Exchange of Subunit Interfaces between Recombinant Adult and Fetal Hemoglobins

EVIDENCE FOR A FUNCTIONAL INTER-RELATIONSHIP AMONG REGIONS OF THE TETRAMER

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The inter-relationship between the interior subunit interfaces and the exterior diphosphoglycerate (DPG) binding region of the hemoglobin tetramer and the effects of a specific N-terminal acetylation on tetramer assembly have been evaluated. Tetrameric fetal hemoglobin F in the liganded state was found to dissociate to dimers much less than previously appreciated, i.e. about 70 times less than adult hemoglobin A (Kd = 0.01 μM and 0.08 μM, for HbF and HbA, at pH 7.5, respectively) over the pH range 6.2–7.5, whereas HbF, in which the N-terminal of the γ-chains are acetylated, dissociates like HbA. To determine whether this feature of HbF could be transferred to hemoglobin A, the single amino acid difference in their αβαγ interfaces and the 4 amino acid differences in their αγαγ interfaces have been substituted in HbA to those in HbF. This pentasubstituted recombinant HbA/F had the correct molecular weight as determined by mass spectrometry, the expected mobility on isoelectric focusing, the calculated amino acid composition, and normal circular dichroism properties, oxygen binding, and cooperativity. Although HbA/F has the same amino acid side chains that bind DPG as HbA, its diminished response to 2,3-DPG resembled that of HbF. However, its tetramer-dimer dissociation constant (Kd = 0.14 μM) was between that of HbA and HbF despite the fact that it was composed entirely of HbF subunit interfaces. The results indicate that regions of the tetramer distant from the tetramer-dimer interface influence its dissociation and, reciprocally, that the interfaces affect regions involved in the binding of allosteric regulators, suggesting flexible long range inter-relationships in hemoglobin.

Some functional properties of HbF (α2γ2), such as its oxygen affinity and its interaction with allosteric regulators such as 2,3-DPG1 differ considerably from the corresponding values of HbA (α2β2) (1). The reasons for these differences, which play a very important physiological role in the transfer of O2 from maternal to fetal blood, are not yet fully understood in structural terms (2, 3). Whether they are due in part to the relative extents of tetramer-dimer dissociations of HbA and HbF is not known because the dissociation constant of HbF has not been reported, although it has been generally assumed to be similar to that of HbA. In this report, we show that these values differ considerably, as reported in preliminary form (4).

Another important function of HbF involves its role in the amelioration of the crises associated with sickle cell anemia by administration of therapeutic agents that result in its increased synthesis; the basis for this therapy is that sickle cell anemia patients with hereditary persistence of fetal hemoglobin have a clinically mild disease. There are two mechanisms by which HbF could inhibit HbS polymerization in the deoxy state where sickling occurs, i.e. through the formation of mixed hybrids of HbF and HbS (α2βγ) or by a direct “sparing” effect on the solubility of HbS by the HbF tetramers themselves (5–8). A prerequisite in the first mechanism is that there be a similar proportion of dimers of oxy-HbF and oxy-HbS (which dissociates like HbA; Ref. 9), which then re-associate randomly in the deoxy state to give HbS and HbF tetramers as well as the mixed hybrid. Indeed, mixed hybrid tetramer formation is demonstrable only with deoxyhemoglobin (10–12) but not with oxyhemoglobin. Thus, the relative extents of dimer formation from HbS and HbF in theoxy state dictate the amount of mixed hybrid in the deoxy sickling conformation, but this value for HbF is not known.

