Xenopus laevis Hemoglobin and Its Hybrids with Hemoglobin A†

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ABSTRACT: Isolated α and β chains from *Xenopus laevis* hemoglobin have been purified. The isolation procedure yields native α chains whose functional behavior has been characterized and compared with that of human α chains. Isolated β chains in the presence of oxygen are characterized by low stability, and hence their functional characterization was limited to the CO binding kinetics. When stoichiometric amounts of the isolated α and β chains are mixed, a tetramer characterized by heme–heme interactions and oxygen affinity comparable to that of the native molecule is readily reconstituted. Moreover, both chains, under appropriate conditions, form stable hybrid tetramers with the partner subunits from human hemoglobin; results on the functional properties of these hybrid hemoglobins are presented and discussed in relation to the stereochemical model of the Root effect.

The role of intersubunit contacts in regulating the functional properties of human hemoglobin has been emphasized by the stereochemical model proposed by Perutz on the basis of crystallographic studies (Perutz, 1970; Baldwin, 1980); such a model has often provided a convincing explanation of the properties of abnormal human hemoglobins that show significant functional alterations related to single amino acid replacements at the subunit interfaces (Valdes & Ackers, 1977; Parkhurst, 1977).

Perturbation of intersubunit contacts may be artificially produced by assembling purified α and β chains from different hemoglobins into hybrid tetramers; hence the functional characterization of hybrid hemoglobins may give additional information on the role played by each subunit in determining the functional behavior of both native and hybrid hemoglobins, as shown for the first time by Antonini et al. (1965), employing dog–human hybrids.

Furthermore, much of the experimental work on the comparative aspects of oxygen transport, carried out by us in the past few years, has been directed toward the understanding of the structural basis of the Root effect, i.e., the reduction of blood oxygen capacity at low pH, first observed by R. Root for fish blood (Root, 1931) and subsequently observed in fish and some amphibian hemoglobins (Brunori, 1975; Condò et al., 1981; Perutz & Brunori, 1982).

This extreme form of Bohr effect, related to the extreme stabilization of the low O_2 affinity, deoxyhemoglobin conformation (T state), leads to reduction of oxygen affinity and absence of cooperativity; the functional properties of Root effect hemoglobin have been characterized in the 1970s both from an equilibrium (Binotti et al., 1971; Brunori et al., 1973) and a kinetic point of view (Noble et al., 1970; Giardina et al., 1973).

The structural basis of the Root effect has been correlated to the replacement of the β (F9) cysteine-93 (normally found in mammalian hemoglobins) by a serine (as often occurs in fish and amphibian hemoglobins; Perutz & Brunori, 1982).

Following this interpretation it would be of great interest to prepare and characterize hybrid tetramers assembled from complementary chains obtained from a Root effect hemoglobin and hemoglobin A (HbA); equilibrium and kinetics properties of such hybrids were first reported in detail for carp–human hybrids (Causgrove et al., 1984; Parkhurst & Goss, 1984; Goss & Parkhurst, 1984).

It may be recalled that, apart from the well-characterized chains of human hemoglobin, only preliminary results have been published on the properties of α and β chains from carp hemoglobin (Parkhurst & Goss, 1982) and that no information is available on native chains from amphibian hemoglobins.

In this paper, we report a procedure for preparing α and β chains from *Xenopus laevis* hemoglobin (Hb) which, like teleost fish hemoglobins, presents a Ser residue in the β (F9) 93-position and is characterized by a Root effect for both O_2 and CO (Perutz & Brunori, 1982; unpublished data from this laboratory).

In addition, we report the preparation of stable symmetric hybrid tetramers containing either α or β chains from *X. laevis* Hb and the human mate chains ($\alpha_2^h\beta_2^x$ and $\alpha_2^x\beta_2^h$); the functional properties of these hybrids have been explored in some detail, and the results are presented below and compared with those reported for human–carp hybrids.

Materials and Methods

Blood was collected from adult *X. laevis* by cardiac puncture in isotonic 0.7% NaCl solution containing 2 mM ethylenediaminetetraacetic acid (EDTA). The cells were washed 3 times by centrifugation at 3000 rpm in isotonic saline. In order to prevent contamination by nucleic acids (originated from rupture of nuclei), cells were lysed by adding 2 volumes of a cold aqueous solution of 10 mM $MgCl_2$, 0.5 mM $CaCl_2$, 5 mM tris(hydroxymethyl)aminomethane (Tris) buffer, and 0.1% Triton X-100; pH was adjusted to 7.8 by addition of dilute HCl.

