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Properties of a recombinant human hemoglobin double mutant: Sickle hemoglobin with Leu-88(β) at the primary aggregation site substituted by Ala

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Abstract

A recombinant double mutant of hemoglobin (Hb), E6V/L88A(β), was constructed to study the strength of the primary hydrophobic interaction in the gelation of sickle Hb, i.e., that between the mutant Val-6(β) of one tetramer and the hydrophobic region between Phe-85(β) and Leu-88(β) on an adjacent tetramer. Thus, a construct encoding the donor Val-6(β) of the expressed recombinant HbS and a second mutation encoding an Ala in place of Leu-88(β) was assembled. The doubly mutated β-globin gene was expressed in yeast together with the normal human α-chain, which is on the same plasmid, to produce a soluble Hb tetramer. Characterizations of the Hb double mutant by mass spectrometry, by HPLC, and by peptide mapping of tryptic digests of the mutant β-chain were consistent with the desired mutations. The absorption spectra in the visible and the ultraviolet regions were practically superimposable for the recombinant Hb and the natural Hb purified from human red cells. Circular dichroism studies on the overall structure of the recombinant Hb double mutant and the recombinant single mutant, HbS, showed that both were correctly folded. Functional studies on the recombinant double mutant indicated that it was fully cooperative. However, its gelation concentration was significantly higher than that of either recombinant or natural sickle Hb, indicating that the strength of the interaction in this important donor–acceptor region in sickle Hb was considerably reduced even with such a conservative hydrophobic mutation.

Keywords: hemoglobin; mutagenesis; protein aggregation; sickle hemoglobin

In sickle cell anemia, distorted red cells in the venous circulation (Herrick, 1910) are due to the intracellular aggregation of deoxyhemoglobin S tetramers (Pauling et al., 1949; Ingram, 1956; Bookchin et al., 1967; Hofrichter et al., 1974; Wishner et al., 1975; Edelstein & Crepeau, 1979; Wellems & Josephs, 1979; Eaton & Hofrichter, 1990). This aggregation is the result of the substitution of a hydrophobic amino acid, Val-6, for the hydrophilic Glu-6 on the β-chain of Hb (Kinemage 1). Because this substitution is on the exterior of the HbS tetramer, it interacts at a hydrophobic site between Phe-85 and Leu-88 on an adjacent HbS tetramer in the deoxygenated state; in the oxygenated state the geometry does not favor this interaction. After this initial event, other intertetrameric lateral and longitudinal interactions strengthen the aggregate. The locations of many of these interactions have been identified by several methods, including procedures that utilize the sparing effect of other mutant Hb on the gelation of sickle Hb (Bookchin et al., 1967). More recently, X-ray diffraction techniques and electron microscopy have provided information on critical contact sites in the aggregate. These methods basically identify the sites of interaction between tetramers in the aggregation process.

Another approach that identifies important sites in the aggregate with the objective of preventing its formation is the use of chemical modifiers directed at certain parts of the Hb tetramer (Cerami & Manning, 1971; Dean & Schechter, 1978; Manning, 1991); anti-gelling effects are sometimes due to indirect effects of these modifiers. Even though useful information has come from this approach, the types of sites modified are limited to those that possess reactivity toward a particular reagent. Most often, these are hydrophilic sites because they are the most chemically reactive. However, the importance of hydrophobic side-chain interactions in HbS aggregation, which contribute to the process in a major way, cannot readily be addressed by this approach.

The availability of recombinant DNA technology now permits evaluation of the contribution of any site on the HbS tetramer to the aggregation process. For sickle cell Hb, the ability to alter hydrophobic side chains is especially important because many of the initial and subsequent stages of aggregation involve hydrophobic interactions (Hofrichter et al., 1974; Wishner et al.,

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Abbreviations: Hb, hemoglobin; TFA, trifluoroacetic acid; r, recombinant; PTH, phenylthiohydantoin.