A question of general interest that we have addressed is whether there exists any functional inter-relationship between the two types of subunit interfaces in hemoglobin, i.e. the dimer α2β1 or α1γ1 interface and the tetramer α1β2 or α1γ2 interface either with each other or with the binding region for the allosteric regulator, 2,3-DPG. The converse of the latter linkage, i.e. the shift induced by DPG on the oxy to deoxy equilibrium and hence on subunit interfaces, has been known for some time (3). Another question that we also address here concerns the role of N-terminal acetylation of proteins, which is poorly understood (see Ref. 13, and references therein). Since there is a form of fetal hemoglobin (Hbf1) also present in human blood in which the N termini of its γ-chains are acetylated, we studied its tetramer-dimer dissociation properties, and we report here that this modification endows fetal hemoglobin with a significant increase in its tetramer-dimer dissociation properties. A possible general function for N-terminal acetylation is discussed.

To answer these questions, we employed a rapid and sensitive method for estimation of tetramer-dimer dissociation constants by high resolution gel filtration on Superose-12 (9). Since the eluted peaks were narrow and symmetrical with little

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1 The abbreviations used are: DPG, diphosphoglycerate; HPLC, high performance liquid chromatography.
skewing, the data derived solely from the peak positions could be subjected to a comprehensive mathematical analysis that yielded measurements of tetramer-dimer dissociation constants ($K_d$) that were in the range of published values obtained by a variety of techniques (14–18). In the present study, we further evaluate this procedure itself, and we used it to study the relative extents of dissociation of tetramers made up of $\alpha_2 \beta_2$ dimers (HbA) compared with $\gamma_2 \delta_2$ dimers (HbF and HbF$\delta$).

This study of a functional inter-relationship between different regions of the tetramer was possible because of the significant difference we found between the tetramer dissociations of HbF and HbA. To accomplish this objective, we constructed a recombinant pentasubstituted hemoglobin (referred to as HbA/F) composed of HbF subunit interfaces side chains but otherwise containing the HbA sequence.

**MATERIALS AND METHODS**

**Hemoglobins**—HbA (sometimes referred to as HbA0) was purified as described previously (9). HbF (sometimes called HbFo), which was kindly donated by Dr. Robert Bookchin (Albert Einstein College of Medicine), was isolated from postpartum umbilical cord blood. After purification it was >95% pure as ascertained by isoelectric focusing and by FPLC on a Mono S column; no acetylated HbF$\delta$ was detected. Amino acid analysis gave the correct composition for $\alpha$-chain (calculated, 15,126; found, 15,126 $\pm$ 3) and $\gamma$-Ac chain (calculated, 16,037; found, 16,042 $\pm$ 7), thus confirming the presence of a single acetyl group per $\gamma$-chain.

**Recombinant Substitution of Interfaces**—The construction of the five mutations was done by combining the amplified sequences bearing the relevant mutations using the strategy shown in Fig. 1. Since the interface residues that were mutated were clustered in two regions (residues 43 and 51 and residues 112, 116, and 125), we designed two pairs of oligonucleotides overlapping each of these regions. Their respective sequences were TTGACTCTTTGGGGATCTGTCCACTGCTGA and GGTACCTGGTCTGGCCATTCACTTTGGCAAAGAATTCACCCCAG-ACGTCGC. A second pair with their corresponding complementary sequence was also synthesized. Two other oligonucleotides were used, each overlapping one extremity of the $\beta$-coding sequence and containing a restriction site. A two-step amplification procedure adapted from the overlap extension method (19) was employed. The first amplification step was done in three different tubes to obtain the three parts of the $\beta$-coding sequence having the complementary mutated zones. The correct size of each amplified DNA was verified on agarose gel and purified using Geneclean (BIO 101, Inc., Vista, CA). During the second amplification step, the three parts resulting from the first amplification step were combined and amplified. The size of the total resulting coding sequence was checked on an agarose gel, purified, and digested with the appropriate restriction enzyme to be inserted into the pGS190 plasmid containing the $\alpha$ wild-type cDNA expression cassette.