Nuclei and stroma were removed by centrifugation at 5000 rpm for 15 min, and the supernatant was either used immediately or stored frozen.

Analytical isoelectric focusing on polyacrylamide gels (IFPA) was performed as described (Drysdale et al., 1971). Samples of 10–30 μg of protein were applied to the gels containing 2% ampholine (pH range 6–8; LKB, Bromma, Sweden); run was performed at 300 V in the cold room until intensity decreased to undetectable values. Electrophoresis on cellulose acetate films was performed in 0.1 M Tris–0.4

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M glycine buffer at pH 8.6 containing 6 M urea and 0.05 M β-mercaptoethanol; in the latter case, samples were composed of globin (Rossi Fanelli et al., 1958) dissolved in the gel buffer.

Dissociation of *X. laevis* hemoglobin into α and β chains was achieved in alkaline buffers (see below) and was made irreversible by addition of *p*-(chloromercuri)benzoate in stoichiometric amounts (with respect to heme concentration); chromatographic separation of chains as well as that of hybrid tetramers was achieved on CM-52 (Whatman) columns equilibrated in 10–50 mM Tris buffer and eluted with pH or NaCl gradients starting from the equilibrating buffer.

Dissociation of tetramers into monomers as a function of pH and incubation time was extensively investigated by using the Pharmacia fast protein liquid chromatography (FPLC) system for high-performance chromatography of biomolecules. The experiments were carried out with a prepacked Mono Q (anion exchanger) column at flow rate 1 mL/min; elution of proteins required 20 min with a pH gradient from 9.7 to 6 in 20 mM Tris + 20 mM [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane (Bistris) buffer.

Carboxyl-terminal sequences were obtained by 3-h digestion of globins with carboxypeptidase A or A + B in 0.1 M ammonium bicarbonate and 0.1% sodium dodecyl sulfate (SDS) (Ambler, 1967); the reaction was stopped by rapidly freezing the solution, which was then lyophilized. The powder redissolved in 10% trichloroacetic acid and filtered was analyzed for amino acids with an LKB 4400 analyzer equipped with a Spectra Physics System I computing integrator.

Human –SH chains were prepared and demercurated as described (Bucci & Fronticelli, 1965; Geraci et al., 1969); the same procedure was applied to the β chains from *X. laevis*.

Oxygen dissociation curves were determined spectrophotometrically (Rossi Fanelli & Antonini, 1958) with a Cary 219 spectrophotometer, at protein concentrations between 3 and 6 mg/mL; to prevent autoxidation of the relatively unstable human–*Xenopus* hybrids, an enzymatic methemoglobin reducing system (Hayashi et al., 1973) was added to the hemoglobin solutions before starting an oxygen binding experiment.

Flash photolysis kinetics was carried out by using the apparatus described by Brunori and Giacometti (1981).

Results and Discussion

Some General Properties of X. laevis Hemoglobin and Chain Separation. Analytical isoelectrofocusing (IFPA) of the hemolysate from *X. laevis* shows the presence of three Hb components, two of which have the same relative proportion and account for about 90% of the total pigment. These two main hemoglobin components have similar isoelectric points (7.0–7.2) and similar functional behavior; thus, in spite of the nonhomogeneous molecular composition, no sign of important functional heterogeneity has been detected by either equilibrium or kinetic techniques; an indication of minor functional heterogeneity is discussed below.

The primary structure of three of the four polypeptide chains of these two main components (two α chains and one β chain) is available (Richardson et al., 1980; Williams et al., 1980). In the case of the two different α chains the amino acid sequences vary by only eight homologous substitutions and no cysteine is present. The β chain, for which the amino acid sequence is known, contains two cysteinyls at positions 19 and 109, but the one normally present in mammals at the β (F9) 93-position is lacking, being substituted for by serine.

From sequence analysis it can be observed that the $\alpha_1\beta_2$ contact is largely conserved (less than 30% substitutions); the differences are the substitutions of the *Xenopus* leucine for human glutamic at the β 101-position, lysine for threonine at α-38, and serine for valine at α-96.

It is important to remark that $\alpha_1\beta_2$ contact is established between the C helix of each chain and the FG corner of the other; approximately 10 residues for each chain are involved both in oxy- (or met-) hemoglobin and in deoxyhemoglobin. The allosteric transition involves changes in the interactions of these residues.

