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Homos and Heteros among the Hemos

Interaction of two unlike subunits is essential for physiological competence in hemoglobins.

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The hemoglobins of higher animals are tetrameric proteins, composed of two pairs of unlike polypeptide chains. In man, one of these, the α chain, is common to several hemoglobins, which differ only in the composition of the other one. Thus, the major component of the adult (A) human red cell consists of two α and two β chains forming the heterotetramer $\alpha_2{}^A\beta_2{}^A$, while, for instance, human fetal (F) hemoglobin has the same two α chains, but two γ chains of different amino acid composition and sequence replace the β chains, giving rise to $\alpha_2{}^A\gamma_2{}^F$

It is the purpose of this article to show that, at least in the case of the hemoglobins, the interaction of two unlike subunits is essential for the allosteric properties which make these molecules physiologically useful. These capabilities depend on the characteristic conformational changes which the protein undergoes with ligand binding and which are absent in hemoglobins of the same molecular weight, but composed of only one kind of subunit. These are therefore functionally useless.

When we first arrived at Columbia in 1960, we asked Helen Ranney, who was already then a recognized authority on hemoglobinopathies, if there were any abnormal hemoglobins which consisted of only the α chains or the β chains which make up normal hemoglobin. Her reply was, "α we don't have, but β we've got." This started a collaboration on HbH, a tetrameric hemoglobin consisting only of the β chains of normal hemoglobin (*1, 2*). It is found in patients with α thalassemia, a condition in which the synthesis of α chains lags behind that of the β chains because of decreased activity of α globin messenger RNA (*3*). Since α chains are common to several different hemoglobins, the complementary chains of those hemoglobins are also produced in excess in α thalassemia and associate to form homotetramers like HbH. Thus, the children of patients with HbH disease often have Hb Bart's ($\gamma_4{}^F$), and Hb $\beta_4{}^S$ has been described in patients where the sickle and the α thalassemia genes are present together (*4*).

Functional Properties

In normal hemoglobin several regulatory mechanisms operate to ensure adequate delivery of oxygen in response to demand. One of these, cooperative oxygen binding, is responsible for the sigmoid shape of the oxygenation curve (the curve obtained when percentage saturation of hemoglobin is plotted against the partial pressure of oxygen, p_{O_2}). The steep middle portion of this curve makes oxygen release suitably sensitive to small drops in oxygen pressure. Equally important are the environmental factors which control the position of the curve along the p_{O_2} axis or the overall oxygen affinity. They are hydrogen ions, carbon dioxide, and the erythrocytic organic phosphate 2,3-diphosphoglycerate (DPG). An increase in the concentration of any of these lowers the affinity of hemoglobin for oxygen and this change is, of course, reversible.

In Figs. 1 and 2 the oxygenation properties of normal hemoglobin are compared with those of $\beta_4{}^A$. It is clear that the oxygen affinity of the homotetramer is very high and its oxygen binding curve shows no cooperativity since it is hyperbolic rather than sigmoid. Ligand binding is independent of *p*H; that is, the so-called Bohr effect is absent (Fig. 1). Furthermore, the oxygenation curve is identical in the absence and presence of DPG (Fig. 2).

In its functional behavior $\beta_4{}^A$ therefore resembles a monomeric heme protein such as myoglobin, although it is structurally a tetramer. As a result, HbH cannot be useful as an oxygen carrier under physiological conditions.

Conformational Changes

In the intervening years, the original hypothesis of Haurowitz (*5*) and of Wyman (*6*) that normal hemoglobin exists in two separate conformations corresponding to the fully oxygenated and fully deoxygenated state has been amply verified by experiment (*7*). The models devised to explain allosteric effects in proteins in general are based on the assumption that such proteins must exist in at least two distinct conformational states. In the case of hemoglobin, the deoxy or T conformation has a low oxygen affinity and the oxy or R state a high one, and the transition between the two is responsible for such allosteric phenomena as cooperative oxygen binding ("heme-heme interaction") and the Bohr effect.