Because of the frequency of mismatches during the two-step amplification by polymerase chain reaction, it was essential to check the fidelity of the amplified products using FPLC on a Mono S column and showed a single band upon isoelectric focusing. It had the correct mass ($\gamma$-chain, 15,995; kindly performed by Dr. Urooj Mirza and Dr. Brian Chait, Rockefeller University). HbF$\delta$, purified from the same source, eluted before HbF on a Pharmacia Mono S column and showed a single band upon isoelectric focusing. It had the correct mass ($\alpha$-chain (calculated, 15,126; found, 15,126 $\pm$ 3) and $\gamma$-Ac chain (calculated, 16,037; found, 16,042 $\pm$ 7), thus confirming the presence of a single acetyl group per $\gamma$-chain.

**Growth Conditions and Purification**—The growth of yeast was monitored and harvested as described previously (20–22). Purification of Hb to homogeneity was achieved on CM-52 as the CO form, as also described previously (21, 22), and subsequently on a Mono S column (Pharmacia) attached to a FPLC system. We refer to this pentasubstituted recombinant hemoglobin as HbA/F.

**Tetramer-Dimer Dissociation Constants**—The dissociation constants ($K_d$) were determined by the hemoglobin concentration dependence of peak positions on a Superose-12 HR10/30 column as it eluted between the positions of cross-linked tetrameric Hb and the natural dimeric Hb Rothschild as described previously in detail (9). The peak position used to determine the $K_d$ values was accurately measured by the Pharmacia FPLC Director software for each liganded (CO or O$_2$) Hb concentration; the mathematical analysis of the curves has been reported (9). The elution positions had a high degree of precision ($\pm$ 0.04 ml) and have been reproducible using the same column over at least a 2-year period. Each analysis on the Superose column took approximately 1 h for completion.

Since small size gel filtration studies on Sephadex supports have been criticized because of the possibility of sample dilution on the column and peak broadening leading to erroneous $K_d$ values (23), we have tested the extent to which this occurs on the Superose support used in our studies. Two types of analysis were performed as described below: one using Hb concentrations within the $K_d$ range (Fig. 6) and another using Hb concentrations higher than the $K_d$ (Table I). The values for peak widths and positions were determined by the FPLC software packages; reproducibility was 1% or better (see Table I).

The buffer routinely used for the study of the tetramer-dimer dissociations of HbA, HbF, HbF$\delta$, and HbA/F was made by adjusting the pH of 150 mM Tris base to pH 7.5 with glacial acetic acid. For lower pH values, the appropriate amount of glacial acetic acid was added. The

**FIG. 1. Strategy for construction of HbA/F expression cassette.**
concentrations of hemoglobins, which were determined by amino acid analysis of hydrolyzed samples, agreed within 3% with the concentrations determined by their visible spectra. For the Kₐ analyses, accurate dilutions were made with the Trias acetate buﬀers.

**HPLC Analysis**—The globin chains of HbA, HbF, and HbA/F were separated by reverse phase HPLC on a Vydac C₄ column attached to a Shimadzu HPLC unit using a gradient of 20–60% acetonitrile containing 0.1% trifluoroacetic acid. The eluent was monitored at 220 nm, and the peak retention times were determined with a Shimadzu CR 501 integrator. Amino acid analysis of the globin chains thus isolated was performed on a Beckman 6300 instrument with a System Gold data handling system.

**Mass Spectrometry Analysis**—Electrospray mass spectrometric analysis of HbA/F was kindly performed on a Finnigan-MAT TSQ-700 triple quadrupole mass spectrometer by Dr. Urooj Mirza and Dr. Brian Chait. Fifty pmol of the hemoglobin sample was loaded onto a desalting protein cartridge (Michrom BioResources, Inc., Auburn, CA) and washed with 1 ml of deionized water. The sample was eluted from the cartridge using a solution of water/acetonitrile/acetic acid, 30/67.5/2.5 (v/v/v) and electrosprayed directly into the mass spectrometer. The flow rate was maintained at 6 μl/min through a 100-μm inner diameter fused silica capillary.