X. laevis hemolysate does not split into chains under conditions comparable to those used for human hemoglobin A (Bucci & Fronticelli, 1965); nevertheless, it binds *p*-(chloromercuri)benzoate (PMB) at pH 7.0 with a –SH/heme ratio of 0.3 (to be compared with 0.5 for HbA). This figure, which is evidently uneven with respect to the symmetric assembly of hemoglobin (corresponding to 1.2 –SH/tetramer) could be explained by the heterogeneity of *Xenopus* hemolysate.

The pH dependence of chains–dimer–tetramer equilibria was studied by means of analytical FPLC; the experiments revealed that at pH lower than 9 the composition of *Xenopus* hemolysate corresponds to the isoelectric focusing pattern, while, when the protein was incubated in alkaline solutions, new chromatographic species with very different isoelectric points (6.2 and 9) appeared and at high pH values (i.e., pH 10–11) replaced completely those resolved at low pH values.

These new chromatographic species have been isolated and, as demonstrated below, they correspond to pure α and β chains. In our hands, only analytical FPLC procedures have been able to separate subpopulations of the α and β peaks.

Complete dissociation of *X. laevis* hemoglobin tetramers can be obtained in about 5 min as the pH is brought to 11.5 by adding dilute NaOH to the protein in Tris buffer; even though the β chains rapidly undergo partial oxidation, the dissociation process is almost completely reversible since reassociation can be obtained by lowering the pH below 9. However, addition of PMB in a 4 to 1 ratio (mercurial to tetramer) prevents chain reassociation when the pH is lowered.

In order to obtain purified chains in higher yields, the following procedure was adopted: after incubation with PMB at pH 11.5 the solution was dialyzed against 0.05 M phosphate buffer plus 0.1% Triton X-100 at pH 6.3 under 1 atm of pure CO and applied on a CM-52 column equilibrated with the same buffer. Triton X-100 was found to be necessary to improve the stability of the dissociated protein; however, even under the best conditions a fraction (approximately 30%) of the protein irreversibly precipitates when the pH is lowered. Elution with a linear pH gradient (6.3–9) gives three well-resolved fractions (see Figure 1) which together account for about 70% of the initial material. Electrophoresis of the globins of these fractions in 6 M urea gives the results reported in the inset of Figure 1. By comparison with human globin, the component with higher cationic mobility (which is also that obtained in larger amount from the chromatographic column) is the α chain, the other component being obviously β. This has been confirmed by determination of the C-terminal residues of these subunits (as obtained by carboxypeptidase digestion).

Analytical FPLC experiments performed with purified α and β chains gave retention volumes identical with those previously measured with partially dissociated hemoglobin (see above) and demonstrated that the products of the alkaline dissociation are actually single chains.

In order to test the reversibility of the alkaline dissociation and PMB reaction, an aliquot of the material incubated with PMB was dialyzed against 0.1 M Tris buffer, pH 8.5, + 1 mM β-mercaptoethanol. Under these conditions, dissociation is completely reversed, α and β chains reconstituting a tetramer

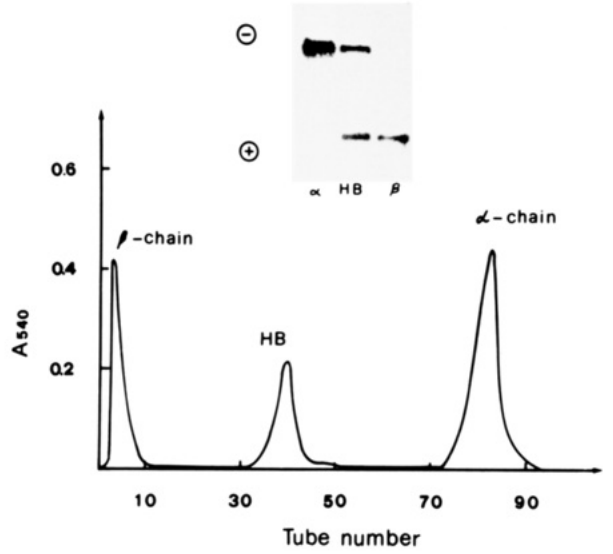

FIGURE 1: Elution profile of *X. laevis* hemoglobin treated with PMB at pH 10.5 in the presence of 0.1% Triton X-100; resin, CM-52 (Whatman); pH gradient 6.3–9. The inset shows the cellulose acetate film electrophoretic pattern obtained on the globins of the three components in Tris–glycine buffer, pH 8.6, + 6 M urea.