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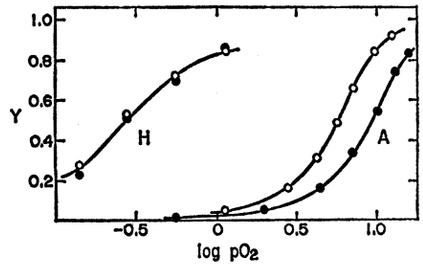

Fig. 1. Oxygenation curves of hemoglobins A and H. The hemoglobins used for these experiments were isolated from the same blood sample. The hemoglobin concentration was 0.1 percent in 0.1*M* phosphate buffer. The ordinate is fractional saturation. (Open circles) *p*H 7.30, (closed circles) *p*H 6.88, temperature 22°C. [Modified from (*1*)]

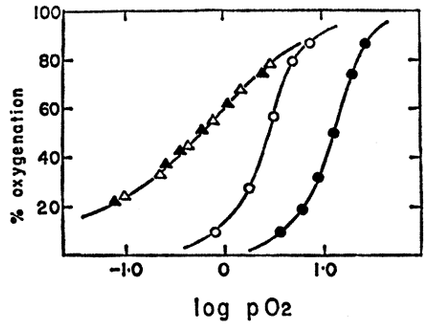

Fig. 2 (left). Effect of DPG on the oxygenation curves of hemoglobins A and H. The hemoglobin concentration was 0.3 percent, *p*H 7.0 (before deoxygenation), and temperature 30°C. (Circles) $\alpha_2^A\beta_2^A$, (triangles) β_4^A, (open symbols) 0.01*M* NaCl, (closed symbols) 0.01*M* NaCl and $1 \times 10^{-4}M$ DPG. [From (*16*)]

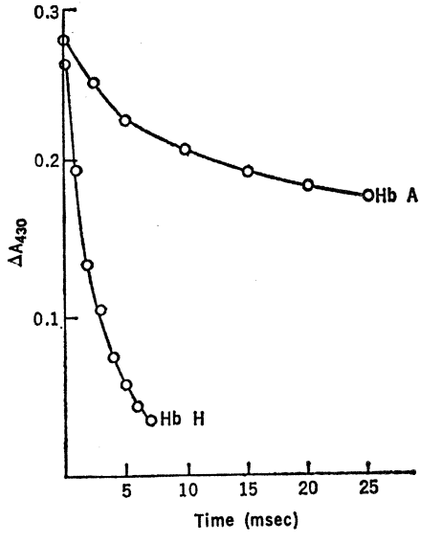

Fig. 3 (right). Reaction of hemoglobin H and hemoglobin A with carbon monoxide after flash photolysis. The ordinate is the change in absorbancy at 430 nanometers. Hemoglobin was $1 \times 10^{-5}M$ (heme basis); carbon monoxide, $4 \times 10^{-5}M$; temperature, 3°C; light path, 3 mm; flash energy, 130 joules. [Modified from (*9*)]

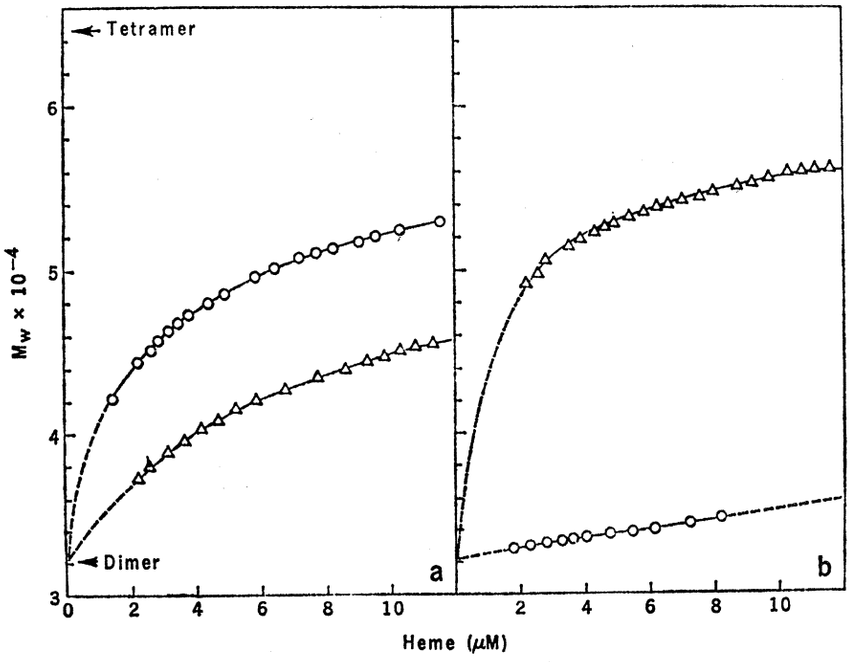

Fig. 4. Dependence of weight-average molecular weight, M_w, on concentration. Experiments on HbA (circles) and β_4^A (triangles) in (a) 0.1*M* phosphate, *p*H 7.0, and (b) 0.1*M* phosphate, *p*H 7.0, plus 2*M* NaCl. Data were recorded after 18 hours of centrifugation at a rotor speed of 30,000 rev/min. The temperature of the rotor was maintained at 20°C. Curves were drawn with fitted values of the tetramer $\rightleftharpoons$ dimer equilibrium constant $K_{4,2}$; for hemoglobin dissociation these values are equal to the concentration (micromolarity) of heme at $M_w = 44{,}000$. The lower curve of (b) corresponds to $K_{4,2} = 4 \times 10^{-5}M$. Each curve contains the results from one representative sedimentation equilibrium analysis. [From (*11*)]

