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The N-terminal Sequence Affects Distant Helix Interactions in Hemoglobin

IMPLICATIONS FOR MUTANT PROTEINS FROM STUDIES ON RECOMBINANT HEMOGLOBIN FELIX*

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The N-terminal 18-amino acid sequence of the β-chain of hemoglobin, as far as the end of the A helix, has been replaced by the corresponding sequence of the γ-chain of fetal hemoglobin with the remaining sequence of the β-chain retained (helices B through H). The γ-β-chain had the correct mass, and its entire sequence was established by mass spectrometric analysis of its tryptic peptides; the α-chain also had the correct mass. This recombinant hemoglobin (named Hb Felix) retains cooperativity and has an oxygen affinity like that of HbA both in the presence and absence of the allosteric regulators, 2,3-diphosphoglycerate or chloride but differs from HbF in its 2,3-diphosphoglycerate response. However, Hb Felix has some features that resemble fetal hemoglobin, *i.e.* its significantly decreased tetramer-dimer dissociation and its circular dichroism spectrum, which measure the *strength* of the tetramer-dimer interface in the oxy conformation and its *rearrangement* to the deoxy conformation, respectively. Even though Hb Felix contains the HbA amino acids at its tetramer-dimer interface, which is located at a distance from the substitution sites, its interface properties resemble those of HbF. Therefore, the N-terminal sequence and not just those amino acids directly involved at the subunit interface contacts with α-chains must have a strong influence on this region of the molecule. The results reinforce the concept of fluid long range relationships among various parts of the hemoglobin tetramer (Dumoulin, A., Manning, L. R., Jenkins, W. T., Winslow, R. M., and Manning, J. M. (1997) *J. Biol. Chem.* 272, 31326–31332) and demonstrate the importance of the N-terminal sequence, especially in some mutant hemoglobins, in influencing its overall structure by affecting the relationship between helices.

The amino acid sequence of a protein contains the information necessary for its proper folding into the native state (1). Predicting patterns of protein folding into secondary and tertiary structures based on the primary structure is currently the focus of intense study in many laboratories. Assembly of such folded polypeptides into specific quaternary structures depends on how the individual subunits interact with one another. In human tetrameric hemoglobins there are two quaternary conformations, oxy (R) and deoxy (T), which are responsible for its functional binding and release of O_2, respectively (2, 3). We describe here how some of these quaternary subunit interactions involving α subunits in adult ($\alpha_2\beta_2$) and fetal ($\alpha_2\gamma_2$) hemoglobins are influenced by the N-terminal sequences of their β and γ subunits.

Adult and fetal hemoglobins have several significant differences in their functional behavior at the quaternary level. These differences include their responses to DPG[1] binding, which affect their ability to carry O_2 (4), the strengths of their tetramer-dimer interactions as recently reported (5), and the circular dichroism and absorption spectra of the aromatic amino acids in their polypeptide chains (6). These well defined and accurately measured properties in adult and fetal hemoglobins are due to unique subunit interactions in the $\alpha_2\beta_2$ and the $\alpha_2\gamma_2$ tetramer, respectively, that originate from the 39 amino acid substitutions between the β- and γ-chains. Therefore, comparative studies of these hemoglobins are potentially informative in establishing a relationship between amino acid sequence and quaternary conformational properties.

The tetramer-dimer interface of fetal hemoglobin undergoes dissociation about 70 times less readily than the corresponding interface in adult hemoglobin (5). In an effort to explain this unexpected property of HbF, we previously expressed a recombinant hemoglobin containing all five amino acid replacements of γ- *versus* β-chains at both sets of subunit interfaces; four of these replacements were at the monomer-dimer interface, and one was at the dimer-tetramer interface with the remaining sequence corresponding to that of the β-chain. This recombinant HbA/F had only a fraction of the significantly decreased tetramer-dimer dissociation properties of HbF, and its response to DPG was unexpectedly like that of HbF rather than that of HbA even though the amino acids at its DPG binding site were the same as those in HbA. These results suggested that a sequence(s) of the protein distant from the tetramer-dimer interface and the DPG binding site affect interactions at these sites. In this study we identify this sequence.

In their comparison of the deoxy structures of HbF and HbA, Frier and Perutz (7) found that the A helix in the N-terminal region of the γ-chains moved away from the H helix and toward the E helix relative to HbA. Based on this observation, we have expressed a recombinant Hb containing the eight amino acid

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[1] The abbreviations used are: DPG, 2,3-diphosphoglycerate; IHP, inositol hexaphosphate; HPLC, high pressure liquid chromatography; FPLC, fast protein liquid chromatography; ESI, electrospray ionization; MS, mass spectroscopy; LC, liquid chromatography.

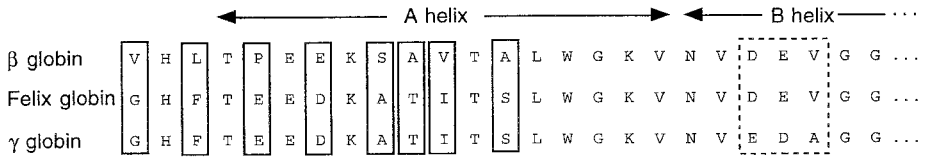

FIG. 1. **N-terminal amino acids of γ-β-chain of Hb Felix, the β-chain of HbA, and the γ-chain of HbF.** In Hb Felix the eight amino acids in the *solid boxes* were replaced in the β-globin sequence to correspond to those in the γ-globin sequence of HbF. The three amino acids at positions 21–23 in the *dashed boxes* are unique to β- or γ-chains.