**Functional Properties**—Oxygen binding curves were determined at 37°C on a modified Hem O Scan instrument (Aminco) after converting the Hb from the CO form in which it was purified to the O₂ form. This was also found for some other hemoglobins (21), the overall profiles in the Soret region, the maximum ellipticity was at 424 nm, which was identical to that of natural HbA. Except for the slightly lower ellipticity at 412 nm, which was also found for some other hemoglobins (21), the overall profiles in the Soret were identical, indicating no adverse effects on the heme pocket by the amino acid replacements.

**HPLC Analysis and Amino Acid Analysis**—Separation of the globin subunits of HbA/F by HPLC (Fig. 2) indicated that the recombinant β'/γ' subunit containing the 5 amino acid substitutions eluted in a much more retarded position than the natural β-chain of HbA. Its elution position was close to that of the γ-chain of HbF. Amino acid analysis of each subunit isolated by HPLC (Table II) gave the expected composition, consistent with the substitution of the 5 amino acids in the recombinant subunit. For the β chain of HbA/F, the presence of one Ile introduced by site-directed mutagenesis was diagnostic since the β-chain of HbA lacks Ile.

**Circular Dichroism**—In the far ultraviolet (220–225 nm), the circular dichroism profiles of the penta-substituted recombinant HbA/F were practically superimposable with those of recombinant HbS and of native HbA that we reported previously (21), indicating that there were no adverse effects of the mutations on the secondary structure of the recombinant Hb. In the Soret region, the maximum ellipticity was at 424 nm, which was identical to that of natural HbA. Except for the slightly lower ellipticity at 412 nm, which was also found for some other hemoglobins (21), the overall profiles in the Soret were identical, indicating no adverse effects on the heme pocket by the amino acid replacements.

**RESULTS**

**Properties of HbA/F**—The recombinant HbA/F was found to be pure by chromatography on a Pharmacia FPLC Mono S column. It showed one sharp band upon isoelectric focusing in the Isolab Resolve pH 6–8 system (20–22), and its migration was consistent with a net gain of four negative charges per tetramer. SDS-polyacrylamide gel electrophoresis, there was a single band at 16 kDa in the same position as the denatured subunits of HbA and HbF.

**Mass Spectrometry**—Mass spectrometric analysis of the recombinant HbA/F gave the expected mass for both subunits. Thus, the substitution in the α₂β₂ interface of Glu-43(β) by Asp and in the α₁β₁ interface Pro-51(β) by Ala, Cys-112(β) by Thr, His-116(β) by Ile, and Pro-125(β) by Glu gives a calculated mass of 15,832, which was the value found by mass spectrometry. The α-chain of HbA/F did not involve any amino acid replacements, and its mass, 15,125, agreed with the calculated value.

| [Hb] loaded | Volume loaded | Peak position | Peak width at half-height |
|------------|---------------|---------------|--------------------------|
| μl         | μl            | ml            | μl                       |
| A          |               |               |                          |
| 20.0       | 100           | 13.26         | 510                      |
| 20.0       | 50            | 13.26         | 510                      |
| 20.0       | 25            | 13.26         | 510                      |
| B          |               |               |                          |
| 10.0       | 100           | 13.26         | 510                      |
| 20.0       | 50            | 13.26         | 510                      |
| 40.0       | 25            | 13.26         | 510                      |
| Average    |               | 13.23 ± 0.04  | 513 ± 7                  |

**TABLE I**

Effect of sample volume and hemoglobin concentration on elution profiles

The experimental details are given under "Materials and Methods."
corresponding values on the x axis when y = 1 (9).

Hemoglobin A—The tetramer-dimer dissociation constant of liganded HbA is shown in Fig. 4A. Its $K_d$ value, 0.68 $\mu$M, obtained by mathematical analysis of the data (inset) is in good agreement with values obtained by several different procedures (e.g. Turner et al. (24) reported a value of 1.1 $\mu$M using the large-zone exclusion method on a Sephadex support, and Benesch and Kwong (17) found 0.7 $\mu$M using a method dependent on heme dissociation from dimers.