The absence of these properties in HbH therefore raised the question what conformational changes (if any) accompany ligand binding by this hemoglobin. Much experimental evidence has confirmed the expectation that β_4^A binds ligands without the change in conformation which is so obvious in the case of HbA. Thus, for example, the reactivity of the β^{93} –SH groups differs substantially between the liganded and unliganded state in normal hemoglobin (*8*). By contrast, in HbH all the –SH groups are fully reactive both in the presence and in the absence of oxygen (*1*). Kinetic evidence likewise supports the conclusion that β_4^A binds ligand without a change in conformation. The rate of recombination with CO after flash photolysis of COHbH showed that only a single fast-reacting species was present. In the case of HbA, on the other hand, this reaction is biphasic, reflecting the conversion of the R to the T conformation (*9*) (Fig. 3).

One of the most striking differences between the R and T conformations of normal hemoglobin is their ability to dissociate into dimers. Only the liganded form breaks up into $\alpha\beta$ half-molecules, either upon dilution or in the presence of high concentrations of neutral salts (*2, 10, 11*). Thus, in the presence of 2*M* NaCl oxy HbA is largely split into dimers, whereas deoxy HbA remains tetrameric. Under the same conditions β_4^A is tetrameric even when fully oxygenated (*2*). The data of Tainsky and Edelstein (*11*) (Fig. 4) illustrate that high salt actually has the opposite effect on the β_4 tetramer since it stabilizes it in the tetrameric form. Evidently the forces which hold the homotetramer together are quite different from the bonds which oppose dissociation into half-molecules in the mixed tetramer.

The hydrogen-tritium exchange of β_4^A was studied by Englander and Englander (*12*) who found that, in contrast to HbA, it shows exactly the same exchange behavior in the oxy and deoxy states. It is noteworthy that the exchange curve of the homotetramer is very close to that of oxy HbA.

The work of Perutz and Mazzarella (*13*) on crystals of HbH is in excellent agreement with the chemical evidence since they found no significant difference in the x-ray diffraction patterns in the presence and absence of oxygen. By contrast, crystals of deoxy HbA cannot combine with oxygen without being destroyed (*5*).

The affinity for organic phosphates, such as DPG, is a particularly sensitive

probe for the conformational state of hemoglobin tetramers. A strong and specific binding site for DPG is located between the N termini of the β chains of HbA in the deoxy conformation. Transition to the oxy conformation destroys this site so that the cofactor is expelled (*14*). This highly selective affinity of DPG for deoxyhemoglobin is, of course, responsible for the decrease in oxygen affinity in the presence of the phosphate ester and is the chemical basis for the physiological effect of DPG in facilitating oxygen release to the tissues (*15*).

Once again, the behavior of the homotetramer toward DPG differs radically. Although β_4^A also binds 1 mole of DPG, its oxygenation curve is completely uninfluenced by the presence of the phosphate (Fig. 2). The thermodynamic consequence—an identical affinity of DPG for β_4^A in the presence and absence of ligand—is borne out by the binding curves in Fig. 5 (*16*). These results again confirm the absence of conformational change with ligand binding, and support the conclusion of Perutz and Mazzarella (*13*) that β_4^A remains in the deoxy conformation even when fully oxygenated. Finally, these findings clearly show that DPG can be bound to a hemoglobin without affecting its oxygen affinity. It is only the differential affinity for the oxy and deoxy conformations, seen in the heterotetramer, which alters the affinity for the ligand and which makes DPG into a cofactor for oxygen release.

Abnormal β_4^A tetramers combine readily with free α chains to form the normal mixed tetramer, $\alpha_2^A\beta_2^A$. It has been shown in several laboratories that this reassembly, although it is relatively slow, is quantitative and leads to a hemoglobin which is indistinguishable from normal HbA, since all allosteric properties are fully restored (*17*) (Table 1). The reaction of single α chains with β_4 tetramers must, of course, be quite complex, and its mechanism has not yet been clarified (*7*, p. 120).