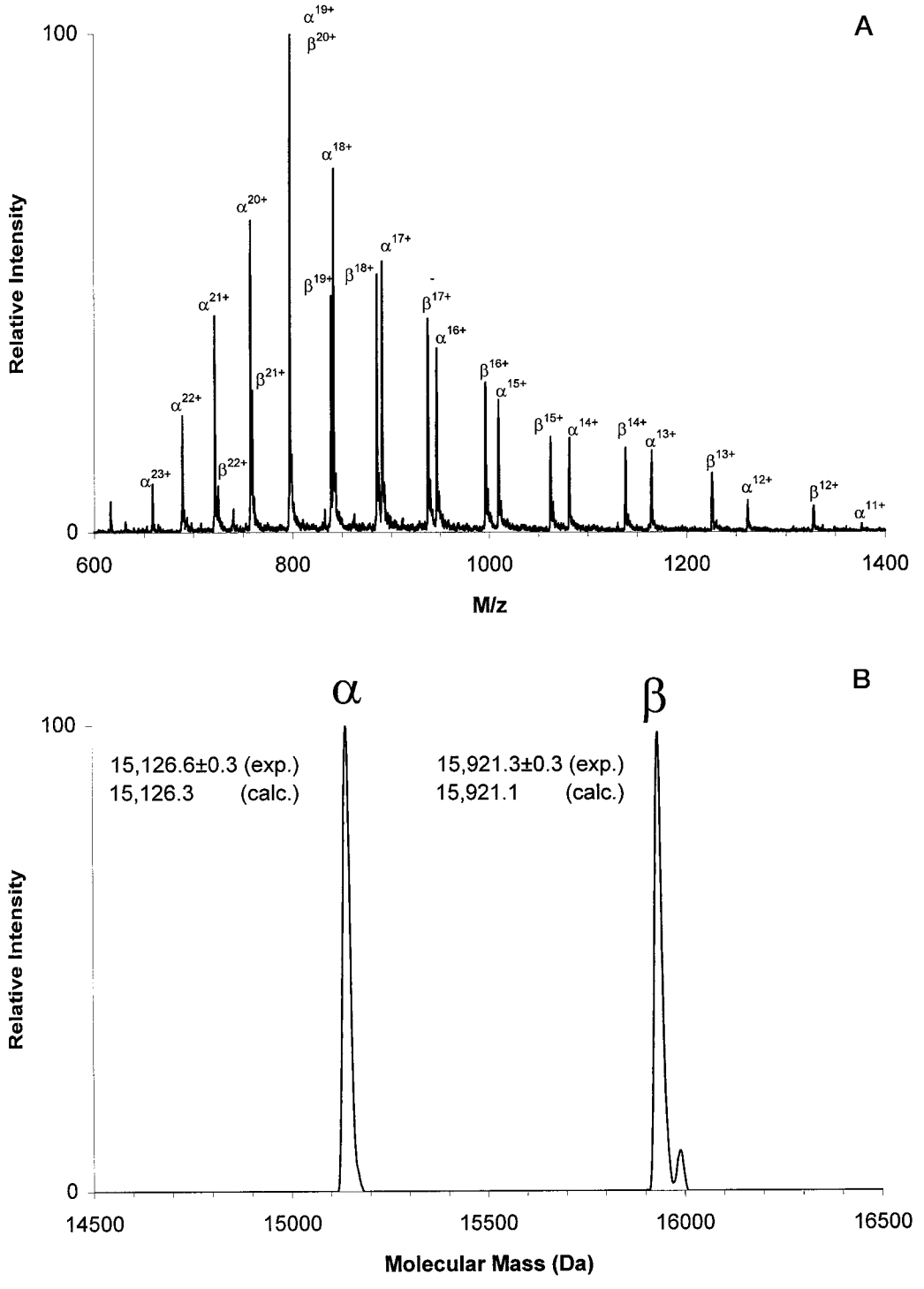

FIG. 2. **Electroscopy ionization mass spectrometric analysis of intact subunits of Hb Felix.** *A*, raw ESI/MS spectrum. *B*, deconvoluted mass spectrum. Deconvolution was performed on charged states ranging from 11+ to 18+. Masses reported are the average values of five independent measurements.

substitutions of the γ-chain of HbF over the span of the first N-terminal 18 amino acids (containing the A helix) but retaining the subsequent 128 amino acids of the β-chain sequence; we refer to it as Hb Felix (see Fig. 1). This recombinant γ-β hybrid tetramer is analogous to the natural gene fusion human variant Hb Kenya (8). In this article we report that Hb Felix shows

