Transposing Sequences between Fetal and Adult Hemoglobins Indicates Which Subunits and Regulatory Molecule Interfaces Are Functionally Related[†]

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ABSTRACT: To correlate amino acid sequence changes with hemoglobin function we are carrying out a detailed recombinant analysis of the adult hemoglobin/fetal hemoglobin (HbA/HbF) systems. The important physiological differences between these two tetramers lie at unspecified sites in the 39 sequence substitutions of the 146 amino acids in their β and γ chains. In this paper, significant differences in the tetramer−dimer dissociation constants (referred to as tetramer "strength" or "stability") of adult (HbA) and fetal (HbF) hemoglobin tetramers have been used to probe the relationship between the allosteric, sliding interface and the effects of the allosteric regulator, 2,3-DPG, in promoting oxygen release. The single amino acid difference at the allosteric interfaces of these two hemoglobins, Glu-43(β) → Asp-43(γ), which is not near the DPG binding site, leads to a significantly lower DPG response, approaching that of HbF. The results are inconsistent with the long-held idea that the replacement of His-143(β) in HbA to Ser-143(γ) in HbF is solely responsible for the lowered DPG response in HbF. On the other hand, the Val-1(β) → Gly-1(γ) replacement near the DPG binding site has no effect on the DPG response. The replacement of His-116(β) by the hydrophobic Ile-116(γ) at the rigid $\alpha_1\beta_1$ interface has a marginal yet detectable effect on the allosteric $\alpha_1\beta_2$ interface. The results, overall, are interpreted using a model involving electrostatic coupling between certain side chains and extend the concept of a long-range relationship between some distant regions of the tetramer that are likely mediated through the central cavity.

Functional tetrameric mammalian hemoglobins have two types of subunit interfaces, a mobile or sliding $\alpha_1\beta_2$ (and $\alpha_2\beta_1$) tetramer−dimer interface and a rigid, or packing, $\alpha_1\beta_1$ (and $\alpha_2\beta_2$) dimer−monomer interface for HbA[1] (*1*). The subunit interactions at the mobile tetramer−dimer interface are critical to the regulatory allosteric mechanism involving the oxy (R) and deoxy (T) conformational states of the hemoglobin tetramer. The contacts between the subunits at all sets of Hb interfaces are well-known (*1*). Likewise, the allosteric regulator 2,3-diphosphoglycerate (2,3-DPG) is known to bind stoichiometrically at the interface between the two β-subunits in deoxyhemoglobin A (HbA) (*2, 3*) to promote oxygen release by shifting the allosteric equilibrium from the oxy (R) to the deoxy (T) conformation, concomitant with rearrangement of the sliding contacts at the tetramer−dimer interface. In fetal hemoglobin F, the same allosteric transition occurs at the $\alpha_1\gamma_2$ interface, but the 2,3-DPG response is less pronounced than in adult hemoglobin A (*4*). This property represents an important physiological difference between these two hemoglobins because it permits the transfer of oxygen from maternal to fetal hemoglobin in vivo. The differences in the response of HbA and HbF to 2,3-DPG have previously been considered to be due solely to the replacement of His-143(β), one of the 2,3-DPG binding residues in HbA, to a Ser residue in HbF. Our results show that the 2,3-DPG response is also a function of the nature of the amino acids at the allosteric tetramer−dimer interface and not simply those at the DPG binding interface.

There are unanswered questions regarding the interactions between the two types of subunit interfaces and the one that binds the allosteric regulator, 2,3-DPG. We have addressed these questions by studying two significant differences between HbA and HbF: (a) their different responses to 2,3-DPG (*4*) and (b) the intrinsic differences in their tetramer strengths at the $\alpha_1\beta_2/\alpha_1\gamma_2$ interfaces in their R states, as manifested by the 68-fold difference in their tetramer−dimer dissociation constants (*5*). The reason(s) for the increased tetramer strength of the liganded state of HbF, compared to that of HbA, is not known. This difference represents a significant free-energy change in these two tetramers, yet

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[1] Abbreviations: Hb, hemoglobin; HbF, fetal hemoglobin; HbA, adult hemoglobin; DPG, 2,3-diphosphoglycerate; R state, liganded conformation; T state, unliganded conformation; K_d, tetramer−dimer dissociation constant; PCR, polymerase chain reaction; HPLC, high-pressure liquid chromatography.

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the similarity in their intrinsic oxygen affinities in the absence of 2,3-DPG does not explain this free-energy difference (*4*−*6*). One purpose of these studies is to locate the source(s) of the increased tetramer strength of HbF by making sequence replacements at selected and critical sites and evaluating how these influence the effect of DPG on oxygen binding. Such an approach will likely facilitate a systematic investigation of hemoglobin thermodynamics such as that described by Turner et al. (*6*); extensive studies on the thermodynamic properties of Hb mutants, with substitutions at various locations at the $\alpha_1\beta_2$ interface, have provided information on the ratio of R to T conformational states in the absence of 2,3-DPG (*6*). However, the effects of point mutations at this interface have not generally been considered to be transmitted to distant regions of the tetramer, or vice versa, i.e., mutations apart from the interface could influence its properties.

In recent studies, designed to understand the source of the increased tetramer strength of HbF in its R state, the single amino acid difference at the mobile, tetramer−dimer allosteric interfaces of HbF and HbA was substituted together with the four amino acid differences at their dimer−monomer interfaces in a pentasubstituted recombinant HbA/F (*5*). In this recombinant Hb, representing all of the subunit interface differences between these two hemoglobins, only a fraction of the decreased dissociation of HbF was restored. Subsequently, we found that a large contribution to the increased tetramer strength of HbF was derived from the A-helix of the γ-subunit, demonstrating the existence of long-range effects in the tetramer, probably mediated through helical rearrangements (*7*, *8*).

