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Remote contributions to subunit interactions: lessons from adult and fetal hemoglobins

For many years, hemoglobin (Hb) has been a model protein for studies of factors that affect the conformational states of proteins[1]. Natural Hb variants and recombinant Hbs that possess single amino acid residue substitutions have provided important information on the contributions of specific sites to interactions at the interface between the dimer pairs involved in the allosteric transition as the Hb tetramer moves between its oxy and deoxy conformations[2]. The recent unexpected finding[3] that the interactions between these dimer pairs are much stronger in fetal Hb [HbF; $(\alpha\gamma)_2$] than in adult Hb [HbA; $(\alpha\beta)_2$; see Fig. 1) has opened new avenues for exploration of sequence–function relationships in proteins.

Replacing sequences from the β subunit of HbA with sequences from the γ subunit of HbF reveals that the amino acid residue contacts at the dimer interface are not, by themselves, sufficient to endow HbF with its enhanced tetramer stability. The N-terminal A helix of the γ subunit, which is at one end of the central cavity in the tetramer at a location distant from this interface, makes a very significant contribution to its strength. The findings demonstrate that long-range communication occurs among regions of the tetramer; this could be relevant to proteins in general.

Differences between adult and fetal hemoglobins

The major physiological function of HbF is to transfer oxygen from maternal to fetal blood. In the red blood cell, the allosteric regulator 2,3-diphosphoglycerate (2,3-DPG) binds between β or γ subunits to promote release of oxygen[4,5]. The difference in the oxygen-binding capacities of HbA and HbF is mainly due to more-efficient binding of 2,3-DPG to HbA than to HbF (Ref. 6). This leads to transfer of oxygen from HbA to HbF (Ref. 6). The β chain of adult HbA and the γ chain of fetal HbF differ at 39 (of 146) residues.

The term tetramer stability, as applied to hemoglobin, refers to the strength of the interactions at the interface between the two dimer pairs, which is where the allosteric transition between deoxy (T) and oxy (R) tetramers occurs (see Fig. 1). Monod, Wyman and Changeux[7] originally described dissociation of the functional state of a protein, which is the tetramer in the case of hemoglobin, as a general mechanism for desensitization. Hb dimers, for example, do not bind oxygen in a cooperative fashion and therefore are not functional. Until recently, HbF and HbA were not considered to differ in tetramer stability. Our recent results, however, show that their stabilities do differ[3] (Fig. 1). The method we have developed employs high-resolution gel filtration and complete analysis of peak positions and widths to extend the sensitivity of determination of subunit-dissociation constants (K_d) by at least an order of magnitude[3,8]. We[3] found that the stability of the liganded HbF tetramer is nearly 100-fold greater (K_d = 0.01 μM) than that of liganded HbA (K_d = 0.68 μM). Shear *et al.*[9] have used this finding to explain the resistance of neonatal red cells to the malaria parasite: HbA is more susceptible to a specific malaria protease than is HbF, presumably because the protease acts on dissociated dimers rather than the tetramer.

Transposing residues at the subunit contact sites in adult and fetal hemoglobins

Frier and Perutz[10] compared the crystal structures of deoxy HbF and deoxy HbA, and found that the two proteins have very similar overall structures and do not differ significantly at the interfaces between either dimers or monomers. Four of five sequence differences between these hemoglobins are at the very tight interface between the α monomer and the β or γ monomer; this region does not change during the allosteric transition[1]. The fifth difference is an Glu→Asp substitution at a site located at the dissociable interfaces between the two αβ dimers (arrows in Fig. 1). We constructed a recombinant HbA/F by replacing all five of these residues in the β chain sequence with residues from the γ chain. The strength of the tetramer formed by this mutant (K_d = 0.14 μM) was between that of HbA and that of HbF (Ref. 3). The single Glu→Asp substitution accounts for the change in K_d: a mutant that has only the Glu→Asp change at its dimer interface has a K_d of 0.20 μM. In a mutant that has a His→Ile substitution at the monomer interface, the strength of the dimer interface was unaffected. These results indicate that sequence(s) apart from the dimer interface itself also determine tetramer strength. Furthermore, although HbA/F contains HbA residues at the 2,3-DPG-binding site, its oxygen-binding properties are similar to those of HbF rather than those of HbA, in the presence of the allosteric regulator. Hence, dimer-interface residues influence how oxygen is released from distant heme moieties, given that amino acids in the HbA sequence are required both at the dimer interface and at the 2,3-DPG-binding site for maximal release[3].