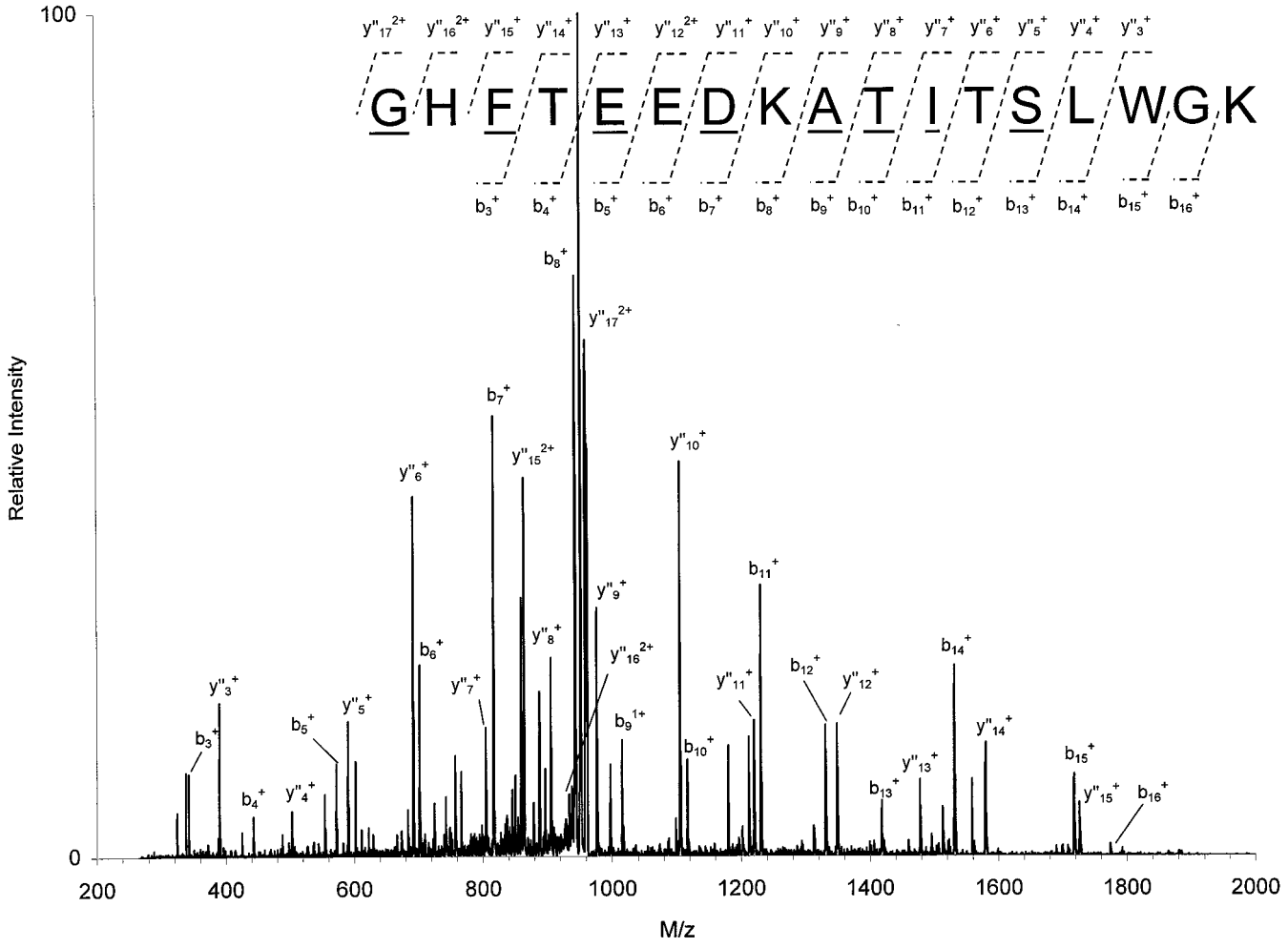

FIG. 3. **MS/MS analysis of the N-terminal 17-residue tryptic peptide from the γ-β-chain of Hb Felix.** *Underlined* residues were mutated. Unlabeled peaks with >5% relative intensities can be attributed to water losses from *b* and *y* ions and to *a*-series ions.

some striking similarities to HbF at its tetramer-dimer interface in the R state, and we suggest that the N-terminal domain influences tertiary interactions between helices that, in turn, affect some quaternary properties involving α subunits. The results are consistent with similar although smaller changes reported for the same domain of several natural hemoglobin variants with single substitutions involving sickle hemoglobin.

MATERIALS AND METHODS

Hemoglobins—HbA was purified as described previously (9, 10) and was >95% pure as ascertained by isoelectric focusing, by FPLC on a Amersham Pharmacia Biotech Mono S column, and by amino acid analysis. Separation of γ-β globin was achieved by HPLC (9, 10).

Mass Spectrometry—Molecular masses of the intact chains were measured by ESI/MS using a Finnigan-MAT TSQ-700 electrospray-triple quadrupole mass spectrometer (ThermoQuest, San Jose, CA). The hemoglobin solution was diluted to 0.1 μM in water-methanol-acetic acid, 49:50:1, v/v/v) and infused at 3 μl/min through a 50 μM (inner diameter) fused silica capillary into the ion source of the mass spectrometer. Desolvation of the protein ions was accomplished by maintaining the heated capillary at 200 °C. One hundred individual spectra were acquired and later averaged to produce the spectrum shown in Fig. 2*A*. The deconvoluted spectrum shown in Fig. 2*B* was obtained from processing the averaged spectrum in Fig. 2*A* with deconvolution software developed at the Rockefeller University Laboratory of Mass Spectrometry and Gaseous Ion Chemistry.

For peptide analysis, 250 pmol of hemoglobin were diluted in 100 mM ammonium bicarbonate buffer solution, pH 8.2, containing 2 mM calcium chloride, and the solution was incubated at 37 °C for 5 min. Modified L-1-tosylamido-2-phenylethyl chloromethyl ketone-trypsin (Boehringer Mannheim) was then added at 0 h (substrate-enzyme ratio at 40:1, w/w) and at 2 h (final substrate-enzyme ratio of 20:1, w/w). The final reaction volume was 25 μl, and the final concentration of hemoglobin was 10 μM. The enzymatic hydrolysis was performed for 4 h at 37 °C with occasional stirring.

