For many years, hemoglobin (Hb) has been a model protein for studies of factors that affect the conformational states of proteins. 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. The recent unexpected finding that the interactions between these dimer pairs are much stronger in fetal Hb (HbF) than in adult Hb (HbA; 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 HbA with its enhanced tetramer stability. The N-terminal A helix of the non-globin chain sequence with the very tight interface between the two αβ dimers (arrows in Fig. 1) has a Kd of 0.01 μM. In a mutant that has only the γ subunit of HbA, the dimer interface itself also determines tetramer stability. 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.

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.

The difference in the oxygen-binding capacities of HbA and HbF is mainly due to more-efficient binding of 2,3-DPG to HbF than to HbA (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 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 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 (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 (Kd) by at least an order of magnitude. We found that the stability of the liganded HbF tetramer is nearly 100-fold greater (Kd = 0.01 μM) than that of liganded HbA (Kd = 0.68 μM). Shear et al. 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.
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. The greatly increased stability of liganded HbF (Ref. 3) is consistent with this tighter structure in the deoxy state. 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. 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 (K₅ₐ₅ = 0.03 μM) as that of HbF (K₅ₐ₅ = 0.01 μM), even though Hb Felix contains HbA residues at its tetramer–dimer interface, which is distant from the A helix. A natural fetal hemoglobin that has altered tetramer strength and a modified A helix

Normal human blood contains a minor HbF component, HbF₁, that is acetylated on the N-terminus of its γ subunit. 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 (K₅ₐ₅ = 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 suggest that there is an inter-relationship 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. The covalent sequence changes in the recombinant γ-β hemoglobin (Hb Felix) and in the mutant in which the subunit-interface sequences were exchanged (HbF/A) further emphasize the importance of the central cavity in transmission of signals as the cavity moves between its larger, deoxy, and the smaller, oxy, conformation. 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. Acknowledgements

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