From the data of Williams and Kim (18), who used the highly precise ultracentrifugal method, a $K_d$ value of 0.3 $\mu$M for HbA at pH 7.0 can be calculated. The correlation between our value for the $K_d$ of HbA at pH 7.5 is consistent with their results. The range of published values for the $K_d$ of HbA is consistent with the validity of the high resolution method described here and the mathematical analysis of the data.

Hemoglobin F—To measure the dissociation constant of liganded HbF, much lower concentrations than those used for HbA were required as suggested by the results in Fig. 3. At pH 7.5, a $K_d$ value of 0.01 $\mu$M was calculated (Fig. 4B), indicating that the HbF tetramer is about 70 times less dissociated to dimers than HbA. The different dissociations for these hemoglobins explain an earlier report that there was no detectable exchange of HbF and HbA subunit interfaces in the pH range (Fig. 6), and it occurs maximally at concentrations of 0.5 $\mu$M in tetramer was applied separately in 100 $\mu$l to Superose-12 as described (9). At these concentrations, HbF is tetrameric and HbA is predominantly dimeric as calculated from their $K_d$ values in Fig. 4. The detector was set at 405 nm.

Effect of Protons on HbA and HbF Tetramer Dissociations—Increased tetramer to dimer dissociation with decreasing pH has been reported previously for HbA (26). We compared this effect for HbA and HbF and found that they each had the same profile with decreasing pH (Fig. 5) and the same 70-fold relative difference in their $K_d$ values persisted throughout the pH range studied. This result also shows the consistency of the Superose-12 gel filtration methods in providing information on relative $K_d$ differences between hemoglobins. The slope of both lines in Fig. 5 is about 1, suggesting the uptake of a proton for each type of tetramer ($\alpha_2\beta_2$ or $\alpha_2\gamma_2$) dissociating to dimers. Its structural basis is currently unknown.

HbA/F—The tetramer-dimer dissociation constant of the recombinant liganded HbA/F (Fig. 4C) was determined to be 0.14 $\mu$M. Thus, in toto, neither the single substitution of Glu-43$^\beta$ to Asp in the $\alpha_1\beta_2$ interface nor the four substitutions in the $\alpha_1\beta_1$ interface were able to transfer completely the dissociation properties of HbF to HbA, suggesting the involvement of other regions of the tetramer in the strength of the tetramer-dimer interface. Because the Glu $\rightarrow$ Asp substitution in the $\alpha_1\beta_2$ interface is conservative, the major contribution may arise from the changes of the amino acids in the $\alpha_1\gamma_1$ interface.

Hemoglobin F1—The presence of an acetyl group on the N terminus of each of its $\gamma$ chains has a profound effect on the tetramer-dimer dissociation of HbF1 (Fig. 4D); liganded HbF1 has a $K_d$ of 0.33 $\mu$M. Hence, it dissociates to dimers to about the same extent as HbA (Fig. 4A), thus reversing the 70-fold decreased tetramer-dimer dissociation of HbF (Fig. 4B). Possible reasons for this behavior are given under "Discussion."

Elution Profiles—There is a detectable increase in peak width for liganded HbF, HbF1, and HbA within the $K_d$ concentration range (Fig. 6), and it occurs maximally at concentra-