Even though it is understood that 2,3-DPG binding in HbA causes a rearrangement of the sliding contacts at the tetramer−dimer $\alpha_1\beta_2$ interface, during the transition to the deoxy state (*1*), the *reverse* process, i.e., how the strength of the contacts at this interface influence oxygen binding in the presence of 2,3-DPG, has not yet been studied in detail. This issue essentially focuses on the extent to which an allosteric regulatory molecule (2,3-DPG) affects the distant site that it influences (the allosteric interface) or whether the nature of the interface itself is a controlling factor. In the present paper, we report results on Hb E43D(β), the only substitution at the mobile interfaces of HbA and HbF. We show that the latter replacement accounts for most of the dissociation properties of the pentasubstituted HbA/F and a significant portion of its decreased DPG response. This study is part of our objective to gain a broader understanding of inter-relationships between all of the regions of the Hb tetramer by introducing substitutions in two well-known hemoglobins, HbA and HbF, at selected sites of the 39 amino acid differences (of the 146 total residues) between β and γ subunits.

The N-terminal residue of the A-helix of the β-subunit is Val, which has a pK_a of 6.6 in the liganded state, but the corresponding Gly on the γ-subunit has a much higher pK_a of 8.1 (*9*). Because unprotonated amino groups bind more CO_2 than the protonated forms, HbF binds less CO_2 than does HbA. Because these N-termini are part of the 2,3-DPG binding site, it was of interest to determine how the Val → Gly substitution, V1G(β), influenced the response to 2,3-DPG in a recombinant Hb that retained all the other β-subunit amino acids. By comparing these effects in the two recombinant hemoglobins described here, E43D(β) and V1G(β), as well as their subunit cooperativity with the corresponding properties in natural HbF and HbA, possible deleterious effects on the sensitive 2,3-DPG response due to the yeast expression system itself can be assessed.

Another important property of HbF is its ability to significantly inhibit the polymerization of sickle Hb both in vitro and in vivo (*10*). The extent to which the greatly decreased dissociation of HbF tetramers to dimers compared to HbS tetramers (*11*), which would reduce formation of the $\alpha_2\beta^S\gamma$ polymerization terminator, needs to be evaluated. Because 2,3-DPG is present in sickling erythrocytes, information on its interaction with HbF is obviously important to understand fully the role of HbF in ameliorating the symptoms of sickle-cell disease.

EXPERIMENTAL PROCEDURES

Materials. Restriction enzymes, alkaline phosphatase, and T4 DNA ligase were from New England Biolabs (Beverly, MA). The DNA polymerases used for PCR were Taq polymerase (Amersham), Vent DNA polymerase (New England Biolabs), or cloned pfu polymerase (Stratagene). The deoxynucleotide set (dNTP) was from Amersham. The oligonucleotides containing the mutations were synthesized by Operon Technologies (Alameda, CA) or by the Protein Sequencing Facility at Rockefeller University (New York, NY). 2,3-DPG and IHP were purchased from Sigma (St. Louis, MO). The Geneclean kit was from Bio 101 (La Jolla, CA). The Qiagen plasmid midi kit was from Qiagen Inc. (Santa Clarita, CA). *Escherichia coli* XLI-Blue was used for cloning manipulations, and *Saccharomyces cerevisiae* GSY112 Cir° was used for expression. The growth conditions in a 20 L New Brunswick Fermentor BioFlo IV and the plasmids used in cloning, pGS189$\alpha\beta$ and pGS189α, have been described previously (*5*, *14*).

Construction of Mutants. The construction of mutants was done by the overlap extension PCR-based mutagenesis strategy (*5*). A *XhoI* fragment of pGS189 containing the β-globin coding sequence was prepared by the Geneclean III kit, after agarose-gel electrophoresis, and used as a template for the first amplification step. The first PCR was performed in two different tubes in a GeneAmp PCR system 2400 (Perkin-Elmer, Norwalk, CT) with 25 cycles of 30 s denaturation at 97 °C, 1 min primer/template annealing at 50 °C, and 2 min (with an increase of 1 s each cycle) extension at 72 °C. The correct size of each amplified DNA was verified by agarose-gel electrophoresis, and it was purified using the Geneclean III kit. During the second amplification step, the two fragments were combined and amplified with 45 °C primer/template annealing temperature to produce the entire β-globin coding sequence containing the desired mutation.

The size of the entire β-coding sequence from the two PCR steps was checked by agarose-gel electrophoresis. This DNA was purified by elution of the appropriate band from the gel, digested with *XhoI*, and inserted into the pGS190α. Because of the frequency of mismatch and the risk of contamination by the template wild-type β cassette during the two-step PCR, the full β cDNA was sequenced after inserting into pGS190α. Both the α and the β cassettes were then excised together from the pGS190$\alpha\beta$ plasmid by

digestion by *NotI*. Because the sizes of these two fragments were similar, *BGLI* was used together with *NotI* to digest the pGS190α*β*. The mutated α*β* cassette was then inserted into the shuttle *E. coli*/yeast plasmid pGS389. Because of the large difference in size between the vector and the insert, a fast-screening method was used with a large number of colonies. Streaked transformants were picked and suspended in 40 μL of lysis buffer. Then, 3 μL of 1 N HCl and 5 μL of loading buffer were added to each tube. The tubes were kept at −80 °C for 15 min and centrifuged at 10000*g* for 10 min. The correct construction was verified by its restriction profile on an agarose gel. After the positive clone had been identified, the pGS389α*β* plasmid was prepared in large quantity (Midi Prep, Qiagen, Chatsworth, CA) and transformed into the GYS112 *S. cerevisiae* strain using the lithium acetate method as described (*5*).