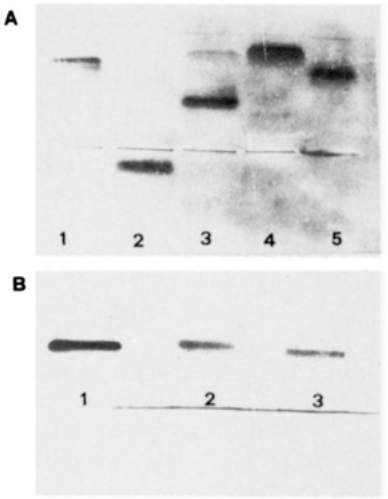

FIGURE 2: Starch gel electrophoresis of hybrid hemoglobins. Panel A: (1) *X. laevis* hemoglobin; (2) α^x chains; (3) hybrid $\alpha_2^x\beta_2^h$ before elimination of β^h excess; (4) β^h –SH chains; (5) HbA. Panel B: (1) *X. laevis* Hb; (2) hybrid $\alpha_2^h\beta_2^x$; (3) HbA.

with electrophoretic mobility identical with that of the native species. It should be remarked that, while the α chains are stable both as oxy and carbon monoxide derivatives throughout the dissociation and isolation procedure, the β chains tend to become met and to precipitate (and thus their quantitative recovery is lower).

Preparation and Isolation of Hybrid Hemoglobins. When isolated α chains from *X. laevis* Hb (α^x) are mixed with a slight excess (1–1.4) of human β –SH chains (β^h), the $\alpha_2^x\beta_2^h$ hybrid was readily obtained in approximately 30 min of incubation at 8 °C (see Figures 2A and 3). The excess of human β chains is then eliminated by chromatography on a CM-52 column (equilibrated in 0.5 M Bistris buffer, pH 7, and eluted with a linear gradient of NaCl up to 0.3 M).

Instability of the isolated β chains from *X. laevis* Hb (β^x) required a different preparation procedure for the complementary hybrid hemoglobin ($\alpha_2^h\beta_2^x$). *Xenopus* hemolysate in dilute Tris buffer at pH 11.5 (where it is fully monomeric) was mixed with a previously prepared suspension of CM-52 resin in 0.01 M Tris-HCl buffer, pH 8.2, containing human

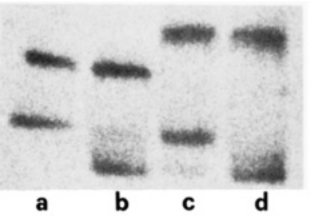

FIGURE 3: Cellulose acetate film electrophoresis in 6 M urea of (a) human globin, (b) globin from the $\alpha_2^h\beta_2^x$ hybrid, (c) globin from the $\alpha_2^x\beta_2^h$ hybrid, and (d) *X. laevis* globin. The lyophilized globins were dissolved in the electrophoresis buffer containing 1 mM β-mercaptoethanol.

Table I: Equilibrium and Kinetic Parameters for O_2 and CO Reaction of *X. laevis* Hemoglobin and Isolated Chain[a]

	$p_{1/2}$ (mmHg)	$n_{1/2}$	l' (M^{-1} s^{-1})
X. laevis Hb	15[b]	2.6[b]	1.7×10^5[c]
reconstituted Hb	12[b]	2.9[b]	1.2×10^5[c]
α chains	0.47[b]	1[b]	3.2×10^6[c]
β –SH chains			1.4×10^6[c,d]
β PMB chains			2.0×10^6[c,d]

[a] Conditions: 0.1 M Tris-HCl buffer, pH 7.5–7.75, +0.1 M NaCl; t = 20 °C. [b] pH 7.75; solution containing 5 mM 2,3-diphosphoglycerate. [c] pH 7.5; solution containing 1 mM P_6 inositol. [d] Values refer to the slow kinetic component (see text), obtained by flash photolysis.

α –SH chains (α^h). The final ratio α^h:α^x was about 4. The mixture was then brought to pH 8.2 by addition of dilute HCl.