1975; Edelstein & Crepeau, 1979; Wellems & Josephs, 1979; Eaton & Hofrichter, 1990). The site and nature of the mutagenesis described in this communication were chosen in order to understand more fully the strength of the initial interaction between deoxy HbS tetramers. Hence, Leu-88 was changed to an Ala residue because such a substitution would not alter the hydrophobic character of this important primary binding site for aggregation. This mutation was achieved with the yeast expression system recently described for sickle Hb (Wagenbach et al., 1991; Martin de Llano et al., 1993a, 1993b). In this system, *Saccharomyces cerevisiae* carrying a plasmid containing the human α- and β-globin cDNA sequences permits correct N-terminal processing and a soluble Hb tetramer is produced (Ogden et al., 1994). The oxygen equilibrium curves of recombinant and natural sickle Hb at high protein concentration were superimposable, and there was a high degree of cooperativity as indicated by Hill coefficients of 3.3 (Martin de Llano et al., 1993a). Furthermore, the gelation properties of recombinant and natural sickle Hb were also the same at the high Hb concentrations that occur in the red cell.

In this communication, in addition to evaluating the effects of the mutation of Leu-88(β) at the primary aggregation site of sickle Hb, we present studies aimed at characterizing the quaternary structure of these recombinant Hb by comparing the circular dichroism properties of recombinant sickle Hb and the recombinant double mutant with those of natural sickle Hb and normal HbA. This part of the study was meant to answer questions about the overall folding pattern of recombinant Hb.

Results

Mutagenesis and growth of yeast

To construct the recombinant Hb double mutant, Hb E6V/L88A(β), site-directed mutagenesis was performed with the recombinant M13mp18 phage containing the cDNA of human sickle β-globin. The presence of both mutations was confirmed by sequencing the β-globin cDNA; no other change was found in the sequence.

Purification and characterization of recombinant Hb E6V/L88A(β)

The recombinant Hb was expressed in yeast, which was grown and collected as described previously (Martin de Llano et al., 1993b). The growth of the yeast was not adversely affected by the presence of the mutant Hb. Purification was achieved on the CM-52 and Synchropak CM-300 HPLC columns as described in Martin de Llano et al. (1993a).

Isoelectric focusing of recombinant Hb

The Hb E6V/L88A(β) double mutant prepared as described above, the recombinant HbS obtained as described previously (Martin de Llano et al., 1993a, 1993b), as well as natural HbA and natural HbS from human red cells were each purified by HPLC and subjected to isoelectric focusing using the pH 6–8 Hb-Resolve system (Isolab). This system is sufficiently discriminating to separate various types of Hb and to detect any impurities, if present. Shown for comparison in Figure 1 are HbA (lane a) and natural sickle Hb (lane b). The degree of separation of HbA and HbS due to the difference of 2 negative charges per tetramer is an indication of the separating power of this system. The double Hb mutant, E6V/L88A(β), behaved as a single band in this system (lane c) and there were no detectable impurities. There was no separation between recombinant HbS (lane d) and recombinant Hb double mutant, E6V/L88A(β) (lane c), consistent with the lack of a charge difference in the substitution. When the 2 single mutants and the 1 double mutant Hb in bands b, c, and d were mixed and subjected to isoelectric focusing, only 1 band was found (lane e).

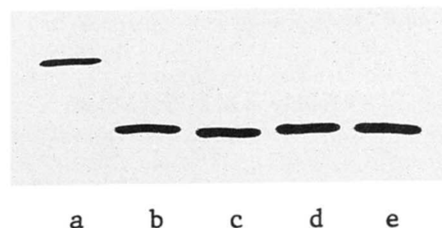

Fig. 1. Isoelectric focusing of natural and recombinant Hb. Hb samples purified by chromatography on a CM-300 HPLC column were subjected to isoelectric focusing. Lane a, natural HbA; lane b, natural HbS; lane c, recombinant Hb E6V/L88A(β) double mutant; lane d, recombinant HbS; lane e, a mixture of HbS, recombinant HbS(L88Aβ), and recombinant HbS. Each lane contained 15 μg of Hb. The anode is at the top and the cathode at the bottom.

Separation of subunits of recombinant HbS(L88Aβ)

The purified Hb double mutant was subjected to HPLC on a Vydac C-4 column in order to separate the α- and β-subunits. As shown in Figure 2, the mutant β-chain elutes earlier from this column than does the β-chain of the recombinant HbS, consistent with the less hydrophobic nature of the second mutation, i.e., Ala for Leu. The symmetry of the eluted peak was consistent with the presence of a single component, as also shown below by other methods of characterization.