Characterization of Mutants. The three-step strategy used to characterize the recombinant hemoglobins has been described elsewhere (*14*). The molecular masses of the intact Hb subunits were measured by ES/MS using a Finnigan-MAT TSQ-700 electrospray-triple quadrupole mass spectrometer (ThermoQuest, San Jose, CA). Fifty nanomolar hemoglobin subunits in water−methanol−acetic acid (49:50:1, v/v/v) were infused at 1 μL min^{-1} through a 50 μm (i.d.) fused-silica capillary into the ion source of the mass spectrometer and electrosprayed at +3.8 kV. The desolvation of protein ions was accomplished by maintaining the heated capillary at 180 °C. One hundred spectral scans were acquired at a rate of 1 scan/s and averaged to produce the final spectrum.

For peptide analysis, 250 pmol of recombinant hemoglobin was diluted in 100 mM aqueous ammonium bicarbonate solution, pH 8.2, and 2 mM calcium chloride and incubated for 5 min at 37 °C. Modified L-1-tosylamido-2-phenylethyl chloromethyl ketone-trypsin (Böehringer Mannheim) was added at zero time and at 4 h (final substrate-to-enzyme ratio at 20:1, w/w), for 8 h total at 37 °C with occasional stirring. The final reaction volume was 50 μL, and the final hemoglobin concentration was 5 μM. The reaction was stopped by adding 50 μL of water−methanol−acetic acid (49:49:2, v/v/v). Aliquots of 1 μL were mixed with 19 μL of saturated solutions of α-cyano-4-hydroxycinnamic acid (*4hcca*) in either water−acetonitrile (1:1, v/v) or water−acetonitrile−TFA (1:2, v/v, 0.1% TFA). Aliquots of 0.5 μL of the latter solutions were deposited onto a gold-coated sample plate and allowed to air dry. The dried areas were washed with 0.5 μL of cold 0.1% aqueous TFA solution prior to undergoing analysis on a matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (model STR Voyager, PerSeptive, Perkin-Elmer, Boston, MA) equipped with delayed extraction and ion reflection.

For mass spectrometric fragmentation of the peptides containing mutated residues, the hemoglobin digest was diluted to 0.05 μM in water−methanol−acetic acid (49:50:1, v/v/v) and infused at 1 μL min^{-1} through a 50 μm (i.d.) fused-silica capillary into the source of a Finnigan-MAT LCQ electrospray-ion trap mass spectrometer (ThermoQuest, San Jose, CA) and electrosprayed at 2.8 kV. The desolvation of peptide ions was accomplished by maintaining the heated capillary at 125 °C. For each peptide precursor ion selected, mass spectrometric fragmentation yielded a series of "b"- and "y"-type ions (*15*).

Tetramer−Dimer Dissociation Constants and Oxygen Binding. The tetramer−dimer dissociation constants (K_d) were measured by a rapid and precise small-zone gel-filtration method on the superose-12 column of an FPLC system (*11*,*16*). Different concentrations of liganded Hb in 100 μL were applied and eluted with 150 mM Tris-Ac buffer, pH 7.5 at a flow rate of 0.4 mL/min. The Hb concentrations used depended on the K_d value for a particular Hb and ranged from 0.01−0.10 μM for HbF to 0.10−3.0 μM for HbA, for example, as described (*5*). The concentration of each Hb was determined by amino acid analysis (*11*, *16*). The eluent was monitored at 405 nm, and the calculations were performed using Grafit. The oxygen binding curves of hemoglobin were determined at 37 °C on a modified Hem-O-Scan instrument (American Instrument Co., Silver Springs, MD) as described previously (*16*−*18*). Prior to the taking of the measurements, the buffer was changed to 50 mM bis-tris-Ac, pH 7.5, by passage through a Sephadex G-25 column (Pharmacia). Hemoglobin was converted from CO to oxy form, and the sample was concentrated using Centricon Microcon ultrafiltration devices (10 000 molecular weight cutoff, Amicon, Beverly, MA) to a final concentration of 0.6−1.0 mM for measurements of O_2 binding with a precision of ±1 mmHg. The measurements were carried out without or with a 5-fold molar excess of 2,3-DPG over Hb, and the Hill coefficient was calculated as described (*17*−*19*).

RESULTS

Relationship between the Rigid $\alpha_1\beta_1$ Interface and the Mobile Allosteric $\alpha_1\beta_2$ Interface. Whether there is communication between the mobile and the rigid interfaces of Hb is an issue that has been previously considered by Fronticelli et al. (*20*); this question has been difficult to study directly because the strength of the $\alpha_1\beta_1$ ($\alpha_2\beta_2$) dimer−monomer interface precludes accurate measurement of its dissociation in either R or T state. In contrast, the large difference in the strengths of the tetramer−dimer interfaces of HbF and HbA in their R states is easily measured (*5*). The limited number of substitutions in both types of interfaces in these two hemoglobins, therefore, presented an opportunity to determine whether a substitution in the rigid interface affected the strength of the mobile interface. Of the four amino acid differences between HbA and HbF at their dimer−monomer interfaces, the His 116 → Ile substitution was considered as the most likely candidate to have a possible effect on its properties with respect to influencing other regions of the tetramer because the other substitutions are more conservative. Glu-43(*β*) is the only difference between HbF and HbA at their allosteric tetramer−dimer interfaces, so we substituted it with the Asp that occurs at this position in HbF.