At this pH, α^x chains (isoelectric point 9–9.2), instead of recombining with the original partners (β^x), are rapidly bound to the resin and may be removed by centrifugation. In the supernatant the β chains from *X. laevis* Hb (β^x) are therefore confronted with a great excess of human α chains. This allows the formation of the expected hybrid molecule ($\alpha_2^h\beta_2^x$), which can be purified of the excess of human α chain by using a CM-52 resin at pH 7.3, either on a column or in "batch". The homogeneity of the hybrid hemoglobin is clearly demonstrated by the electrophoretic pattern reported in Figures 2B and 3.

Functional Properties of Isolated Chains. The oxygen binding properties of the isolated α^x chains (see Table I) show very high oxygen affinity ($p_{1/2}$ = 0.47 mmHg), absence of Bohr effect (from pH 6.5 to pH 8.5), and no heme–heme interactions ($n_{1/2}$ = 1). The same type of information for the reaction with oxygen is not yet available for the β^x chains, which in the isolated form are stable only as the carbon monoxide derivative.

Isolated chains were studied by flash photolysis to characterize the kinetics of carbon monoxide binding. Their kinetic behavior may be summarized as follows: (i) In the case of α^x chains after full photolysis (100% photodissociation), the CO recombination corresponds to a simple process, in complete agreement with the noncooperative oxygen binding behavior. Under the same conditions CO binding to the β chains is heterogeneous with approximately 25% of a faster component ($l' = 1.3 \times 10^7$ M^{-1} s^{-1}) which could not be separated chromatographically. (ii) Upon partial photolysis (~20% photodissociation), the time course of CO recombination remains identical for both chains, as expected. (iii) The apparent second-order rate constant is 3.2×10^6 M^{-1} s^{-1} for the α^x chain and 1.4×10^6 M^{-1} s^{-1} for the main component of the β^x –SH chain (see Table I).

This functional behavior compares well with that of human hemoglobin chains (Antonini & Brunori, 1971), which are characterized by noncooperative ligand binding, very high oxygen affinity ($p_{1/2}$ = 0.4–0.45 mmHg), and a very high rate constant for CO combination (4×10^6 M^{-1} s^{-1}). It should be recalled, however, that both chains from carp hemoglobin

Table II: Effect of pH on Oxygen Equilibria of Hybrid Hemoglobins[a]

	pH 6.0		pH 7.0		pH 8.5	
	$p_{1/2}$[b]	n_{max}[c]	$p_{1/2}$	n_{max}[c]	$p_{1/2}$	n_{max}[c]
X. laevis Hb[d]	152	1	89	1.7	8	2.5
$\alpha_2^x\beta_2^h$ hybrid	20[e]	1.5	17.5	1.5	4	1.6
$\alpha_2^h\beta_2^x$ hybrid	41.5	1	28	1.2	6.3	1.6
human Hb	63	2.2	42	2.4	3	2.6

[a] Conditions: 0.1 M Tris or Bistris buffer containing 0.1 M NaCl and 3 mM P_6 inositol; t = 20 °C; enzymatic methemoglobin reducing system was added to all samples. [b] In units of mmHg. [c] The value for the Hill coefficient corresponds to the maximum observed slope of the Hill plot, instead of that measured at 50% oxygen saturation, due to asymmetry of the oxygen binding curves (see text). [d] Solution containing 1 mM β-mercaptoethanol. [e] Owing to the acid Bohr effect, the highest $p_{1/2}$ value is not this one but that observed at pH 6 ($p_{1/2}$ = 32 mmHg and $n_{1/2}$ = 1.5).

(Parkhurst & Goss, 1982) display second-order rate constants for CO combination lower (by approximately 10 times) than those characteristic of human α and β chains; this finding has been interpreted by Parkhurst and Goss along the model developed for the quantitative analysis of the functional properties of Lamprey hemoglobin (Andersen & Gibson, 1971).

In addition, isolated α and β chains from *X. laevis* can readily reconstitute a native tetramer when mixed in stoichiometric amounts after PMB removal. The rate constant for CO combination of the reconstituted tetramer is very close to that of the total hemolysate (Table I), although the fraction of quickly reacting form observed upon full photolysis is slightly higher. Moreover, the reassembly of a native tetramer, upon mixing stoichiometric amounts of the CO derivatives of α^x and β^x chains, is confirmed by the oxygen equilibrium data on reconstituted hemoglobin which regains cooperativity and low affinity (Table I). As a matter of fact, the reassembled tetramer displays a Hill coefficient ($n_{1/2}$ = 2.9) consistently higher than that of the hemolysate ($n_{1/2}$ = 2.6); this may support a higher degree of homogeneity with respect to the starting hemoglobin sample, probably resulting from the loss of protein which, during preparation, occurs preferentially at the expenses of the β chains.