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**Table II**

**Amino acid composition of HbA/F chains**

| Amino acid | $\alpha$-Chain Calculated | $\alpha$-Chain Found | $\beta$-Chain Calculated | $\beta$-Chain Found | $\gamma$-Chain Calculated | $\gamma$-Chain Found |
|------------|--------------------------|----------------------|--------------------------|----------------------|--------------------------|----------------------|
| Lys        | 11                       | 10.9                 | 11                       | 11                   | 11.4                     | 11.4                 |
| His        | 10                       | 9.5                  | 9                        | 8                    | 7.6                      | 7.6                  |
| Arg        | 3                        | 2.9                  | 3                        | 3                    | 2.8                      | 2.8                  |
| Asp        | 12                       | 13.7                 | 13                       | 14                   | 14.5                     | 14.5                 |
| Thr$^a$    | 9                        | 8                    | 7                        | 8                    | 7.4                      | 7.4                  |
| Ser$^b$    | 11                       | 11.3                 | 5                        | 5                    | 6.7                      | 6.7                  |
| Glu        | 5                        | 7                    | 11                       | 11                   | 11                       | 11                   |
| Pro        | 7                        | 7.3                  | 7                        | 5                    | 5.5                      | 5.5                  |
| Gly        | 7                        | 8.6                  | 13                       | 13                   | 12.7                     | 12.7                 |
| Ala        | 21                       | 18.6                 | 15                       | 16                   | 14.6                     | 14.6                 |
| 1/2Cys$^c$ | 2                        | 0.5                  | 2                        | 1                    | 0.5                      | 0.5                  |
| Val$^d$    | 13                       | 11.5                 | 18                       | 18                   | 13.9                      | 13.9                 |
| Met$^e$    | 2                        | 0                    | 1                        | 1                    | 0                        | 0                    |
| Ile        | 0                        | 0                    | 0$^f$                    | 0$^f$                | 1$^d$                     | 1$^d$                |
| Leu        | 18                       | [18]$^g$             | 18                       | 18                   | [18]$^g$                 | [18]$^g$             |
| Tyr$^b$    | 3                        | 2.3                  | 3                        | 3                    | 0.7                      | 0.7                  |
| Phe        | 7                        | 6.7                  | 8                        | 8                    | 7.4                      | 7.4                  |

$^a$ These amino acids are partially or completely destroyed during acid hydrolysis.

$^b$ During the 20 h of acid hydrolysis, Val-Val sequences are incompletely hydrolyzed.

$^c$ The amino acids were calculated relative to the value found for Leu, which was set at 18.

$^d$ The values for Ile are diagnostic for a difference between the $\beta$ chain and the $\beta$$\gamma$ chain.

$^e$ $^f$ $^g$ Numbers in brackets represent values obtained by mathematical analysis of the data.
tions close to their $K_d$ values determined from the peak position (Fig. 6, arrows). The minimum peak widths occur when dimers or tetramers are the predominant species. Bell-shaped curves are found between these two extrema, i.e. at concentrations where maximal equilibration between dimers and tetramers occurs (the $K_d$ value). Various possibilities for this behavior are under study ranging from a simple mechanism involving compensating trailing of dimers and tetramers leading to increased peak widths in a symmetrical fashion to give a bell-shaped curve but not affecting peak position. Alternatively, a more complex dynamic mechanism involving the dissociation-association process itself could be involved. In either instance, the 70-fold difference between HbF and HbA is apparent whether peak positions or half-heights are considered. When concentrations of HbA higher than its $K_d$ value were injected in different volumes onto the column, the eluted peak positions and widths were practically constant (Table I). Erroneous $K_d$ values due to changes in peak positions and widths on Sephadex supports as a function of the sample volume and concentration (23) are not encountered to a significant extent on the Superose supports under these conditions.