Mass spectrometry analysis

The recombinant Hb double mutant was subjected to mass spectrometry as described previously (Beavis & Chait, 1989). The

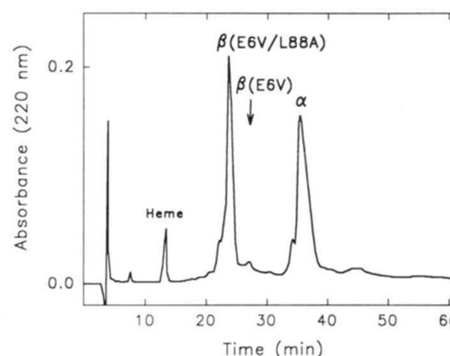

Fig. 2. Separation of α- and β-chains of recombinant HbS(L88Aβ). The double mutant Hb was applied to a Vydac C-4 HPLC column and eluted as described in the text.

measured mass of the α-chain was 15,126 (calculated value: 15,126). The mass of the β-chain from the double mutant was 15,794 (calculated value: 15,796) compared to a mass of 15,838 for the natural sickle β-chain. This difference of 42 mass units is consistent with the substitution of an Ala for a Leu on the β sickle chain.

Tryptic digestion of mutant β-chain

For further confirmation of the mutation of Leu-88(β) to Ala, the purified mutant β-chain was subjected to tryptic digestion and then to analysis by HPLC on a Vydac C-18 column. This peptide map, shown in Figure 3B, was compared to the peptide map of the β-chain of recombinant sickle Hb shown in Figure 3A. There was 1 peptide missing from the tryptic peptide map of the β-chain of rHbS (Fig. 3A, arrow) and a new component in the peptide map of the E6V/L88A(β) globin chain (Fig. 3B, *). Amino acid analysis showed that this peptide was derived from residues 83–104 of the β-chain; there was no tryptic cleavage after Lys^{95} because of the presence of an Asp at position 94. This purified peptide was subjected to sequencing and the results indicated that the mutation was indeed at position 88 because the PTH derivative of Ala was found in high yield during Edman degradation (Fig. 4).

Absorption spectra

The absorption spectra of the recombinant Hb double mutant, recombinant HbS itself, natural HbA, and natural HbS were virtually identical for both unliganded Hb (Fig. 5A) and liganded

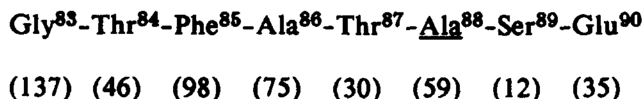

Fig. 4. Sequencing of the mutant peptide from recombinant HbS(L88Aβ). The mutant peptide was isolated by HPLC after tryptic digestion as described in the legend to Figure 3. This peptide was subjected to 8 cycles of Edman degradation. The underlined Ala residue is the site of the substitution at position 88. The numbers in parentheses refer to the number of picomoles of PTH-amino acid found at each cycle of Edman degradation.

Hb (Fig. 5B); see also Antonini and Brunori (1971). There was, however, a minor shift of less than 0.5 nm in the Soret region at 429 nm and a 10% increase in the ultraviolet absorption for deoxy HbS and rHbS (L88Aβ). For the CO-Hb samples, the spectra were identical (data not shown).

Circular dichroism studies

The circular dichroism spectra of the recombinant HbS and recombinant double mutant E6V/L88A(β) were compared to those of natural HbA and HbS isolated from human red cells. The spectra of liganded Hb are shown in Figure 6, in 3 different spectral regions. The regions from 200 nm to about 290 nm are due mainly to the globin portions of Hb and the regions from 290 nm to 650 nm are a reflection of the heme contribution to

