Mutational analysis of sickle haemoglobin (Hb) gelation

Xianfeng Li*, Juha-Pekka Himanen†, Jose Javier Martin de Llano‡, Julio Cesar Padovan§, Brian T. Chait§ and James M. Manning*[1]

*Department of Biology, Mugar 414, Northeastern University, 360 Huntington Avenue, Boston, MA 02115, U.S.A., †Memorial Sloan Kettering Cancer Center, 1300 York Avenue, New York, NY 10021, U.S.A., ‡Instituto de Investigaciones Citologicas, Fundacion Valenciana de Investigaciones Biomedicas, Amadeo de Saboya, 4, 46010 Valencia, Spain, and §Rockefeller University, Laboratory for Mass Spectrometry and Gaseous Ion Chemistry, 1230 York Avenue, New York, NY 10021, U.S.A.

The use of recombinant Hb has provided the advantage that any amino acid substitution can be made at sites not represented by natural mutants or that cannot be modified by chemical procedures. We have recently reported the expression of human sickle Hb (HbS) in the yeast *Saccharomyces cerevisiae* that carries a plasmid containing the human α- and β-globin cDNA sequences; N-terminal nascent protein processing is correct and a soluble correctly folded Hb tetramer is produced. The yeast system produces a recombinant sickle Hb that is identical by about a dozen biochemical and physiological criteria with the natural sickle Hb purified from the red cells of sickle-cell anaemia patients. Most importantly, the gelling concentration of this recombinant sickle Hb is the same as that of the HbS purified from human sickle red cells. The misfolding of Hb reported for the *Escherichia coli*-expressed protein is not apparent for Hb expressed in yeast by any of the criteria that we have used for characterization. These findings indicate that this system is well suited to the production of HbS mutants to explore those areas of the HbS tetramer whose roles in the gelation process are not yet defined and to measure quantitatively the strength of such interactions at certain inter-tetrameric contact sites in the deoxy-HbS aggregate. This article reviews our studies on a number of sickle Hb mutants, including polymerization-enhancing HbS mutants and polymerization-inhibiting HbS mutants.

Introduction

Sickle-cell anaemia results from a single point mutation in the gene encoding β-globin, whereby the Glu-6(β) residue in HbA is replaced by Val in sickle Hb (HbS) [1,2]. This hydrophobic side chain initiates a process by which the densely packed deoxyhaemoglobin tetramers inside the red cells interact with other side chains to form long polymeric fibres that distort the cells into a characteristic sickle shape [3]. 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 favour 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 [4]. More recently, X-ray diffraction techniques [5] and electron microscopy [6–8] have provided information on critical contact sites in the aggregate. These methods identify the sites of interaction between tetramers in the aggregation process. However, the quantitative contributions to polymerization of these sites, i.e. their relative strengths, are unknown and are addressed here.

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 [9–11]. Even though useful information has come from this approach, the types of site 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 be readily addressed by this approach. However, methods using recombinant DNA can provide such information, as described here.

Experimental

Reagents and plasmids

Some of the methods used have been published [12–15] and updated procedures are described here. The restriction endonucleases, alkaline phosphatase and DNA ligase were from Boehringer Mannheim (Indianapolis, IN, U.S.A.) or

Abbreviations used: DPG, 2,3-diphosphoglycerate; ESI–MS, electrospray ionization MS; HbS, sickle Hb; IEF, isoelectric focusing; MALDI–TOF-MS, matrix-assisted laser desorption ionization–time-of-flight MS; TFA, trifluoroacetic acid.

[1] To whom correspondence should be addressed.

New England Biolabs (Beverley, MA, U.S.A.). The DNA-sequencing kit and the PCR reagent kit were obtained from U.S. Biochemical Corp (Cleveland, OH, U.S.A.). The Geneclean kit was from Bio 101 (La Jolla, CA, U.S.A.). The Qiagen plasmid midi kit was from Qiagen (Santa Clarita, CA, U.S.A.). The ^{35}S-labelled dATP was from Amersham (Arlington Heights, IL, U.S.A.). The nucleotides used to make the mutations were synthesized by Operon Technologies (Alameda, CA, U.S.A.) or by the Protein Sequencing Facility at the Rockefeller University (New York, NY, U.S.A.). pBluescript II SK(+) was from Stratagene (La Jolla, CA, U.S.A.). CM-cellulose 52 was from Whatman (Maidstone, Kent, U.K.), and HPLC columns (C_4 and C_{18}) were from Vydac (Southborough, MA, U.S.A.). Tosylphenylalanyl-chloromethane (TPCK')-treated trypsin, dextran, 2,3-diphosphoglycerate (DPG) and IHP were purchased from Sigma (St Louis, MO, U.S.A.). Galactose (containing less than 0.1% glucose) was from Pfanstiehl (Waukegan, IL, U.S.A.) or U. S. Biochemical Corp. All the other reagents were of the highest purity available. The construction of pGS189 and pGS389 plasmids has been described elsewhere [12–14].