**Functional Properties and Effect of 2,3-DPG**—In the absence of 2,3-DPG, the oxygen affinities of HbA, HbF, and HbA/F were nearly the same at 37° within experimental error and each showed significant cooperativity with $n$ values ranging from 2.1 to 2.7 for all samples (Table III). The slightly lower oxygen affinity of HbF compared with HbA found by tonometry (27) is beyond the sensitivity of the instrument used here. Nevertheless, our values are completely reproducible and the extent of responses to 2,3-DPG for HbA and HbF reported in Table III are similar to those obtained by tonometry (27). We tested a 10-fold excess of 2,3-DPG to Hb to ensure that the maximum effect of the allosteric regulator would be achieved. A significant lowering of the oxygen affinity of HbA was found, but a smaller effect on HbF was noted, consistent with previous reports (1, 27). The effect of 2,3-DPG on HbA/F at 2.5-, 5.0-, and 10.0-fold (data shown) DPG/Hb ratios was virtually the same as that for HbF and unlike that for HbA, even though the amino acids in its DPG binding site are the same as those in HbA. These results demonstrate that the response to the allosteric regulator, which binds to a site between the two $\beta$-chains, is also controlled to a significant extent by the subunit interface or other interactions.

**DISCUSSION**

The opportunity arose to determine whether there existed an inter-relationship between different regions of the hemoglobin molecule because adult hemoglobin A and fetal hemoglobin F were found to dissociate to dimers to significantly different extents. This observation permitted us to determine whether there was a relationship between the tetramer-dimer interface and the dimer-monomer interface. The well known difference between HbA and HbF in their ability to bind the allosteric regulator 2,3-DPG allowed us to study the effects of the subunit interfaces on the allosteric binding site for DPG. Both objectives were addressed by the experiments with the recombinant HbA/F. The yeast expression system used to produce HbA/F has been shown by a variety of criteria to yield recombinant hemoglobins with the same biochemical and functional properties as their native counterparts (20–22, 27–29). Furthermore,
the tetramer-dimer dissociation constants that we reported using small zone gel filtration on Superose-12 are the same within experimental error for native HbS ($K_d = 0.4 \, \mu M$) and recombinant HbS ($K_d = 0.7 \, \mu M$) and are in the same range as the 0.2 $\mu M$ value reported by Williams and Kim using the ultracentrifuge (18) although different pH values were used. Other $K_d$ values that we have reported (9) using this procedure for the natural variant Hb Yakima and the chemically modified carboxymethyl Hb are in agreement with those found by Benesch and Kwong (17), who used a procedure based on heme loss and by Turner et al. (24), who used large zone filtration on Sephadex. The differences in $K_d$ values reported here for HbA, HbF, HbF1, and HbA/F may be regarded as significant and due to effects of the substitutions themselves and not to any anomalous properties of the recombinant HbA/F or to the procedure used to measure these values.

The recombinant HbA/F is composed of the same amino acids in its tetramer-dimer interface and dimer-monomer interfaces as in HbF, but otherwise it has the remainder of the HbA sequence. HbA/F has not undergone significant structural rearrangements as shown by its circular dichroism properties, which are identical to those of natural and recombinant hemoglobin studies earlier (21). However, it possesses two properties that are not quantitatively characteristic of either hemoglobin A or F, i.e., its tetramer-dimer dissociation and its response to 2,3-DPG. These changes represent an indirect effect on the Hb tetramer brought about by the total subunit interface exchange, suggesting that reciprocal long range effects exist between the interior interfaces that can translate to the exterior region such as the DPG site. Indeed, the well known shift of the oxy (R) to deoxy (T) equilibrium induced by DPG binding can be considered as the corollary to this effect. The reduced binding of 2,3-DPG to HbF has generally been considered to be due to the specific replacement of His-143 in HbA to a Ser in the HbF $\gamma$-chains, but we demonstrate here that the interior subunit interfaces also play a significant general role in this process since HbA/F retains His-143. Furthermore, the tetramer-dimer $K_d$ of HbA/F was found to be between that of HbF and HbA even though the recombinant Hb has the HbF interfaces, indicating that regions other than the subunit interfaces themselves affect subunit dissociation of the tetramer. Fronticelli et al. (16) reported evidence consistent with propagation of an effect of amino acid substitution within the $\alpha_1\beta_1$ interface to the $\alpha_1\beta_2$ interface. Although the dissociation of $\alpha_\beta$ and $\alpha_\gamma$ dimers into their monomeric constituents is known to be different (30), the actual tetramer-dimer dissociation constant of HbF has not been reported. However, it has been widely assumed to be similar to that of HbA, but no systematic investigation has ever been reported to our knowledge. The decreased dissociation of HbF described here probably accounts for the greater stability of the low affinity quaternary conformation of HbF relative to HbA (31). Thus, the difference in dissociation properties between the
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two co-existing hemoglobins when viewed in relation to the 20% decrease in maternal HbA concentration and the 50% increase in fetal hemoglobin concentration during gestation (32) would result in a greater percent of HbA dimers relative to the HbF dimers. Consequently, the ratio of HbF to HbA tetramers may result in more O2 release from HbF compared with HbA. Other physiological factors are surely involved in facilitating O2 transfer (32), but the tetramer-dimer dissociation process described here likely also plays a role.