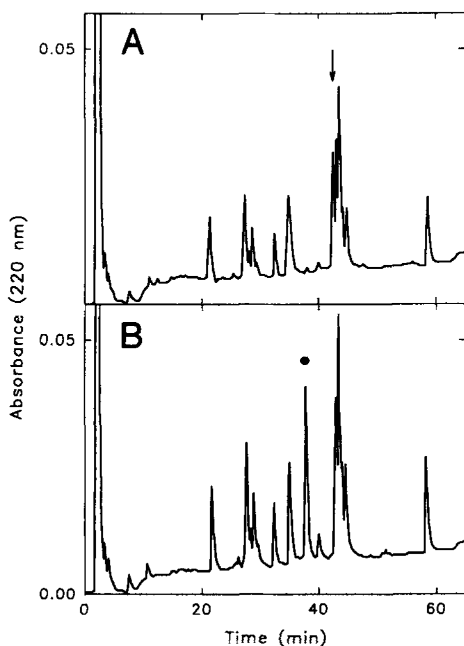

Fig. 3. Tryptic map of the β-chain isolated from recombinant HbS and from recombinant HbS(L88Aβ). The β-chain separated by HPLC (Fig. 2) was carboxymethylated, digested with trypsin, and applied to a Vydac C-18 column as described in the text. A peptide from the E6Vβ chain (A, arrow) does not appear in the digest of the E6V/L88A(β) chain (B). A new peptide appears in B (*).

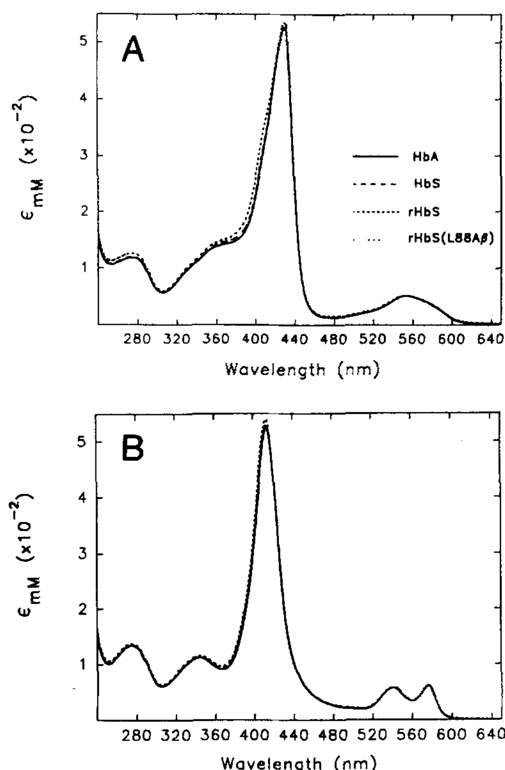

Fig. 5. Absorption spectra of natural and recombinant Hb. **A:** Unliganded (deoxygenated) Hb samples. **B:** Liganded (oxygenated) Hb samples.

the circular dichroism properties as it is bound to the globin. In general, the results showed that there was overall close conformity of each recombinant Hb to their natural counterparts, although there were some minor differences. Of particular relevance was the far ultraviolet region of the spectra with the minimum at 222 nm, which is a measure of the overall protein conformation (Fasman et al., 1966). The ellipticity values at 222 nm are independent of the presence of heme on the protein except to the extent that heme contributes to correct folding. Comparison of all 4 spectra in Figure 6 indicated that some minor differences existed between the individual Hb. However, even for natural HbS and HbA, there are small differences at 222 nm that are likely intrinsic to these different proteins. The differences observed for the recombinant HbS single mutant and the recombinant Hb double mutant were no greater than the small differences between the natural HbA and natural HbS.