The bacteria and yeast strains used in our study and their growth conditions have been described [12–14]. The *Escherichia coli* strain XLI-Blue was used in most of the procedures. Luria–Bertani media and Luria–Bertani agar plates, which contained antibiotics when required, were used. GSY 112 cir° was the yeast strain used to harbour the mutant pGS389 plasmid.

Site-directed mutagenesis

The methods used for site-directed mutagenesis were different for each individual HbS mutant. A PCR-based strategy [16] was used to generate the desired mutation sites. In earlier studies, the method of Kunkel [17] was used to create the mutations. Therefore the protocol for generating each individual recombinant HbS mutant will be given separately in the text.

Purification of recombinant HbS mutants

The purification of the recombinant HbS mutants was accomplished by chromatography on CM-cellulose 52, with a linear gradient consisting of 150 ml of equilibration buffer and 150 ml of a second buffer. The equilibration buffer was 10 mM potassium phosphate, pH 6.0, and was the same for all recombinant HbS mutants. However, the second buffer varied in both potassium phosphate concentration and pH depending on the nature of the amino acid replacement in each individual HbS mutant.

Yeast expression system

pGS389 is a yeast/*E. coli* plasmid containing the human α- and β-globin cDNA species and the 2 μm yeast element. It can replicate in *E. coli*, where the selection is facilitated by the use of its ampicillin resistance gene. Transcription of the globin genes is induced by growing the cells in a medium containing galactose. pGS189 is a derivative of Bluescript II SK(+) plasmid containing the human α- and β-globin cDNA species and replicates only in *E. coli*. The recombinant pGS389 were transformed into *Saccharomyces cerevisiae* GSY112 cir° strain, with the use of the lithium acetate method [12–14]. The transformants were selected by using a complete medium, first without uracil, then without uracil and leucine. To express each recombinant mutant the yeast strain was grown in 12 litres of YP medium in a new Brunswick Fermentor BioFlo IV (Edison, NJ, U.S.A.) for 3–4 days, with ethanol as the carbon source, until a D_{600} in the range 5–10 was reached. The promoter controlling the transcription of the globin genes was induced for a maximum duration of 20 h by the addition of galactose to a final concentration of 3% (w/v) . The collection and breakage of the cells after bubbling with CO gas has been described [12–14]. The use of a fermenter for the large-scale production of yeast cells greatly simplified the procedure for the preparation of medium, the addition of ethanol during cultivation and the induction of the Hb mutant by galactose.

Analytical methods

SDS/PAGE of the recombinant mutants was performed on the Phast System (Pharmacia, Uppsala, Sweden). The protein bands were stained with Coomassie Brilliant Blue R-250. Isoelectric focusing was performed on the pH 6–8 or pH 7–10 Hb-Resolve system from Isolab (Akron, OH, U.S.A.). The α- and β-globin chains from recombinant haemoglobins were separated by reverse-phase HPLC on a Vydac (Hesperia, CA, U.S.A.) C_4 column (250 mm $\times$ 4.6 mm) with a gradient of acetonitrile containing 0.1% (v/v) trifluoroacetic acid (TFA). The eluate was monitored at 220 nm. Amino acid analysis of globin chains isolated by this procedure was performed on a Beckman 6300 instrument with a System Gold data-handing system (Beckman Instruments, Palo Alto, CA, U.S.A.). The spectrum of each HbS mutant was recorded on a Shimadzu 1601 UV–visible spectrophotometer (Columbia, MD, U.S.A.).