After proteolysis, aliquots of 1 μl were withdrawn, transferred to

TABLE I
Properties of natural and recombinant hemoglobin tetramers

For circular dichroism measurements, each hemoglobin was converted to the ferric form at pH 6.0 as described in the text. The molar ellipticity values given are at the minimum for each Hb shown in Fig. 6. The Hb concentrations were in the range of 9–10 μM as determined by spectral measurements; when present, the IHP concentration was 200 μM. Tetramer-dimer dissociation constants (K_d) and oxygen binding properties were measured as described in the text.

Hemoglobin	N-terminal sequence	Interface subunits	Tetramer dimer dissociation (K_d)[a]	Circular dichroism values at 287 nm	
				Without IHP	With IHP
			μM		
HbA	β	α + β	0.68	−6,260	−17,650
HbA/F	β	α + γ	0.14	−17,300	−18,300
Hb Felix	γ	α + β	0.03	−19,200	−35,300
HbF	γ	α + γ	0.01	−34,450	−52,450

	No additions		DPG:Hb (10:1)		Cl:Hb (125:1)	
	P_{50}	n	P_{50}	n	P_{50}	n
	mm Hg		*mm Hg*		*mm Hg*	
Hb Felix	7	2.0	24	2.1	14	2.4
HbA	9	2.7	24	2.7	14	3.1
HbF	8	2.8	11	2.5	13	2.9

[a] The accuracy of these values is ±10%.

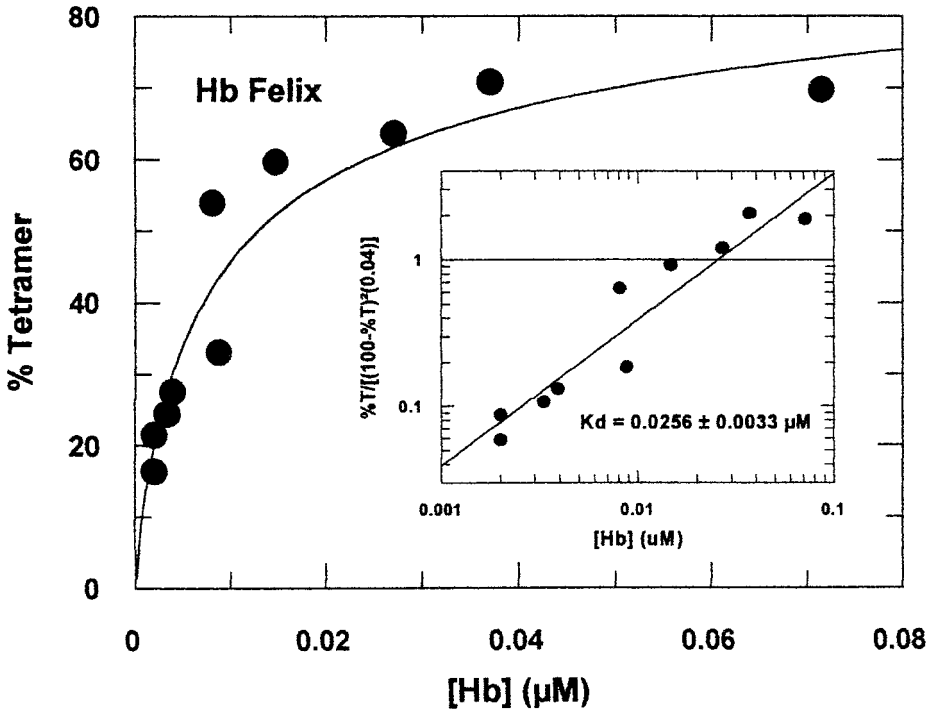

FIG. 4. **Tetramer-dimer dissociation constant of Hb Felix.** The tetramer-dimer equilibrium data are shown in the main part of the figure. The K_d value, calculated by the method in Ref. 9, is 0.03 μM (*inset*).

0.65-ml Eppendorf tubes, and dried in a Speedvac rotary concentrator (Savant, Farmingdale, NY) for 5 min at room temperature. The aliquots were resuspended in saturated solutions of α-cyano-4-hydroxy-cinnamic in either water-acetonitrile (1:1, v/v) or 0.1% aqueous trifluoroacetic acid-acetonitrile (1:1, v/v) (11). Aliquots of these solutions were spotted onto a gold-coated sample plate and analyzed in a matrix-assisted laser desorption ionization time of flight mass spectrometer, model STR Voyager (Perkin-Elmer) equipped with delayed ion extraction and ion reflection. In addition, aliquots of 1 μl of the enzymatic hydrolysate were subjected to LC-MS in an ESI ion trap mass spectrometer (see below).

Mass spectrometric fragmentation of the tryptic peptides was performed using a Finnigan LCQ electrospray ion trap mass spectrometer (ThermoQuest, San Jose, CA) either by direct infusion of the total proteolytic digest in a mixture of water-methanol-acetic acid (49:50:1, v/v/v) or by on-line LC-MS/MS. MS/MS spectra of the peptides containing the mutated sites were acquired after the proteolytic mixture was directly infused into the ion source at 1 μl/min through a 50 μM (inner diameter) fused silica capillary. HPLC was performed on an Ultrafast Microprotein Analyzer (Michrom Bioresources, Inc., Auburn, CA). Separation was achieved using a Vydac C_8 column (15 × 1-mm inner diameter, 300 Å, 5 μM) with water-acetonitrile-0.1% aqueous trifluoroacetic acid standard buffers. Peptides (10 pmol loading) were eluted with an increasing concentration of acetonitrile at a flow rate of 50 μl/min. The post-column effluent was split 15:1 yielding a flow rate of 3 μl/min into the ion source of the mass spectrometer. For desolvation, the heated capillary in the ion trap mass spectrometer was maintained at 125 °C.