The increase in O2 affinity at low pH for both HbA and HbF (acid Bohr effect, reverse Bohr effect) is well documented, but whether its structural basis is related to R-state and T-state tetramers is unknown. Moreover, the acid Bohr effect is less pronounced (~20%) for HbF than for HbA (1). Whether or not this is due to the decreased tetramer R-state stability for HbA compared with HbF into the acid pH range (Fig. 5) awaits further study.

Several investigators (33, 34) have attributed the greater stability of the HbF tetramer compared with HbA as due to increased electrostatic interactions as well as to increased hydrophobicity upon conversion of the deoxy to the oxy conformation. Our results on the greatly increased hydrophobic nature of the ψ/γ recombinant chain during HPLC analysis compared with that of the ψ-chain show that this increased hydrophobicity also pertains to the individual heme-free subunits during reverse phase chromatography in organic solvents. Furthermore, since the ψ/γ recombinant subunit behaves much more like the γ-subunit than the ψ-subunit during HPLC, such increased hydrophobicity can be ascribed to a small number of amino acids, in this instance 5 or perhaps fewer. The substitution of His-116(ψ) to Ile is a prime candidate for such a role.

The occurrence of α-N-acetyl groups on the N-terminal residues of many proteins not only represents an obstacle to sequence determination but has also been a long standing enigma since its biological function is not known. For the hemoglobin system, HbF, represents that portion of the nascent γ-chain that is incompletely processed by the deacetylase system. The likely reason for the 30-fold increased dissociation of HbF, compared with HbF is the presence of α-N-terminal acetyl groups at the juncture where αγ dimers assemble to form the tetramer. This explanation is qualitatively similar to the 2-fold increased tetramer dissociation of HbA when it is carboxymethylated on its N-terminal residues (9). The even greater effect of the acetyl groups in promoting tetramer dissociation compared with the carboxymethyl groups would appear to be their ability to abolish completely the positive charge at the N termini, whereas carboxymethyl groups do not abolish charge. The function of the N-terminal acetylation in the embryonic hemoglobin Portland (ζζγδ) in its chloride response (35) is consistent with this concept, i.e. that acetylation modulates protein interactions of biological significance. In view of the effect of acetylation of histone lysine ε-amino groups in decreasing its association with DNA leading to active gene expression (36, 37), the role of α and ε amino group modification may share a common mechanism in influencing either protein-protein or protein-nucleic acid interactions.

The overall conclusions are that localized modifiers such as specific acetylation at certain critical sites where subunits assemble and other segments of the hemoglobin sequence apart from the tetramer-dimer interface itself influence its overall dissociation process as well as the binding site for the allosteric regulator 2,3-DPG between the β-subunits. The results in general convey the concept of flexible long range effects between the same or different subunits and reciprocal inter-relationships in hemoglobin. Further studies are in progress to define the location(s) of these latter segments, to assess their contribution to the dynamics of the subunit interfaces themselves, and to elucidate further the role of protein acetylation.

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