Characterization of Recombinant Hemoglobins. The entire cDNA coding region of the three mutants was sequenced and found to be correct. The purified mutants showed one peak upon FPLC chromatography on Mono-S and one band by isoelectric focusing. The masses of the α- and *β*-subunits of H116I(*β*), which are shown in Figure 1A, B, were correct, i.e., 15 126.6 ± 0.8 for the α-subunit (theory: 15 126.3) and 15 843.3 ± 0.8 for the *β*-subunit (theory: 15 843.2). MALDI-TOF/MS analysis of the tryptic peptides of both α- and *β*-subunits (Figure 1C) indicates 97% coverage for both subunits. In Figure 1C is shown the MS/MS spectrum of

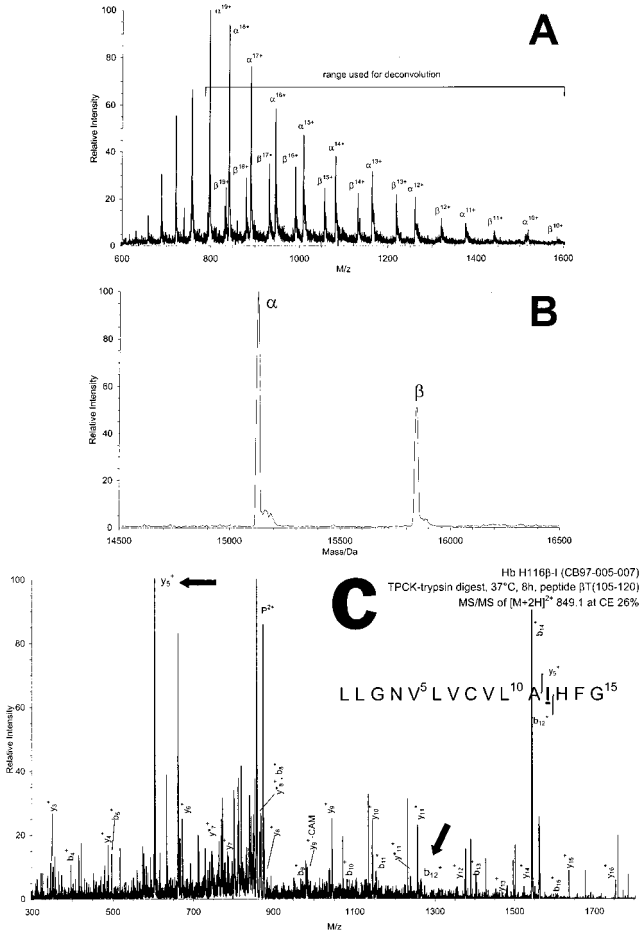

FIGURE 1: ES/MS of hemoglobin (H116I(β). Sample analysis is shown in panel A. Transformed mass spectrum is shown in panel B. MS/MS spectrum of peptide βT (105−120) from hemoglobin (H116I(β) (panel C). The substitution is underlined.

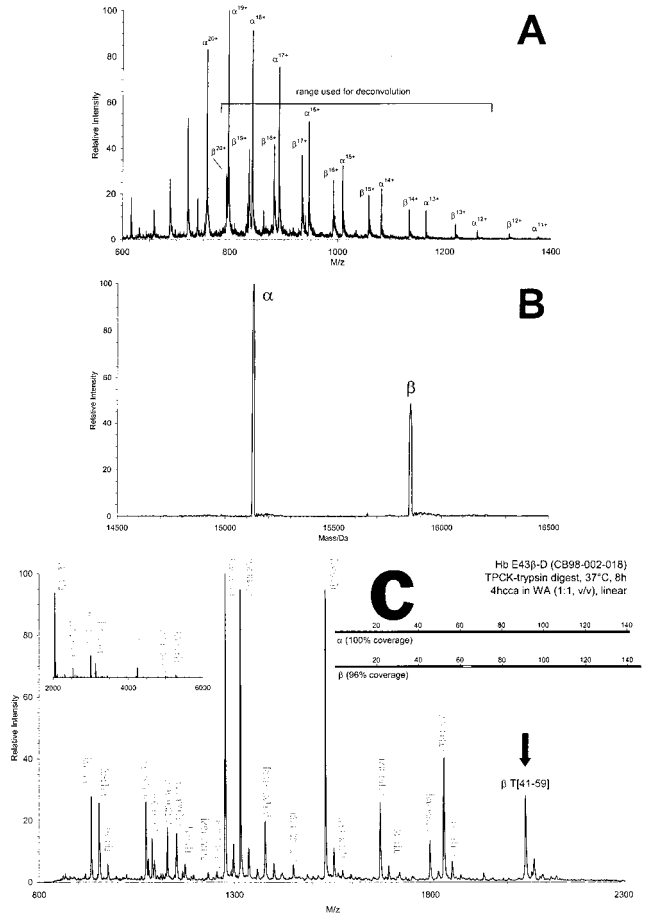

FIGURE 2: ES/MS of hemoglobin E43D(β). Sample analysis is shown in panel A. Transformed mass spectrum is shown in panel B. MALDI-TOF/MS spectrum of the mutant tryptic peptide from hemoglobin E43D(β) (panel C) (arrow).

the tryptic peptide βT (105−120) containing the mutation site at position 116. These results indicate that the correct mutation His116 → Ile had been introduced and no others were found.

For mutant E43D(β), ESI/MS analysis of the intact subunits showed that these had the correct masses, i.e., 15 126.4 ± 0.4 for the α-subunit (theory: 15 126.3) and 15 853.5 ± 0.3 for the mutant β-subunit (theory: 15 853.2) (Figure 2A, B). MALDI-TOF/MS analysis of the tryptic peptides showed that the correct mutant peptide, βT (*41*−*59*), was obtained (Figure 2C).