Construction of Hb Felix—Eight amino acids were replaced at the N-terminal region of the β globin subunit by the corresponding amino acids of the gamma globin subunit (see Fig. 1). The mutagenesis procedure was a two-step amplification method adapted from the overlap extension method described previously (5). Because the eight residues to be mutated are clustered on the same region of the β-chain, it was possible to mutate this region using two oligonucleotides overlapping the eight positions. Because this region is relatively long, the oligonucleotides were designed not to be totally complementary but sufficiently so to hybridize to each other in the second amplification reaction. Their sequences are: ACT GAA GAG GAC AAG GCT ACC ATT ACT TCC CTG TGG GGC AAG and GGT AGC CTT GTC CTC TTC AGT AAA GTG CCC CAT GGT TTA TTT. The nature of the mutated codons has been selected to produce as little change as possible compared with the β codons to optimize the primer hybridization during the amplification. Each of these oligonucleotides primed an elongation framed with its mate oligonucleotide that contains a *Xho*I site on the extremities of the expression cassette and was then cloned into the pGS190α plasmid using the *Xho*I site. This plasmid contains an α globin expression cassette already cloned (5). DNA sequence analysis was performed to check both the efficiency of the mutagenesis procedure and the absence of any extra mutation that might be produced due to the problem of *Taq* polymerase infidelity. Both α and β cassettes were extracted together from the pGS190 plasmid by a *Not*I digestion and inserted into the pGS389 yeast expression plasmid. The resulting pGS389 Felix plasmid was prepared in large quantity (Midi Prep, Qiagen) and transformed into

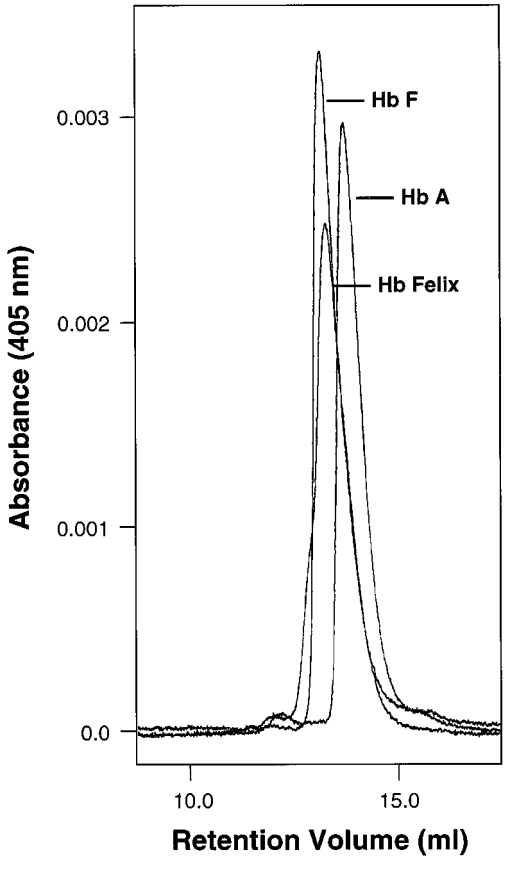

FIG. 5. **Elution of HbA, HbF, and Hb Felix.** Each Hb (0.2 μM) was applied to Superose 12 as described in Ref. 9. The elution profiles were then superimposed by the FPLC director software program.

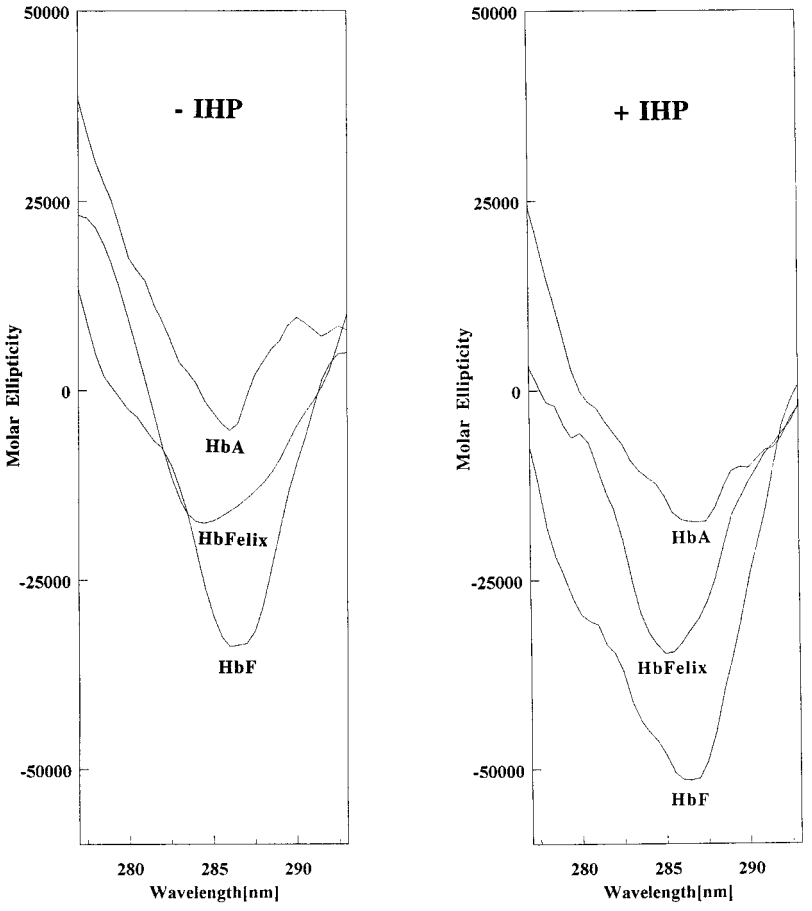

FIG. 6. **Circular dichroism properties of HbA, HbF, and Hb Felix.** Spectra were recorded in the presence (*right*) or absence (*left*) of IHP as described in the text.

the GSY112 *Saccharomyces cerevisiae* strain as described previously (5).