An investigation of the effect of DPG on the rate of the reaction could perhaps provide a lead as to the slow step, since, in the presence of oxygen, DPG is bound by one of the reactants, but not by the product

$$\beta_4^A\text{DPG} + 4\alpha^A \rightarrow 2\alpha_2^A\beta_2^A + \text{DPG}$$

Anaerobically, on the other hand, additional DPG will be bound as the reaction proceeds

$$\beta_4^A\text{DPG} + 4\alpha^A + \text{DPG} \rightarrow 2\alpha_2^A\beta_2^A\text{DPG}$$

Other Homotetramers

The formation of homotetramers is not confined to β^A chains. Two tetramers composed of mutants of this chain, β_4^S and β_4^C, have been prepared and crystallized in our laboratory. In the β^S chain the sixth amino acid from the N terminal end is valine (Val) instead of glutamic acid (Glu), and the β^C chain carries a lysine in the same position. These homotetramers also lack the allosteric properties of their corresponding heterotetramers and are therefore useless for oxygen transport under physiological conditions.

The amino acid substitution in HbS causes a dramatic decrease in the solubility of the deoxy form of this hemoglobin. It was therefore particularly interesting to see if this property is preserved in the homotetramer. The results in Fig. 6 clearly show that this is not so since β_4^S shows no decrease whatever in solubility on deoxygenation (*18*). The presence of twice as many mutant chains as in the parent hemoglobin is evidently not enough for "sickling." The most likely assumption is that the α chains in HbS provide receptor sites for the polymerization process. This is borne out by the decreased sickling tendency in $HbS_{Memphis}$, where the β^6 Glu → Val substitution is accompanied by a substitution of glutamine (Gln) in the α chain: α^{23} Glu → Gln (*19*).

The homotetramer γ_4^F has been studied in some detail (*20, 21*). Again, all the usual allosteric functions of fetal hemoglobin were found to be missing, but in this case the special characteristic of the parent hemoglobin, its resistance to alkali denaturation, is at least partially preserved in the homotetramer (Fig. 7). Moreover, the rate of denaturation is even significantly af-

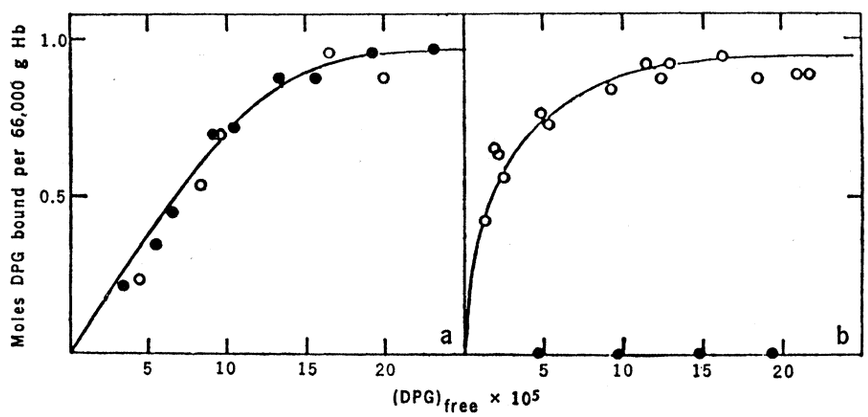

Fig. 5. Binding of DPG by (a) β_4^A and (b) hemoglobin A. All measurements were in 0.1*M* NaCl, *p*H 7.3, at 22° ± 2°C. (Open circles) Deoxyhemoglobin; (closed circles) oxyhemoglobin. [From (*16*)]

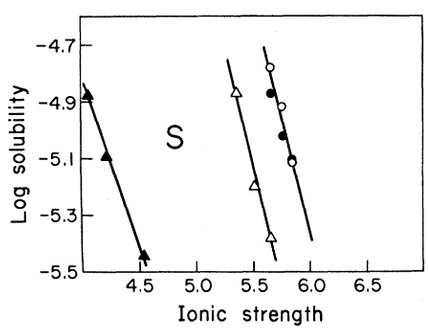

Fig. 6 (above). Solubility of HbS and β_4^S. The solubility (moles per liter) is plotted against the ionic strength of the solvent, phosphate buffer at *p*H 6.8. (Triangles) $\alpha_2^A\beta_2^S$, (circles) β_4^S, (open symbols) oxygenated, (closed symbols) deoxygenated. [From (*18*)]