For mutant V1G(β), ESI/MS analysis of the intact subunits showed that these had the correct masses, i.e., 15 126.1 ± 0.4 for the α-subunit (theory: 15 126.3) and 15 825.1 ± 0.3 for the mutant β-subunit (theory 15 825.1) (Figure 3A, B). Analysis of the tryptic peptides that the correct BT1 mutant peptide was obtained (Figure 3C).

Conformation of Recombinant Hemoglobins. The amino-acid substitution of Ile(γ) for His(β) at position 116 at the dimer−monomer interface, the substitution of Asp(γ) for Glu(β) at position 43 of the tetramer−dimer interface, and the substitution of Gly(γ) for Val(β) at position 1 did not result in any global conformational change in the recombinant hemoglobins as ascertained from their circular dichroism (CD) spectra in the far ultraviolet, which was identical to that of HbA (*17*). The visible CD spectra in the Soret region were also practically identical to those of HbA and to the other recombinant hemoglobins that we have also described earlier (*17*−*19*).

Tetramer−Dimer K_d of H116I(β). The tetramer−dimer dissociation constant of H116I(β) was found to be 0.58 μM (Figure 4A), which is very close to the 0.68 μM value found for HbA (*5*). All of the experimental points fit a smooth hyperbolic curve, indicative of normal dissociation of tetramers to dimers. The K_d value can be read directly from the inset to Figure 4A, where the experimental line crosses the horizontal line at log 1. These results show that the properties of the $\alpha_1\beta_2$ interface are influenced to a slight extent by this nonconservative substitution at the $\alpha_1\beta_1$ interface.

Effect of Replacing the Single Difference at the Allosteric Interface of HbA and HbF on Tetramer−Dimer Dissociation. The only amino acid difference between HbA and HbF at their allosteric interfaces is a Glu(β) → Asp(γ) substitution at position 43. The pentasubstituted HbA/F mutant described previously (*5*) contains this replacement together with the four amino acid substitutions at the $\alpha_1\beta_1$ interface; it has a tetramer−dimer K_d of 0.14 μM (*5*). The E43D(β) recombinant Hb was found to have a K_d value of 0.21 μM (Figure 4B), which accounts for most of the tetramer−dimer strength of HbA/F. The E43D(β) mutant is slightly more electronegative than HbA on isoelectric focusing.

Effect of Replacing Val by Gly at the DPG Binding Site. The mutant V1G(β) was slightly more positively charged

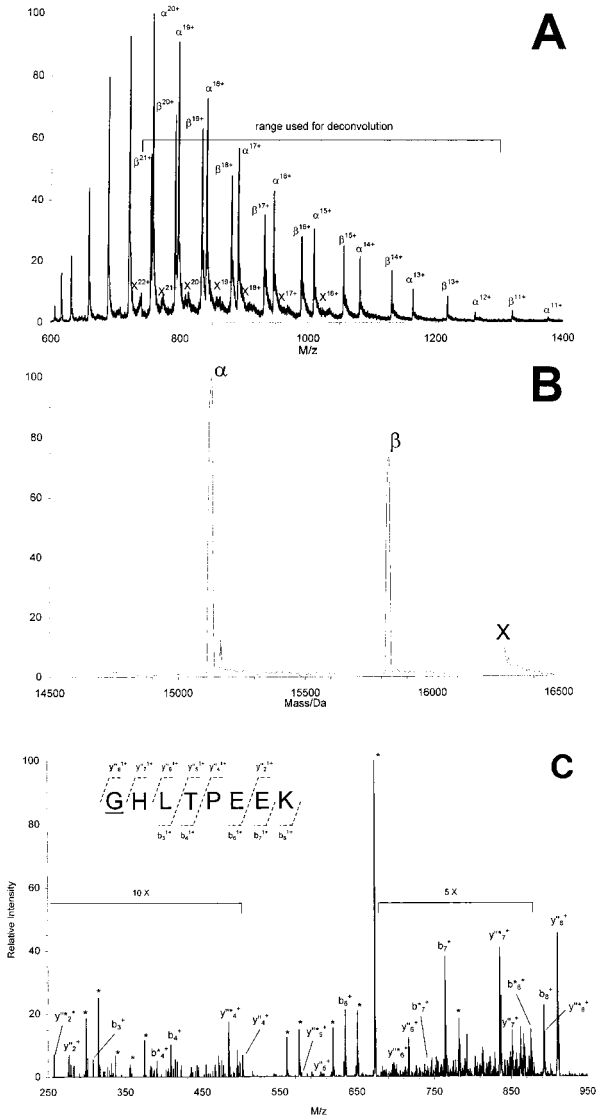

FIGURE 3: ES/MS of hemoglobin V1G(β). Sample analysis is shown in panel A. Transformed mass spectrum is shown in panel B. MS/MS spectrum of peptide BT1 is shown in panel C.

than HbA, as shown by its migration on isoelectric focusing, consistent with its higher pK_a (*9*) described above. This substitution at the N-terminus of the A-helix of the β/γ subunit is one of the eight replacements in Hb Felix (*7*) and provides a more positive charge to one end of the central cavity. The tetramer−dimer K_d of Hb V1G(β) is 0.18 μM (Figure 4C), indicative of a significantly higher tetramer strength than that of HbA (Table 1). This accounts for part of the high tetramer strength of Hb Felix and is in accord with the findings of Perutz et al. (*21*) on the effects of increased positive charge in the central cavity.