Tetramer-Dimer Dissociation Constants—The dissociation constants (K_d) were determined on a Superose-12 HR10/30 as described previously (5, 9); the concentration dependence of the peak positions was measured on serial dilutions of the hemoglobin as it eluted between the standardized positions of cross-linked tetrameric Hb and the natural dimeric Hb Rothschild. Peak elution volumes, which were accurate to less than 1%, were found to be a function only of Hb concentration and were independent of the sample volume injected, indicating the absence of dilution on the column. K_d values (± 10%) were determined from these peak positions, which were accurately measured by the Amersham Pharmacia Biotech FPLC Director software for each liganded (CO or O_2) sample (9). These values for HbA were completely consistent with reported values (5, 9). The elution buffer was 150 mM Tris acetate, pH 7.5.

Circular Dichroism—The ferric (met) forms of the hemoglobins were prepared by treatment of the samples with potassium ferricyanide (6, 13). Circular dichroism studies were performed with a Jasco 715 instrument in the presence or absence of a 20-fold molar excess of inositol hexaphosphate (IHP) to determine the molar ellipticity of the band at 287 nm, which is characteristic of T-state hemoglobins (6, 13). Circular dichroism measurements were also performed in the visible region corresponding to the heme absorption and in the far ultraviolet region corresponding to the polypeptide chain.

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 O_2 form (5, 10, 11). This instrument has been shown in our laboratory to give reliable results for a number of chemically modified and recombinant hemoglobins (5, 10, 11). The Hb concentration used for these measurements was 0.5–0.8 mM (tetramer) in 50 mM bis-Tris-Ac, pH 7.5; at these concentrations HbA and HbF are predominantly tetrameric. When DPG was present, its final concentration was in 10-fold molar excess over hemoglobin. When chloride was present, its final concentration was approximately 125-fold molar excess over hemoglobin. Determinations were performed in duplicate with a precision of ± 1 mm Hg.

RESULTS

Characterization—Hb Felix was expressed in yeast and purified by methods described previously (5, 9–11). After chromatography on CM-52, FPLC analysis on a Mono S column showed a single component. Iso-electric focusing indicated a single band whose position was consistent with the one additional negative charge introduced by the mutagenesis on the N-terminal sequence (Fig. 1).

Mass Spectrometry—The α and γ-β globin subunits of Hb Felix were analyzed by mass spectrometry to check whether Hb Felix had the expected amino acid replacements. The molecular mass of the γ-β subunit of Hb Felix was determined by ESI/MS to be 15,921.3 ± 0.3, which is in excellent agreement with the calculated value of 15,921.3 (Fig. 2*B*). The observed molecular mass of the α-chain was 15,126.6 ± 0.3, in agreement with the calculated value of 15,126.3.

Although correspondence between the measured and calculated masses of the globin chains is a necessary condition for establishing correct expression, it is not sufficient. Sufficiency is established at two levels: (*a*) at the first level, we obtain MALDI/MS and LC-ESI/MS maps of the proteolytic products produced by tryptic digestion of the globins and (*b*) at the second level, we isolate peptide ions that contain the mutated amino acid residues and fragment these ions to yield sequence information. Isolation and fragmentation of these ions of interest are performed in an ESI ion trap mass spectrometer. In the present case, the combination of MALDI/MS and LC-ESI/MS peptide mapping experiments yielded information on 99% of the α-chain sequence and 98% for the γ-β-chain sequence (data not shown). The only portions of the primary structure of the two globin chains that were not observed by mass spectrometry were a single lysine residue in the α-chain ($Lys^{\alpha 61}$) and one dipeptide in the γ-β-chain ($Val^{\beta 60}$-$Lys^{\beta 61}$), neither of which contained mutations.

To test whether the amino acid substitutions in the γ-β subunit were located at the correct sites, an N-terminal 17-

residue tryptic peptide containing all eight putative mutations in the γ-β-chain was subjected to LC-ESI/MS/MS analysis (Fig. 3). Inspection of Fig. 3 shows that all eight mutated residues (underlined residues in the figure) were accounted for by the fragmentation pattern, verifying the correctness of the expressed γ-β globin chain.

Edman Sequencing—N-terminal protein sequencing of the γ-β globin subunit isolated by HPLC (10, 11) was carried out for 25 cycles to include the juncture of the γ and β sequences. The results indicated that the eight amino acid substitutions shown in Fig. 1 were correct as established by the mass spectrometric results above and that the DEV sequence at positions 21–23 was present, showing that the cross-over from the γ-globin sequence to the β-globin sequence was successfully achieved (the γ-subunit contains an EDA sequence at positions 21–23).

Tetramer-Dimer Dissociation—Using a high resolution gel filtration method that we recently described (9) for accurately measuring the extent of subunit dissociation, the tetramer-dimer dissociation constant (K_d) of liganded HbA was found to be 0.68 μM, whereas the K_d of HbF was discovered to be much lower than previously appreciated, *i.e.* 0.01 μM (5) (Table I). It should be stressed that the K_d value for liganded HbA obtained by FPLC using small zone gel filtration on Superose 12 is completely consistent with values reported by a variety of other methods as already described in detail (5, 9). Other studies have independently found that this method gives accurate values for various protein equilibrium states (12).