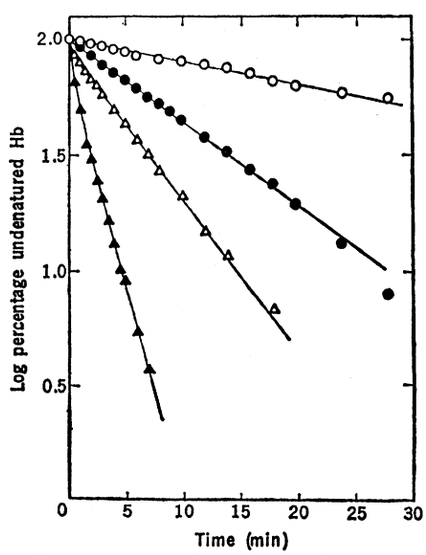

Fig. 7 (right). Alkali denaturation of HbF and γ_4^F. The temperature was 10°C and the final NaOH concentration was 0.095*N*. (Circles) $\alpha_2^A\gamma_2^F$, (triangles) γ_4^F, (open symbols) oxygenated, (closed symbols) deoxygenated. [From (*20*)]

fected by ligand binding in γ_4^F as is the case with HbF itself. This seems to be the only example so far where a molecular property of a homotetramer changes with oxygenation. It is probable that the original interpretation of Haurowitz *et al.* (*22*) that resistance to alkali denaturation is related to the fit between the heme and the surrounding globin architecture was correct. The increase in the resistance to alkali of fetal hemoglobin and γ_4^F with ligand binding therefore probably reflects the tighter binding of the heme when the bonding of the Fe changes from ionic to covalent. This change is, of course, a local one in the immediate vicinity of the hemes and need not be related to the alteration in quaternary structure which forms the basis of allosteric effects.

At this time, at least four homotetramers of hemoglobin (β_4^A, β_4^S, β_4^C, and γ_4^F) have been investigated, and no evidence of allosteric mechanisms has been found in any of these. Since these molecules cannot serve as physiological oxygen carriers, a complete failure of α chain synthesis would be lethal and is therefore never encountered. By contrast, life can be supported in the complete absence of β chains, as in Cooley's anemia, where they are replaced by γ chains, but in the form of the $\alpha_2^A\gamma_2^F$ heterotetramer.

Discussion

The most striking difference between the normally occurring heterotetramers of hemoglobin and the identical chain hemotetramers is that the latter do not change conformation with ligand binding. It remains to be seen how closely this single conformation resembles either the R or the T state of normal hemoglobins. Several lines of evidence suggest a T state. Both the existence of a strong DPG binding site and the resistance to dissociation into dimers are characteristic of deoxyhemoglobin and not of oxyhemoglobin. Furthermore, the diffraction pattern of crystals of β_4^A resembles that of deoxy HbA. On the other hand, some other properties, especially the great affinity for oxygen and the high reactivity of all eight –SH groups as well as the rate of tritium exchange, are typical of the R state. As a first approximation, therefore, the molecule behaves as though the individual subunits were in the R state but their quaternary arrangement resembled the T conformation.

Table 1. Oxygenation parameters at 30°C (*17*). The symbol p_{50} denotes the partial pressure of oxygen at 50 percent saturation; n is the exponent in the Hill equation $[HbO_2]/[Hb] = Kp^n$, where K is a constant and p is the partial pressure of oxygen.

Hemoglobin	$\log p_{50}$		n	$\frac{\Delta \log p_{50}}{\Delta pH}$
	pH 7.02	pH 7.53		
HbA: $\alpha_2^A\beta_2^A$	+1.22	+0.88	2.7	−0.67
β_4^A	−0.34	−0.32	1.0	0
$\beta_4^A + 4\alpha \rightarrow \alpha_2^A\beta_2^A$	+1.22	+0.88	2.7	−0.67

The binding of a single DPG molecule by homotetramers like β_4^A deserves special comment. By analogy with HbA where the single DPG site is formed by the N termini of two β chains, β_4^A could have been expected to bind 2 moles of DPG per mole of tetramer. However, the four β subunits, although all alike, are quite asymmetric and therefore need not form two identical N terminal binding sites.

The major conclusion from the study of the homotetramers—that is, that interaction between unlike subunits is crucial for the functional integrity of hemoglobin—also derives a great deal of support from the behavior of certain hemoglobins with a mutation at the α-β interface. In these cases, for example, hemoglobins Hirose, Yakima, Kempsey, and Kansas, the abnormal amino acid substitution interferes with the interaction of α and β subunits which controls the equilibrium between the two alternative quaternary structures (*23*). This, in turn, leads inevitably to a loss of the allosteric properties which are the hallmark of a normally functioning hemoglobin.

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