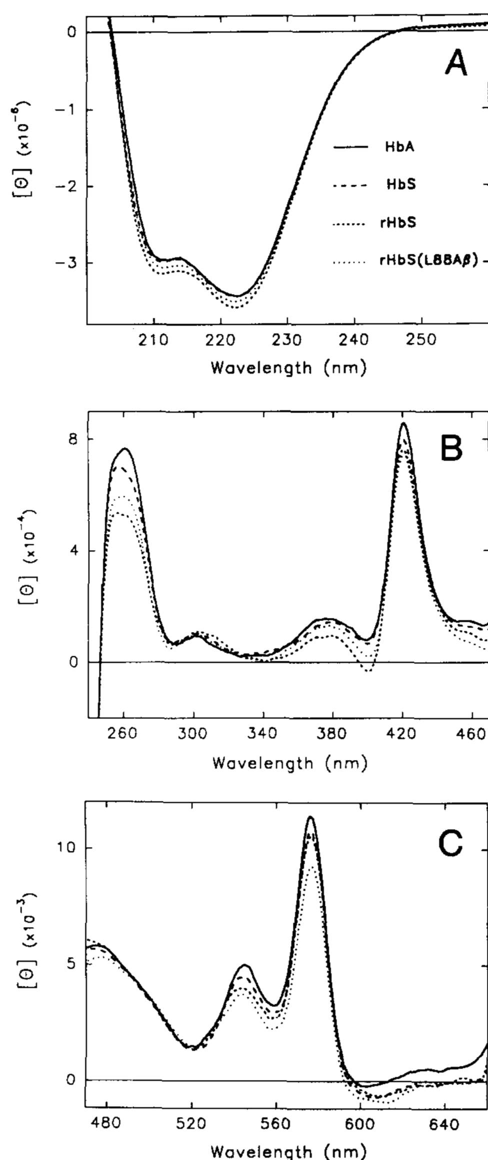

Fig. 6. Circular dichroism spectra of liganded natural and recombinant Hb. The spectra were recorded as described in the text.

For unliganded Hb samples, the circular dichroism spectra showed just minor differences for all 4 samples (data not shown).

Oxygen equilibrium curve

The shape of the oxygen equilibrium curve of the recombinant double mutant was the same as that found previously for natural HbS and recombinant HbS (Fig. 7). The degree of cooperativity of the recombinant double mutant E6V/L88A(β) (n = 3.3) was the same as that found for recombinant sickle Hb reported earlier (Martin de Llano et al., 1993a, 1993b). These results are also consistent with the conclusion that the recombinant double mutant L88Aβ expressed by the yeast system is fully functional.

Gelation of recombinant HbS double mutant E6V/L88A(β)

The substitution of Ala for Leu at position 88 of HbS had a significant effect on the gelation behavior of the protein (Fig. 8). The experimental points for natural and recombinant HbS are from Martin de Llano et al. (1993a) and fit on the same line. Thus, whereas the gelation concentration of both natural and recombinant HbS was 23.7 g/dL, the E6V/L88A(β) double mutant gels at a 30% higher concentration, i.e., 31.2 g/dL. Thus, this rather conservative mutation, which maintains the hydrophobic nature of the acceptor site, has quite significant effects on the gelation of sickle Hb. The data in Figure 8 also show that HbA does not gel at protein concentrations up to 50 g/dL.

Discussion

There are several systems currently available for producing normal or sickle recombinant Hb (Nagai et al., 1985; Baudin-Chich et al., 1990; Wagenbach et al., 1991; Adachi et al., 1992, 1993 Bihoreau et al., 1992; Coghlan et al., 1992; Looker et al., 1992; Martin de Llano et al., 1993a, 1993b; Shen et al., 1993; Vallone et al., 1993). Previous reports have shown that the recombinant sickle Hb produced in yeast closely resembles natural Hb, i.e.,

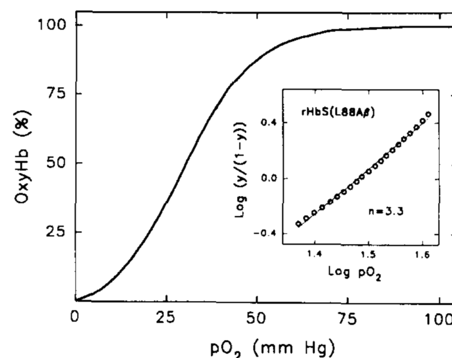

Fig. 7. Oxygen dissociation curve of recombinant Hb double mutant E6V/L88A(β). The recombinant Hb (16.6 g/dL) was in 0.1 M potassium phosphate buffer, pH 6.8, at 37 °C. The curves were recorded on a modified Hem-O-Scan apparatus. Duplicate analyses gave P_{50} values that agreed within ±1 mm Hg. The n value **(inset)** was calculated from this curve.