Hb Felix, which possesses those amino acids of HbA directly involved in contacts with α-subunits at its distant C and FG helices, dissociates to dimers with the profile shown in Fig. 4; a K_d value of 0.03 μM was calculated from these points (Fig. 4, *inset*, and Table I), which is very close to the K_d of 0.01 μM for HbF (5).

This very low K_d of Hb Felix was verified by the results in Fig. 5 where equivalent concentrations of HbA, HbF, and Hb Felix (0.2 μM of each) were applied separately to the Superose-12 column. Hb Felix elutes much closer to HbF than to HbA, consistent with its low K_d value. These results indicate that the N-terminal amino acid substitutions in Hb Felix by themselves lead to a tightening of its structure analogous to that reported by Frier and Perutz for HbF (7).

Circular Dichroism Properties—Perutz *et al.* (6) found that the extent of the transition from the oxy (R) to the deoxy (T) conformation of the ferric (met) form of HbA could be measured by the magnitude of a negative ellipticity band at 287 nm in the circular dichroism spectrum. Wind *et al.* (13) reported that this transition was even more pronounced for HbF than for HbA and that it was enhanced much more for HbF than for HbA by IHP, which displaces the oxy to deoxy equilibrium in favor of the latter conformation. We have reproduced their results for these two hemoglobins (Fig. 6 and Table I). Hb Felix has negative ellipticity at 287 nm between those of HbF and HbA both in the presence and absence of IHP (Fig. 6 and Table I), consistent with its low tetramer-dimer dissociation constant reported above (Figs. 4 and 5). A significant part of the circular dichroism properties is attributed to $Trp^{\beta 37}$, which is an integral part of this interface (6). We interpret these differences as qualitatively consistent with the quantitative differences between HbA, HbF and Hb Felix reported above and shown in Figs. 4 and 5. For comparison, HbA/F, which has the five γ-β replacements at the subunit interfaces, had an ellipticity in the absence of IHP intermediate between HbA and HbF (Table I), consistent with its K_d value of 0.14 μM (5). In the presence of IHP, the ellipticity at 287 nm did not increase significantly in contrast to the effects found for Hb Felix.

The circular dichroism spectra in both the far ultraviolet, which is a measure of the polypeptide chain backbone conformation, and in the visible region corresponding to heme absorption showed that these properties of Hb Felix were identical to those of both HbF and HbA, indicating that the eight amino acid substitutions did not have any adverse global effects on the overall structure of the recombinant Hb Felix. This conclusion is consistent with its normal response to DPG described next.

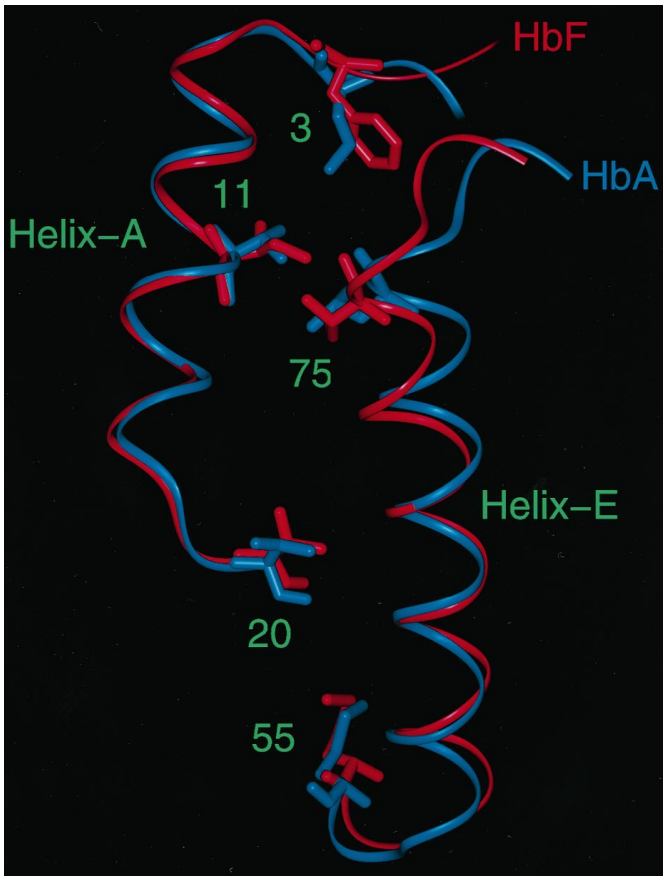

FIG. 7. **Helices A and E of β-globin of HbA (*red*) and γ-globin of HbF (*blue*).** The coordinates for HbA and HbF, taken from the Brookhaven Protein Data Bank, were published in Refs. 25 and 7, respectively.

Functional Properties—The tetramer-dimer dissociation and the circular dichroism results above are indicative of quaternary structural properties in the oxy (R) conformational state. To determine how Hb Felix behaved in the transition from the deoxy (T) to the oxy (R) state, the effect of DPG on oxygen binding was determined (Table I). HbA and Hb Felix, which have the same sequences in their DPG binding sites, respond to DPG to the same extent and do so more than HbF, as already reported (4, 5). Chloride is equally effective in lowering the oxygen affinity of all three hemoglobins, indicating that the amino acid substitutions have little effect on those chloride binding sites that affect the O_2 equilibrium. The Hill coefficient (n) of Hb Felix either in the presence or absence of allosteric regulators shows significant cooperativity (Table I), showing that the amino acid substitutions at the N terminus of the γ-chain joined to the remainder of the β-chain did not have a deleterious effect on the molecule.