The A helix of hemoglobin Felix

The major difference between the native deoxy structures of HbF and HbA reported by Frier and Perutz[10] is the position of their N-terminal A helices and the small N-terminal non-helical tails of the γ and β subunits relative to the central

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cavity: the A helix of the γ subunit and its tail protrude further into the cavity than do those of the β subunit (Fig. 1a,b), which endows HbF with a tighter overall structure[10]. The greatly increased stability of liganded HbF (Ref. 3) is consistent with this tighter structure in the deoxy state[10]. We constructed a hybrid γ–β subunit in which eight different residues of the A helix (residues 1–18) of the β subunit were replaced with the corresponding residues from the γ subunit; the remaining sequence (residues 19–146) was that of the β subunit. The correct sequence of the γ–β hybrid was established by mass spectrometry[11]. The tetramer formed by the α and γ–β hybrid subunits, which we term Hb Felix, responds normally to 2,3-DPG and exhibits normal cooperativity, which indicates that the α subunits and hybrid γ–β subunits form fully functional tetramers. Interestingly, the tetramer strength of Hb Felix (Fig. 1c) is about the same (K_d = 0.03 μM) as that of HbF (K_d = 0.01 μM), even though Hb Felix contains HbA residues at its tetramer–dimer interface, which is distant from the A helix.

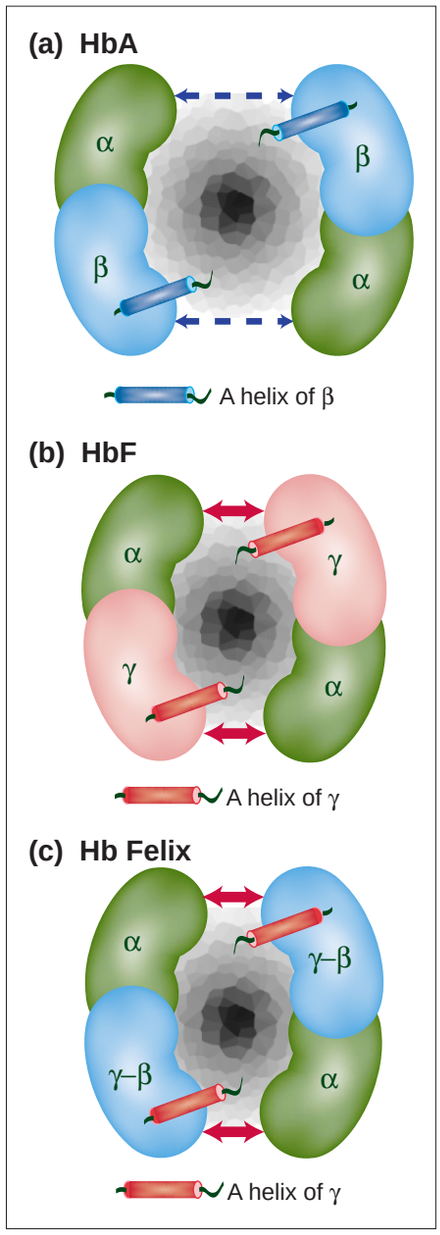

Figure 1
Tetramer strength of ligand-bound hemoglobin (Hb), as shown by the length and density of the arrows between the dimer pairs. **(a)** Adult Hb (HbA). **(b)** Fetal Hb (HbF). **(c)** Hb Felix. The N-terminal segments of the A helices of the β, γ and γ–β-hybrid subunits are shown extending into the central cavity; in HbF and Hb Felix, the helix protrudes further than in HbA. The A helix is distant from the dimer interface in the three-dimensional structure. 2,3-DPG binds between β or γ subunits across one end of the central cavity (shaded area).

A natural fetal hemoglobin that has altered tetramer strength and a modified A helix

Normal human blood contains a minor HbF component, HbF_1, that is acetylated on the N-terminus of its γ subunit[12]. This removes a positive charge at the N-terminus; the function of this acetylation is not known. We found that this acetylation considerably weakens the HbF tetramer; its dissociation constant (K_d = 0.33 μM) mimics that of HbA rather than that of HbF (Ref. 3) – that is, acetylation of HbF at the N-terminus of its γ subunit negates its increased tetramer stability. This observation is consistent with the conclusion that the N-terminal segment has a significant influence on subunit interactions between dimer pairs. Given that the distance between the N-terminal A helix and the allosteric tetramer–dimer interface is large, the results obtained for both recombinant Hb Felix and natural HbF_1 suggest that there is an interrelationship between regions of the Hb tetramer. Our findings are consistent with an important function for the N-terminal region in affecting interactions elsewhere in the sequence.

Role of the central cavity in long-range effects

The central cavity in hemoglobin (shaded areas between dimer pairs in Fig. 1) consists of a continuum of chloride-binding regions through which the effects of non-covalent anion binding on one region of the central cavity are propagated to other regions, including the subunit interfaces[13–16]. The covalent sequence changes in the recombinant γ–β hemoglobin (Hb Felix) and in the mutant in which the subunit-interface sequences were exchanged (HbA/F) further emphasize the important role of the central cavity in transmission of signals as the cavity moves between its larger, deoxy, and the smaller, oxy, conformations. Studies of other proteins that have common functions but differ in sequence (i.e. proteins that are analogous to the fetal–adult hemoglobin system) are likely to improve our general understanding of such long-range effects in proteins.

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