Data Analysis. The procedure for measuring tetramer−dimer dissociation constants (K_d) has a precision of 10−15% (*11*, *16*). An evaluation of the K_d values of the seven hemoglobins in Table 1, numbers (1, 2), (3, 4, 5), and (6, 7), as separate groups, reveals that they are significantly different. This conclusion was confirmed by comparing the gel filtration elution profiles of HbF, HbA, and E43D(β) at equivalent concentrations (0.5 μM), at which HbF is completely tetrameric but HbA contains both tetramers and dimers. The results (Figure 4D) indicate that E43D(β) elutes between HbF and HbA, confirming its intermediate K_d determined independently (Figure 4B). Mutant V1G(β) behaved in the same fashion (data not shown).

Table 1: Summary of Tetramer−Dimer Dissociation Constants[a]

no.	hemoglobin	K_d (μM)	location of amino-acid substitutions
1	HbA	0.68	none
2	H116I(β)	0.58	$\alpha_1\beta_1$ interface
3	E43D(β)	0.21	$\alpha_1\beta_2$ interface
4	V1G(β)	0.18	DPG site at central cavity
5	HbA/F	0.14	$\alpha_1\beta_2$ and $\alpha_1\beta_1$ interfaces
6	Hb Felix	0.03	A-helix of the γ-subunit
7	HbF	0.01	none

[a] The K_d values of each liganded Hb were determined as described in the text.

Effects of Mutations on Oxygen Binding. The oxygen-binding properties of the recombinant mutants V1G(β), E43D(β), HbA/F (*5*), Hb Felix (*6*), and natural HbA and HbF in the presence or absence of 2,3-DPG are shown in Table 2. The recombinant hemoglobins E43D(β) and V1G-(β) are cooperative, as demonstrated by the Hill plots in Figure 5. The values for the reduced DPG response of HbF compared to HbA, shown in Table 2 (5 mmHg vs 13 mmHg, respectively), have been known for many years (*4*). The recombinant E43D(β) hemoglobin has a reduced DPG response of 7 mmHg (Table 2, Figure 5). The reduction in 2,3-DPG response of the pentasubstituted mutant HbA/F (4 mmHg) compared to HbA (*5*) is, therefore, due in large part to the single amino acid replacement at the $\alpha_1\beta_2$ interface, E43D(β). Hb E43D(β) has a response to 2,3-DPG that approaches the limiting value of HbF, even though it contains all of the HbA binding residues at its DPG binding interface. These results indicate that coordination is required between amino acids at the binding site for the allosteric regulator and those involved in the conformational switch at the allosteric $\alpha_1\beta_2$ and $\alpha_1\gamma_2$ interfaces.

Recombinant V1G(β) has a normal response to 2,3-DPG of 14 mmHg, practically identical to that of HbA (Table 2), showing that the procedures used here to express these recombinant hemoglobins do not compromise such a sensitive functional feature as the effect of 2,3-DPG on the conformational equilibria of HbA.

DISCUSSION

Even though the substitutions in the recombinant hemoglobins HbA/F and Hb E43D(β) do not directly involve the DPG binding interface, these hemoglobins are closer to HbF than to HbA in their DPG responses. The long-held notion that in HbF, the substitution of His-143(β) by Ser at the DPG binding site is *solely* responsible for its lowered DPG response requires reevaluation in light of these results. Recently, Fang et al. (*22*), using proton NMR on a recombinant Hb in which His-143(β) was substituted by Ser, also reported results consistent with this conclusion.

In their structural comparison of hemoglobin A and F, Frier and Perutz (*23*) found that the eight substitutions in the A helix of the β vs γ subunits caused the A helix to move toward the E helix, resulting in a tighter overall structure for deoxy HbF. Hb Felix, which contains the A helix of the γ-subunit (*7*) but all of the HbA amino acids at its allosteric interface, has a DPG response like that of HbA (Table 2). Thus, even though the strength of the allosteric