Functional Properties of Hybrid Hemoglobins. Preparation of stable hybrid tetramers assembled with one chain from *X. laevis* hemoglobin and the partner subunit of human HbA (see above) allows one to test the role played by subunit interfaces and by β (F9) Ser-93 in the allosteric properties of these unnatural tetramers. Available data on the functional properties of these hybrid molecules are reported in Table II and Figure 4.

The oxygen binding equilibria of both hybrids have been determined in the presence of the enzymatic, phosphate-containing methemoglobin reduction system as described by Hayashi et al. (1973), because of the intrinsic instability of the $\alpha_2^h\beta_2^x$ hybrid toward autoxidation. For this reason, it has not been possible to obtain results on the stripped hybrids; on the contrary, in order to minimize the effect of glucose 6-phosphate (present in the enzymatic system) on oxygen affinity, all the experiments were carried out in the presence of 3 mM inositol hexakis(phosphate) (P_6 inositol), which was shown to better manifest the Root effect in *X. laevis* hemoglobin (Perutz & Brunori, 1982).

Figure 4 reports the oxygen binding isotherms of the hybrids $\alpha_2^x\beta_2^h$ (A) and $\alpha_2^h\beta_2^x$ (B) at different pH values and in the presence of P_6 inositol.

The two hybrid hemoglobins bind oxygen cooperatively over the pH range explored (with the exception of $\alpha_2^h\beta_2^x$ at pH <7.0). This result indicates that the novel αβ contacts, though

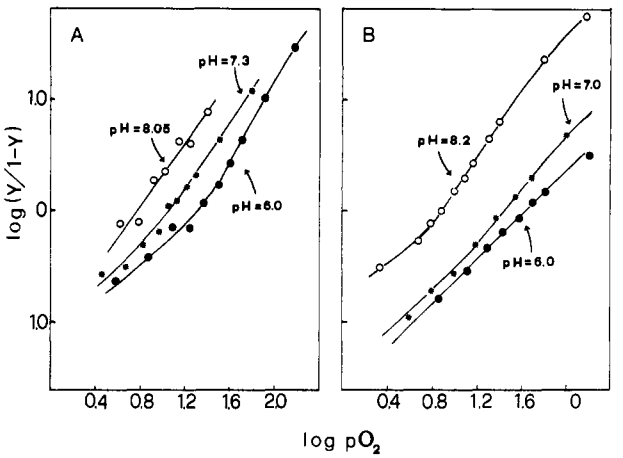

FIGURE 4: Oxygen binding isotherms of the human–*Xenopus* hybrid hemogobins. (Panel A) $\alpha_2^x\beta_2^h$ hybrid: (○) pH 8.1, (*) 7.3, and (●) 6.0. (Panel B) $\alpha_2^h\beta_2^x$ hybrid: (○) pH 8.2, (*) 7.0, and (●) 6.0. Other conditions: buffer 0.1 M Tris or Bistris containing 0.1 M NaCl; P_6 inositol, concentration 3 mM throughout; enzymatic methemoglobin reducing system was always used; t = 20 °C.

unnatural, allow competent fit of the surfaces and are designed to keep the deoxygenated hemoglobin in a low-affinity conformation, thus leading to a quaternary oxygen-linked allosteric transition. Human–*Xenopus* hybrids do not display a marked tendency to dissociate into subunits upon oxygen binding, as shown by electrophoresis. Interestingly, both hybrid hemoglobins display a markedly asymmetric Hill plot; this could be due to intramolecular heterogeneity of human and *Xenopus* chains in the T-state tetramer and, in any case, does not allow us to simulate satisfactorily these experiments with the two-state model. Due to the asymmetry of the Hill plot the values of the Hill coefficient reported in Table II correspond to n_{max} instead of $n_{1/2}$.

The αβ contacts in the hybrid hemoglobins are however not able to impose such a strong constraint as in both parental hemoglobins which are always characterized by lower affinity and higher cooperativity. In this respect, it is noteworthy that the substitutions which are expected to happen at the $\alpha_1\beta_2$ contact could involve disruption of some hydrophobic and hydrogen bond interactions; in particular, in the $\alpha_2^x\beta_2^h$ hybrid the substitution of lysine for threonine could be expected to introduce a salt bridge with β Tyr-145 thus disturbing the arrangement of the β FG corner; furthermore, displacement of this residue toward the exterior is thought to be one of the triggering mechanisms of the allosteric transition (Perutz, 1970), and thus stabilization of this external position of β Tyr-145 is expected to stabilize the R state even in unliganded hemoglobin, with reduction of cooperativity and increased ligand affinity.