CD spectra

CD spectra were recorded on an Aviv-modified Cary 60 instrument (Sugar Land, TX, U.S.A.) for our earlier studies and on a Jasco J-715 Spectropolarimeter (Easton, MD, U.S.A.) for our more recent studies. The Hb samples in 0.1 M potassium phosphate buffer, pH 7.0, were filtered through 0.2 μm filters before their spectra were recorded. The concentration of the sample was approx. 30 μM. Spectra from 660 to 450 nm were recorded in a cuvette with a 1 cm lightpath; spectra from 470 to 290 nm and from 260 to 200 nm were recorded in a with a 0.1 cm lightpath. When

the deoxy form was being analysed in CD studies, the absorption spectra were recorded in the same cuvette before and after recording the CD spectra to confirm that no oxygen had leaked into the cuvette. The ellipticity, [θ], is expressed in degrees $\cdot$ cm^2 $\cdot$ dmol^{-1} on a haem basis.

MS analysis

Simple strategy This strategy, which we employed in our earlier experiments, consisted of a single electrospray ionization MS (ESI–MS) measurement to determine the molecular mass of the mutant of interest. Thus ESI–MS analysis of the purified recombinant mutant Hb tetramers was performed with a Finnigan-MAT TSQ-700 triple quadrupole mass spectrometer (ThermoQuest, San Jose, CA, U.S.A.) [18,19]. Hb sample (50 pmol) was loaded on a desalting protein cartridge (Michrom Bioresources, Auburn, CA, U.S.A.) and washed with 1 ml of deionized water. The sample was eluted from the cartridge with a solution of water/acetonitrile/acetic acid (30:68:2, by vol.) and electrosprayed directly into the mass spectrometer. The flow of the eluting solution was maintained at 6 μl/min through a fused silica capillary with an inner diameter of 100 μm.

The measured molecular masses of both globin chains were as compared to with the molecular masses calculated for the designed mutant. Although correspondence of the measured and calculated molecular masses (to within the accuracy of the measurement) is a necessary requirement to confirm that the mutant was made correctly, it is strictly not sufficient. Therefore a second strategy was devised to characterize the designed mutant(s) more thoroughly.

Improved strategy This more stringest test involved three steps: (1) accurate ESI–MS measurement of the intact Hb chains, (2) enzymic digestion of the chains, followed by matrix-assisted laser desorption ionization–time-of-flight MS (MALDI–TOF-MS) analysis of the resulting proteolytic peptides and (3) ESI–MS/MS (or tandem MS) of selected peptides (usually those containing the mutated residues). We now recommend the use of this more complete approach for characterization and provide here an illustrative example of the 'improved strategy' for the characterization of the E6V(β)/E121R(β) double-mutant Hb.

The molecular masses of the intact chains were measured by ESI–MS with a Finnigan-MAT TSQ-700 electrospray-triple quadrupole mass spectrometer (ThermoQuest, San Jose, CA, U.S.A.) [18,19]. The Hb solution was diluted to 0.1 μM in water/methanol/acetic acid (49:50:1, by vol.) and infused at 3 μl/min through a 50 μm (internal diam.) 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. Spectra were acquired from m/z 600 to m/z 1400 in 1 s, utilizing a total of less than 1 pmol of protein for the acquisition of 100 individual scans over a period of approx. 2 min. After acquisition, scans were integrated and yielded the averaged spectrum shown in Figure 1(A). No background subtraction or smoothing was performed on the data set. A deconvoluted spectrum (shown in Figure 1B) was obtained by processing the averaged spectrum in Figure 1(A) with deconvolution software developed at the Rockefeller University Laboratory of Mass Spectrometry and Gaseous Ion Chemistry.

For peptide analysis, 250 pmol of Hb were diluted in 100 mM ammonium bicarbonate buffer, pH 8.2, containing 2 mM $CaCl_2$; the solution was incubated at 37 °C for 5 min. Modified tosylphenylalanylchloromethane-treated trypsin (Boehringer Mannheim) was then added at 0 h (substrate-to-enzyme ratio 40:1, w/w) and at 2 h (final substrate-to-enzyme ratio 20:1, w/w). The final reaction volume was 25 μl and the final concentration of Hb was 10 μM. The enzymic hydrolysis was performed for 4 h at 37 °C with occasional stirring. After proteolysis, aliquots of 1 μl were withdrawn, transferred to 0.65 ml Eppendorf tubes and dried in a Speedvac rotary concentrator (Savant, Farmingdale, NY, U.S.A.) for 5 min at room temperature. The aliquots were resuspended in saturated solutions of α-cyano-4-hydroxycinnamic acid in either water/acetonitrile (1:1, v/v) or 0.1% (v/v) aqueous TFA/acetonitrile (1:1, v/v) [20]. Aliquots of these solutions were spotted on a gold-coated sample plate and analysed in a MALDI–TOF mass spectrometer, model Voyager STR (Perkin–Elmer, Boston, MA, U.S.A.) equipped with delayed ion extraction and ion reflection. MALDI–TOF spectra were acquired both in linear mode and in reflection mode, the latter providing isotopic resolution on every peak in the spectrum. Data collection was based on the addition of 200 spectra into a single acquired spectrum, which was externally calibrated and smoothed after acquisition. In addition, aliquots of 1 μl of the enzymic hydrolysate were also subjected to liquid chromatography–ESI–MS in an electrospray-ion-trap mass spectrometer (see below).