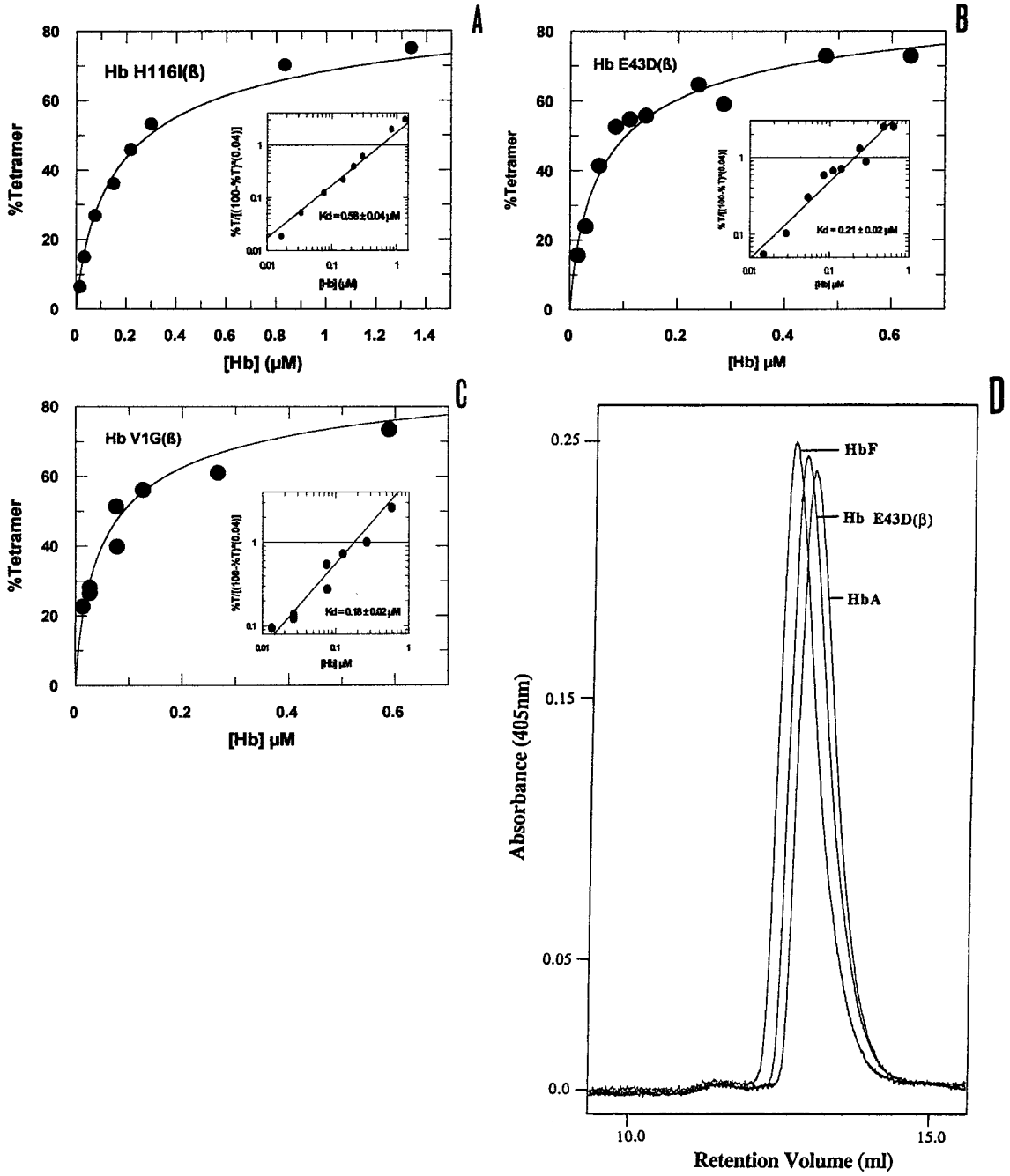

FIGURE 4: Tetramer−dimer dissociation constant of HbH116I(β) (panel A). The tetramer−dimer equilibrium data are shown in the main part of the figure. The K_d value, calculated as described in refs 11 and 16, is 0.58 μM (inset). Tetramer−dimer dissociation constant of HbE43D(β) (panel B); the K_d value shown in the inset is 0.21 μM. Tetramer−dimer dissociation constant of HbV1G(β) (Panel C). The K_d value shown in the inset is 0.18 μM. Elution positions of HbA, HbF, and HbE43D(β) (panel D). Each Hb at a concentration of 0.5 μM was applied to superose 12 as described in refs 11 and 16. The dilution on the column at peak half-height is about 5-fold (see ref 5), so that the effective concentration of each Hb was approximately 0.1 μM, at which HbA is mainly dissociated to dimers but HbF is predominantly tetrameric. HbE43D(β) elutes between these two hemoglobins according to its K_d value, determined in Figure 4B. The elution profiles were superimposed by the FPLC Director software program.

interface in Hb Felix in the oxy (R) conformation (in the absence of DPG) approaches that of HbF (*6*), the allosteric effector can still exert its full effect in the deoxy (T) to oxy (R) conformational switch because the amino acid contacts at its allosteric interface are the same as those in HbA. This result is consistent with the conclusion above on the lack of a complete DPG response for the E43D(β) mutant, i.e., that coordination between the DPG binding site and the allosteric interface is required with respect to the nature of amino acids present in order to attain a full DPG response.

The properties of Hb Felix and of HbA/F together account for both the increased tetramer strength and the lowered DPG response of HbF compared to HbA. These two recombinant hemoglobins have one-third (13/39) of the amino acid differences between β and γ subunits. The remaining 26 substitutions may be needed for other functions of HbF that are different from those in HbA, e.g., its different chloride and CO_2 responses or perhaps to some undiscovered function.

During the shift of the allosteric interface upon DPG binding in HbA, the central cavity in the T state widens at