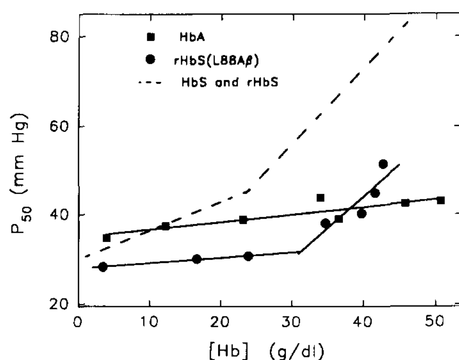

Fig. 8. Gelation of the recombinant Hb double mutant E6V/L88A(β). The plots show the oxygen affinity (P_{50}) of natural HbA and recombinant HbS(L88Aβ) at different concentrations of Hb. The P_{50} values were calculated from the oxygen dissociation curves of Hb samples in 0.1 M potassium phosphate, pH 6.8, at 37 °C obtained on a Hem-O-Scan apparatus. Most determinations are duplicates with a precision of ±1 g/dL. The dashed line shows the behavior of both natural and recombinant HbS, which are identical as reported in Martin de Llano et al. (1993a) and reproduced here for purposes of comparison. For normal HbA, there was no abrupt rise in the P_{50} as a function of Hb concentration up to a concentration of 50 g/dL, indicating lack of gelation.

its chemical and biochemical properties are the same as those of sickle Hb purified from the red cells of sickle cell anemia patients (Martin de Llano et al., 1993a, 1993b). In order to investigate the overall conformation of the recombinant Hb expressed in the yeast system, a comprehensive study employing circular dichroism was undertaken as described in this communication. Because few studies on the overall structure of recombinant Hb have been performed to date, it was considered important to address this point, especially with respect to the properties of double mutants of sickle Hb in which the overall structure of the HbS tetramer plays an important role in the aggregation process. The circular dichroism results in the present communication indicate that the overall structures of both recombinant HbS and the recombinant L88Aβ mutant Hb expressed in yeast were practically the same as those of natural sickle Hb. The ellipticity reading at 222 nm is usually taken as a sensitive indicator of conformation of the folding of the protein backbone (Fasman et al., 1966). The data in this communication for these ellipticity values indicated that the 2 natural Hb and the 2 recombinant Hb had the same degree of secondary structure. The small differences in the regions of the heme absorbances for the recombinant Hb did indicate some minor differences, but these were insufficient to affect the functional properties of the recombinant Hb (Fig. 7). There were also some minor differences between the natural HbA and HbS in various parts of the circular dichroism spectra, which were of the same order of magnitude as the differences between the recombinant and natural Hb. These differences are likely intrinsic to HbA and HbS. In general, the results in this communication agree with previous reports on the circular dichroism properties of Hb (Sugita et al., 1971) and indicate that the yeast expression system produces recombinant Hb that are folded correctly.

Another reason for the choice of Ala as replacement amino acid for Leu-88 was that the hydrophobic side chain at residue 88 is near the heme but the extent of their interaction, if any, and any possible role of Leu-88 in stabilizing the heme moiety was not known (Kinemage 1). Hence, a hydrophobic substitution was chosen especially because the 2 known natural Hb mutants, which have Arg or Pro at this position, are unstable. In none of our studies on the E6V/L88A(β) double mutant did we find any evidence for heme loss even after the recombinant mutant had been subjected to a number of procedures including cation exchange chromatography in several systems, dialysis, and ultrafiltration.