The identical effects of DPG on Hb Felix and on HbA probably indicate that the positively charged side chains involved in DPG binding have not changed their relative positions in these two hemoglobins. Thus, Gly^1 and His^2 of the γ-β subunit, which are part of the N-terminal flexible tail just before the A helix (Fig. 7), can probably assume multiple positions and interac-

tions. The other two DPG binding sites of HbA and Hb Felix, Lys^{82} and His^{143}, are at the beginning of helix F and the end of Helix H, respectively, and their relative positions are apparently not affected by the N-terminal amino acid replacements. Furthermore, Hb Felix and HbA contain the same amino acids at their tetramer-dimer interfaces where the quaternary rearrangement during the oxy/deoxy transition takes place upon DPG binding. These results also show that the recombinant Hb Felix is folded like HbA in these regions of the molecule.

DISCUSSION

The movement of helical domains in proteins is now an established principle, but its magnitude can vary considerably. For example, G proteins of which rhodopsin is a model (14) show well defined movements of certain helices in site-specific recombinants. A more extreme example of structural rearrangement with pathological consequences occurs in the prion proteins (15), which undergo drastic changes from a predominantly α-helical to a β-sheet structure caused by single amino acid substitutions. The current level of our understanding of the sequence/structure relationship in proteins and its ramifications is not sufficiently advanced to explain the range of these tertiary structural changes.

For hemoglobin, changes in the relative position of the A helix have already been reported for several naturally occurring variants with single amino acid substitutions, although they do not lead to effects as large as those reported here for Hb Felix. For example, the replacement of $Glu^{\beta 6}$ by Val in the A helix of sickle hemoglobin results in polymerization of tetramers initiated by the interaction of the donor $Val^{\beta 6}$ with an acceptor site at the EF helical corner of an adjacent tetramer. From their recently solved 2-angstrom x-ray structure of HbS, Royer and colleagues (16) found that this movement of the A helix actually facilitated donor to acceptor binding. There are several other reports of the flexibility of the A helix in the β-chain of hemoglobin, although they are less extensive than the changes in natural HbF reported by Frier and Perutz (6) and those for recombinant Hb Felix described here. Thus, Lesecq *et al.* (17) found that replacement of $Glu^{\beta 7}$ by Ala in an HbS double mutant affected its interaction with $Lys^{\beta 32}$ of helix H. In HbC ($Glu^{\beta 6} \rightarrow$ Lys) (18), and the cyanomet valence hybrids (19), the A helix is displaced away from the E helix. These changes in HbS and HbC only slightly affect the tetramer-dimer interface because the K_d for HbS is 0.42 μM (9) and that for HbC is 0.26 μM,[2] which are just slightly less than the 0.68 μM value for HbA (5). Nevertheless, these observations are consistent with the larger change in the K_d value of Hb Felix with its multiple substitutions.

Frier and Perutz (7) reported that in the deoxy conformation of HbF, the A helix moved toward the E helix and away from the H helix compared with HbA (Fig. 7) leading to a tighter structure of HbF compared with HbA. It is likely that these same relative helix changes also occur in the unsolved oxy structure of HbF.[3] The tighter structure of HbF is consistent with its decreased tetramer-dimer dissociation constant (K_d) at this interface and its smaller elution volume compared with HbA during high resolution gel filtration reported recently (5). The findings with Hb Felix suggest that it also has a tightened structure even though it has the HbA amino acids at this interface, suggesting a predominant role for the N-terminal segment of the γ-β-chain in influencing its overall structure. In contrast, the N-terminal β-sequence of HbA/F does not endow it with such a tightened structure as measured by its intermediate K_d and circular dichroism values (Table I).

Because the crystal structure of Hb Felix is not yet known, we used molecular modelling to suggest a mechanism. Comparison of the overall HbA and HbF structures shows that Phe^3 and Thr^{10} of the γ-chain, which are bulkier than their Leu^3 and Ala^{10} counterparts on the β-chain, make unfavorable contacts with Val^{133} and Val^{126}, respectively, on helix H of Hb Felix (not shown). To relieve such stress, the A helix would need to move away from the H helix. Strong interactions between the A and H helices have been demonstrated in myoglobin (20), but a possible contribution to protein folding has been questioned by Brunori and colleagues (21), who reported that the A helix and part of the H helix could be removed in mini-myoglobin with retention of its folding pattern. HbA residues Leu^3, Val^{11}, and Leu^{75} are replaced by Phe^3, Ile^{11}, and Ile^{75} in HbF (Fig. 7). In HbA, these residues face each other directly, whereas in HbF their orientations are offset. This would allow helices A and E in HbF to approach closer to each other than in HbA as seen in Fig. 7 leading to a tighter structure.

There have been suggestions of inter-relationships among regions of hemoglobin. Thus, Turner *et al.* (22) comparing various single point mutations in natural Hb variants and in chemically modified hemoglobins discussed interactions between different sites in hemoglobin. Fronticelli *et al.* (23) found evidence that the effects of an amino acid replacement at the $\alpha_1\beta_1$ interface could be propagated to the $\alpha_1\beta_2$ interface. An important conclusion of the present study is that amino acid substitutions involving the entire nascent N-terminal domain can lead to significant changes in its relationships with other helices that affect functional quaternary subunit interactions and the properties of the entire protein. The results reinforce the concept of a fluid long range inter-relationship among different regions of the hemoglobin tetramer (5, 24) and show that the N-terminal region has a significant role in this process.

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