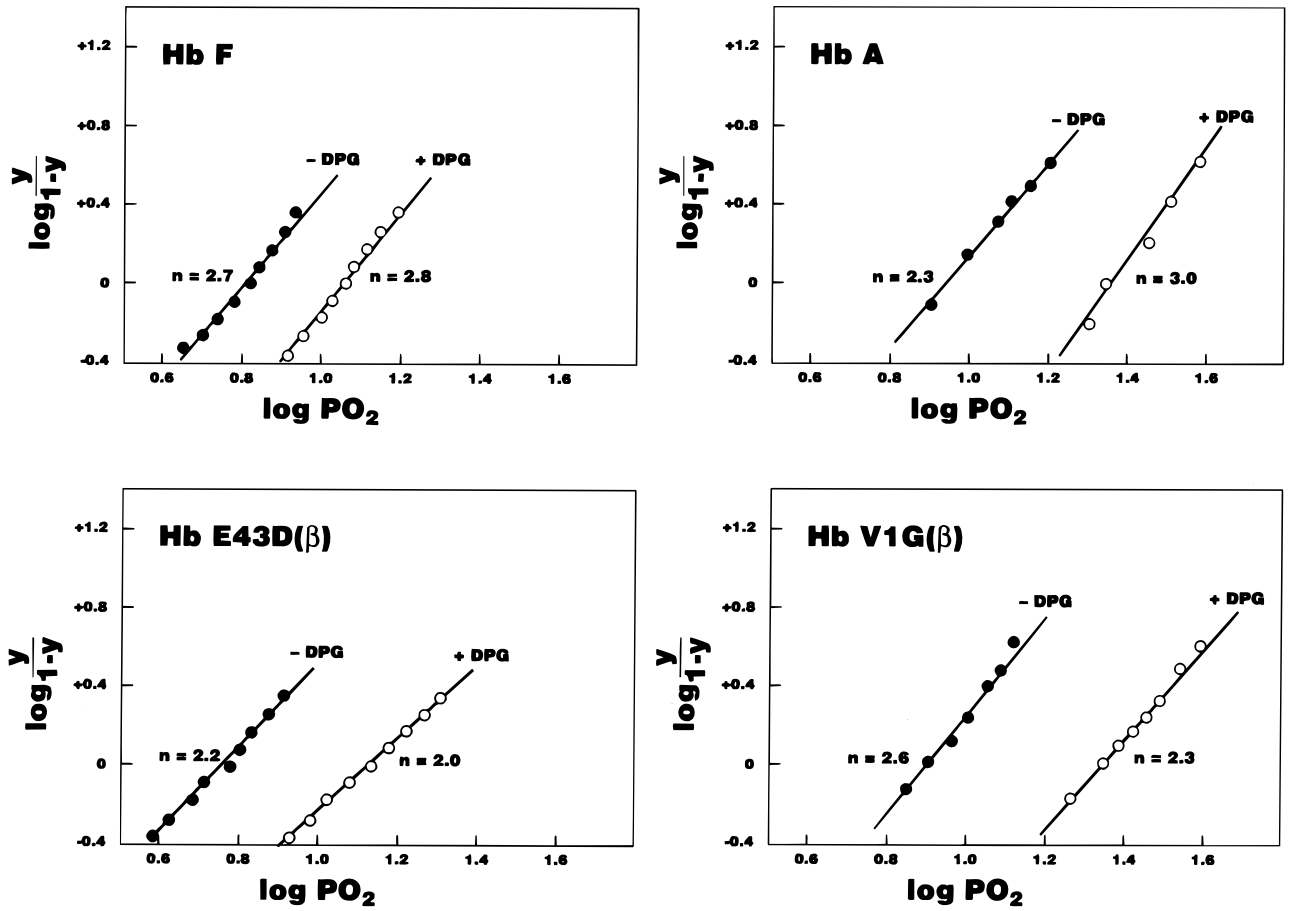

FIGURE 5: Hill plots of hemoglobins. The difference in P_{50} values (the log PO_2 where log $Y/1 - Y = 0$) is log 1.35 − log 0.94 = 14 mmHg for HbA; log 1.34 − log 0.90 = 14 mmHg for Hb V1G(β), i.e., the same DPG response for each Hb. The corresponding values for HbF are log 1.05 − log 0.81 = 5 mmHg and for Hb E43D(β) log 1.12 − log 0.75 = 7 mmHg, indicating that both have a lowered DPG response of similar magnitude and different from that of HbA and Hb V1G(β). Both natural HbA and natural HbF, as well as the two recombinant hemoglobins, are cooperative. The O_2 binding properties of the hemoglobins in the two left panels are intrinsically different from those in the two right panels.

Table 2: Effects of DPG on Oxygen Binding Properties[a]

		Without DPG		With DPG	
no.	hemoglobin	P_{50} (mmHg)	*n*	P_{50} (mmHg)	n
1	HbA	9	2.3	22	3.0
2	V1G(β)	8	2.6	22	2.4
3	E43D(β)	6	2.2	13	2.0
4	HbA/F	8	2.5	12[b]	2.7
5	Hb Felix	7	2.0	24	2.1
6	HbF	7	2.7	12	2.8

[a] The Hb concentrations were 0.5−0.8 mM (as tetramer) in 50 mM bis-tris-Ac, pH 7.5. P_{50} values were determined at 37°C. When present, the molar excess of 2.3-DPG was 5-fold over Hb tetramer. The precision of the P_{50} values is 1 mmHg for 3−5 separate measurements. [b] Taken from (5) and normalized to HbA and HbF

one end to accommodate optimal DPG binding. The involvement of the central cavity in the allosteric transition has recently been found to include chloride binding (*21*, *24*, *25*). In HbA, the presence of Glu-43(β) at the allosteric interface permits maximal oxygen release by allowing a complete conformational rearrangement at this interface, induced by DPG, but its replacement by Asp-43(γ) lowers the P_{50} to 13 mmHg rather than to 22 mmHg, the normal value for HbA. This result suggests that the central cavity may not open to its maximum unless all of the corresponding HbA amino acids are present at the distant $\alpha_1\beta_2$ interface, even if the HbA amino acids are present at the DPG binding interface. Further studies using this approach of exchanging segments of hemoglobin sequence to determine the effects on O_2 binding, DPG response, and the strength of subunit interactions should help answer the questions involving long-range interactions in proteins.

NOTE ADDED IN PROOF

We define electrostatic coupling as the intrinsic polarity between those charged amino acid side chains that are strategically positioned to influence overall protein structure and function yet may be distant from one another. In hemoglobin, enhancement of electrostatic coupling occurs either by increasing net positive charge at the nonhelical N-terminal segment at one end of the central cavity, as in mutant V1G(β), by increasing positive charge within the central cavity itself (*21*), or by increasing net negative charge at the allosteric subunit interface, as in mutant E43D(β). As a result of such increased polarity through the central cavity connecting the N-terminal tail and the allosteric subunit interface, the tetrameric structure is tightened, i.e., it dissociates to dimers less readily at this interface. Removal of positive charge at an N-terminal residue, as in the acetylated variety of fetal hemoglobin, decreases this polarity and loosens the tetrameric structure so that it is more dissociable

(*5*). Electrostatic coupling between remote sites may be a property common to many oligomeric proteins for affecting the strength of subunit interactions and potential assembly with other macromolecules.

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