The results in this communication also provide information on the strengths and tolerances of the acceptor site for sickle Hb in its interaction with the donor site, Val-6(β). Thus, the large increase in the gelling concentration from 23.7 g/dL to 31.2 g/dL brought about by the substitution of the isobutyl side chain of a leucine by the methyl group of an alanine suggests that the van der Waals contacts in the acceptor site that determine the efficiency of the primary interaction and subsequent aggregation of sickle Hb are quite rigorous. Substitution of Leu by Ala apparently introduces a considerable degree of flexibility so that the interaction of the Val-6 on the donor tetramer of sickle Hb with the acceptor region between Phe-85 and Leu-88 are considerably weakened. In accord with this concept are the results of Baudin-Chich et al. (1990) who found that substitution of the hydrophobic side chain of Val-6(β) by an Ile residue led to an enhancement in gelation. The slope of the initial stage of the aggregation is steeper for HbS than for the double mutant (Fig. 8). Indeed, the latter resembles that of HbA. On the other hand, the slope of the aggregation stage, i.e., after the gelation point, is the same for both HbS and the double mutant. These results could indicate that the initial stage of the aggregation, i.e., the nucleation process, is greatly affected by the Leu-88(β) substitution, but that once aggregates are eventually formed, gelation occurs similar to that of HbS although at a significantly higher concentration. An understanding of the strengths of interaction in both the primary and secondary interactions in the sickle Hb aggregate is likely to be important in future clinical interventions for sickle cell anemia.

Materials and methods

Reagents and enzymes

The restriction endonucleases, T4 DNA ligase, and alkaline phosphatase were purchased from Boehringer Mannheim and T4 polynucleotide kinase was from New England Biolabs. The DNA sequencing kit and T7 DNA polymerase (Sequenase version 2.0) were purchased from United States Biochemical. The oligonucleotides were synthesized by Operon Technologies (Alameda, California). To create the Leu-88 → Alaβ in the β-globin gene, the oligonucleotide, 5'-AGTGCAGCTCACTAGC TGTGGCAAAGGTG, was used; the underlined bases were used to create the mutation. All the other reagents were of the highest purity available.

The bacteria and yeast strains used in this study and their growth conditions have been described previously (Martin de Llano et al., 1993b). The *Escherichia coli* strain XL1-Blue was used in most of the procedures, and the strain BW313 was used in the site-directed mutagenesis step. LB media and LB agar plates, which contained antibiotics when required, were used. GSY 112 *cir*^{0} was the yeast strain used to harbor the mutant pGS389 plasmid. The growth of the bacterial cells was at 37 °C. Yeast cells were grown at 30 °C on rich medium YPD or, when

transformed with the pGS389 plasmid, on complete minimal medium without uracil or without uracil and L-leucine; liquid cultures (1-1.2 L) were shaken at 300 rpm in 2-L Erlenmeyer flasks.

Site-directed mutagenesis

The construction of a M13mp18 recombinant phage contain the β-globin cDNA has been described previously (Martin de Llano et al., 1993a, 1993b). After mutagenesis, a new phage containing the sickle β-globin cDNA was obtained. *E. coli* strain BW313 was transformed with this phage containing the mutation Glu → Val at the sixth position of the β-globin chain. The oligonucleotide described above was used in order to create the new mutation Leu-88 → Alaβ by the procedure described by Kunkel (1985). Hence, the new recombinant phage contains the double mutant Glu-6 → Val/Leu-88 → Ala. Sequencing by the dideoxy method of the single-stranded DNA obtained from this phage confirmed the presence of both mutations and no other change was observed in the sequence of the β-globin cDNA.

A procedure analogous to that described previously (Martin de Llano et al., 1993b) was used to create a pGS389 plasmid for expression in yeast harboring the α-globin cDNA and the new double mutant β-globin cDNA (pGS389 Glu-6 → Val, Leu-88 → Ala). Yeast cells GSY112 *cir*^{0} were transformed with this plasmid by using the lithium acetate method (Martin de Llano et al., 1993b). The yeast harboring the plasmid (GSY112 [pGS389 Glu-6 → Val/Leu-88 → Ala]) were selected as previously described (Martin de Llano et al., 1993b).

Growth of yeast, expression and isolation of the recombinant double mutant

The growth of the transformed yeast cells and the induction of expression of the recombinant Hb have been described previously (Wagenbach et al., 1991; Martin de Llano et al., 1993b). The culture was bubbled with CO in order to convert the oxygenated form of the rHbS to the more stable CO form, thus precluding oxidation of the heme moiety. The cells were then harvested, washed, and resuspended in the extraction buffer described previously (Martin de Llano et al., 1993b). After breaking the cells with glass beads in a Bead Beater (Biospec Products, Bartlesville, Oklahoma), the supernatant was dialyzed and chromatographed on CM-52 (Martin de Llano et al., 1993a, 1993b). The main peak containing Hb was further purified on a Synchropak CM-300 HPLC column, as described previously (Martin de Llano et al., 1993a) except that a larger column (250 × 10 mm), which was operated at a higher flow (2.5 mL/min) and a slightly different gradient, was used. After purification the recombinant Hb was stored at −80 °C in the CO form.

