structure in the crystal and in solution.

Apart from this dynamic aspect of the heme-pocket structure, the low-temperature region of Figs. 2 and 3 (I/a,b), i.e. the so-called outer-sphere relaxation (10),

which reflects the overall accessibility of the heme-pocket mouthpiece for the solvent protons, is much greater in the α than in the β chain, a conclusion in line with the former one.

We now turn to the analysis of the allosteric effects induced by IHP binding to the hybrid hemoglobins. Figure 4 confirms convincingly that IHP also binds to the species hybrid methemoglobin $\{\alpha^{H}\beta^{C}\}_2^{(III)}$ bearing the *canine* β chains. The induced increase in the pmr rates (see the two sets of experimental points, circles) within the higher-temperature region is about twofold, just as it was observed with native human methemoglobin (4) (see the two dotted lines).

The chain specificities of this allosteric effect may be derived by comparison of Curves II in Figs. 2 and 3, with the corresponding Curves I/b. In both cases, $\alpha^{H(III)}$ and $\beta^{H(III)}$, the addition of IHP induces a pmr rate-enhancement indicative of a loosening of the corresponding heme-pockets, as discussed in Ref. (4) in detail.

It is not easy to quantitate, from these pmr data and in structural terms, just how large the IHP effect is for each of the two subunits. It is quite certain, however, that the pmr enhancement for the α chain is at least as pronounced as, if not even more than, it is for the β chain. (We assume that the IHP binding-site for the $\{\alpha^{H\cdot III}\beta^{C(III)}\}_2$ hybrid is similar to that determined for native human hemoglobin (14).) This is a direct demonstration of a powerful molecular mechanism across the subunit interfaces: A change in the quaternary structure induced by IHP binding (15) results in alterations of subunit tertiary structure tens of angstroms away from the binding site, as evidenced by pmr enhancement at the α-heme-pocket mouthpiece, i.e., in subunits which do not bind IHP.

Notwithstanding several quantitative details yet to be solved, two main conclusions emerge from these studies: (a) The pmr data obtained with solutions also show the structural nonequivalence of the heme-pockets in the α and β subunits of human methemoglobin in that the former one is a much more open (and more flexible) structure; (b) the allosteric effector (IHP) causes a loosening of the heme-pockets in both types of subunits although it binds only to the one of them, but their nonequivalence is maintained also in the quaternary T-state on binding IHP.

This nonequivalence in pmr behavior of the two subunits bears importance for the interpretation of pmr data obtained with oligomeric protein molecules when the structural equivalence of the paramagnetic metal ion environment in the protomers cannot be proven a priori. A case in point is the comparison of the Mb and Hb data in Ref. (16) where a mean relaxation rate for both Hb subunits was measured assuming tacitly that they do not differ substantially. In the light of the present results, the conclusions drawn in Ref. (16) regarding different stereochemistry within the heme-pocket(s) of Mb and Hb are meaningless. However, the present study does confirm that the overall spatial characteristics of the heme-pocket in myoglobin differ from those in the α and β chains of hemoglobin, although the type of the temperature dependence of pmr for the α chain is more like that for myoglobin. The final check of the position of the "next-neighbor" water molecule which was suggested to be different for myoglobin and hemoglobin (16) could, in principle, be obtained by measurements at very low Larmor frequencies at which one would expect the fast-exchange condition to be fulfilled.

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