The other substitutions could probably introduce similar modifications in the described $\alpha_1\beta_2$ interactions, though it is very difficult to speculate on their nature in the absence of crystallographic information.

Moreover, it may be seen that cooperativity of the $\alpha_2^x\beta_2^h$ tetramer, although generally low, and in any case lower than that of both parental hemoglobins, is virtually independent of pH, the maximum Hill coefficient being always close to 1.6. The $\alpha_2^h\beta_2^x$ hybrid, on the contrary, binds oxygen cooperatively only at pH 7.0, while at lower pH values this hybrid, though tetrameric, shows no cooperative interactions (the Hill coefficient being 1 at pH 6, and $p_{1/2}$ = 43 mmHg).

It is interesting that the only other studied hemoglobin hybrids between human and a Root effect hemoglobin, i.e., carp hemoglobin, display similar oxygen binding properties

(Causgrove et al., 1984): cooperativity is very low ($n_{1/2}$ = 1.5–1) for both hybrids and comparable to that of human *Xenopus* hybrids; oxygen affinity is approximately intermediate between that of parent hemoglobins and in any case higher than that of the Root effect hemoglobin; finally, at pH 6 and in the presence of 1 mM P_6 inositol both hybrid hemoglobins approach noncooperative ligand binding (T-state stabilization, Root effect) even though oxygen saturation in air is close to unity.

In this respect, it is important to underline that the molecular mechanism of the Root effect involves primarily pH dependent stabilization of the T state (n = 1; Brunori et al., 1973; Perutz & Brunori, 1982), and it is observed in human–carp and human–*Xenopus* hybrids; α and β chains in the T-state hemoglobin may in turn be heterogeneous (as in the case of trout or carp hemoglobin) so that one of them does not bind oxygen significantly in the physiologically relevant oxygen pressure range (Brunori et al., unpublished results).

These results are certainly not inconsistent with the model suggested by Perutz and Brunori for the structural basis of the Root effect in fishes and (some) amphibian hemoglobins (Perutz & Brunori, 1982). This model attributes great significance to the presence of serine at the β (F9) 93-position and suggests a secondary role for the structure of the α chains. It therefore predicts that a hemoglobin having a serine at the β (F9) 93-position should display a Root effect, while a hemoglobin containing cysteine at the same position should not. Data in Figure 4 indicate that the $\alpha_2^h\beta_2^x$ hybrid has a very marked effect of pH on oxygen binding, being noncooperative and having low affinity at pH 6; the difference in behavior with the other hybrid is self-evident.

That other residues in the primary structure of both chains play also a role in determining the presence of the Root effect, as well as the amplitude of the Bohr effect, is also established.

It is indeed interesting to compare this conclusion with other data available in the literature and relevant to Root effect hemoglobins. As reported (Perutz & Brunori, 1982; Brunori et al., 1982), one of the hemoglobin components of rat hemolysate displays a marked Root effect, although position β (F9) 93 is occupied by a cysteinyl residue. It has been also demonstrated that the mutant Hb *Nymphaea* (Cys-93 $\beta \rightarrow$ Ser) derived from the human β chain gene by site-directed mutagenesis does not exhibit the Root effect (Nagai et al., 1985). Furthermore, there is at least one direct evidence that another residue is crucially involved in the Root effect; in fact, removal of the β C-terminal histidine in carp hemoglobin markedly reduces the Root effect but does not prevent the hemoglobin from assuming the T state at pH 6 with added P_6 inositol (Parkhurst et al., 1984); it will be observed that even in this case the β chain appears to be mainly responsible for the Root effect.

Thus it seems that the presence of a Ser at β (F9) 93 is not an absolute requirement for a Root effect hemoglobin, and in fact, different structural features do cooperate to express a similar functional behavior. On the other hand, an important even though still conjectural role of serine in providing extra stabilization is consistent with structural analysis (Perutz & Brunori, 1982) and with the data reported in Figure 4. Thus it may be said, paradoxically, that although serine at β (F9) 93 is neither necessary nor sufficient (see above), it is nevertheless important in the manifestation of the Root effect.

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