Other Hb

Recombinant sickle Hb was isolated as described previously (Martin de Llano et al., 1993a, 1993b). Natural HbA and HbS were isolated from the red cells of normal individuals and patients with sickle cell anemia, respectively, using procedures described previously (Martin de Llano et al., 1993a, 1993b). The last step in the purification of all these Hb was the chromatography on the CM-300 HPLC column (Martin de Llano et al., 1993a).

Characterization

The purity of each Hb was determined by SDS-PAGE electrophoresis, by isoelectric focusing, and by mass spectrometry analysis (Beavis & Chait, 1989; Martin de Llano et al., 1993a, 1993b). Separation of the Hb chains was achieved on a Vydac C-4 column using the system described previously (Martin de Llano et al., 1993b). The isolated chains were carboxymethylated (Crestfield et al., 1963) and digested with trypsin using a chain:trypsin ratio of 50:1 (w/w) for 18 h at 37 °C in 0.1 M ammonium bicarbonate. After 18 h, an additional portion of trypsin was added to bring the final chain:trypsin ratio to 50:1.5 and the incubation was continued for an additional 2 h. After lyophilization, the sample was redissolved in 0.1% TFA and a pH of 2 was maintained with additional TFA, if necessary.

For separation of tryptic peptides, the sample was loaded onto a Vydac C-18 column equilibrated in 15% solvent B and eluted at 1 mL/min with a gradient of solvent B from 15% to 55% for 60 min, and then to 100% for 30 min (solvent A: 0.1% TFA; solvent B: 0.1% TFA, 80% acetonitrile). The fractions containing the mutated peptide, which was found by comparison with the pattern of the β-globin chain digest from rHbS, were pooled. An aliquot was used to check the amino acid composition and the remaining sample was subjected to N-terminal sequencing on an Applied Biosystems gas-phase system.

Circular dichroism spectra

The Hb samples in 0.1 M potassium phosphate, pH 7.0, were filtered through 0.2-μm filters before recording their spectra. The CO form was converted to the oxy form as described previously (Manning, 1981). The oxy form was converted to the deoxy form by bubbling the sample with N_2 inside a glove bag. The sample was then transferred to the appropriate cuvette inside the glove bag and it was sealed with a rubber septum (Aldrich).

Circular dichroism spectra were recorded on an Aviv-modified Cary 60 instrument. The concentration of the sample was approximately 30 μM. Spectra from 660 to 450 nm were recorded in a 1-cm-lightpath cuvette; spectra from 470 to 290 nm were recorded in a 0.1-cm-lightpath cuvette. Spectra from 260 to 200 nm were recorded in a 0.1-cm-lightpath cuvette using 10 μM Hb. When analyzing the deoxy form in circular dichroism studies, the absorption spectra were recorded in the same cuvette before and after recording the circular dichroism spectra to confirm that no O_2 had leaked into the cuvette. The ellipticity, θ, is expressed in deg cm^2 per dmol on a heme basis.

Functional studies of recombinant Hb(L88Aβ)

Just prior to determination of the oxygen dissociation curve, the CO form of the purified Hb samples was dialyzed against the appropriate buffer and converted to the oxy form (Manning, 1981; Martin de Llano et al., 1993b). When necessary, samples were concentrated using Centriprep, Centricon, or Microcon ultrafiltration devices (10,000 MW cut-off, Amicon) to about 0.5 mM concentration in tetramer. The oxygen dissociation curves of the Hb in the presence or absence of allosteric effectors at 37 °C in the presence of 0.1 M potassium phosphate, pH 6.8, were obtained on a modified Hem-O-Scan instrument. Aggregation studies were performed with the same instrument

by measuring the effect of Hb concentration on the oxygen affinity by the method of Benesch et al. (1978), as described previously (Martin de Llano et al., 1993a, 1993b).

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