Mass spectrometric fragmentation of the tryptic peptides was performed with a Finnigan LCQ electrospray-ion-trap mass spectrometer (ThermoQuest, San Jose, CA, U.S.A.) either by direct infusion of the total proteolytic digest in a mixture of water/methanol/acetic acid (49:50:1, by vol.) or by on-line liquid chromatography-MS/MS. MS/MS spectra of the peptides containing the mutated sites were acquired after the proteolytic mixture was infused directly into the ion source at 1 μl/min through a 50 μm (internal diam.) fused silica capillary. A single spectrum was acquired that represented a set of 100 spectra averaged before acquisition. HPLC analysis of the enzymic hydrolysate was performed with an Ultrafast Microprotein Analyser (Michrom Bioresources, Auburn, CA, U.S.A.). Chromato-

graphic separation was achieved with Vydac C_8 or C_{18} columns (each 15 mm × 1 mm internal diam., 30 nm (300 Å), 5 μm particle size) with standard buffers: water/acetonitrile (95:5, v/v) for solvent A [with 0.05% (v/v) TFA] and water/acetonitrile (5:95, v/v) for solvent B [with 0.045% (v/v) TFA]. The column was run isocratically for 5 min at 5% (v/v) solvent B, during which time the effluent was diverted from the ion source to avoid contamination of the mass spectrometer with buffers and salts used in the enzymic hydrolysis. Peptides (10 pmol loading) were eluted with a linear gradient of solvent B (5–65%, v/v) in 30 min at a flow rate of 50 μl/min. The post-column effluent was split 15:1, yielding a flow rate of approx. 3 μl/min into the ion source of the mass spectrometer. The HPLC effluent was scanned from m/z 300 to m/z 2000 in approx. 2 s and MS/MS fragmentation was performed on the most intense ion of each full scan whenever the ion intensity was greater than a preset value of 10^5 counts. MS/MS fragmentation scans were performed predominantly on doubly charged ions at a relative collisional energy of 30%; each scan took 2–3 s for completion. The length of the full scans and the MS/MS scans allowed an average of three full scans and three MS/MS scans for each typical chromatographic peak, with an average peak width at base of approx. 15 s. During the chromatographic run, the heated capillary in the ion-trap mass spectrometer was maintained at 125 °C for desolvation of the peptide ions.

Measurement of tetramer–dimer dissociation

This measurement was performed on the liganded recombinant haemoglobins on a Superose-12 HR 10/30 column with a Pharmacia (Uppsala, Sweden) FPLC system [21]. The absorbance of the eluate was measured at 405 nm with the Pharmacia on-line mercury lamp detection system with a 5 mm flow cell. K_d was calculated by using the GraFit program as described [21], with the parameters (V_d and V_t) that were determined from the standard dimeric Hb Rothschild and tetrameric cross-linked Hb. The K_d values of recombinant mutants D75Y(α)/E6V(β)/E121R(β) and D6A(α)/D75Y(α)/E6V(β)/E121R(β) were calculated by curve-fitting the experimental data with parameters V_d/V_t floating because of the increased net basic charge on these mutant haemoglobins.

Functional studies

The oxygen binding curves of the haemoglobins were determined at 37 °C on a modified Hem-O-Scan instrument (American Instrument Co., Silver Springs, MD, U.S.A.). Before measurements were made, the purified Hb sample was dialysed in 50 mM Bis-Tris, pH 7.5, and converted to the oxy form [12–14]. These samples were concentrated by using CentriPrep, Centricon and MicroCon ultrafiltration devices (10000 molecular mass cut-off; Amicon, Beverly, MA, U.S.A.) to a final concentration of 0.6 mM. To measure the effect of anions on the oxygen affinity of these Hb mutants, an aliquot of a solution of 5.6 mM DPG or 2.5 M NaCl in 50 mM Bis-Tris, pH 7.5, was added to the Hb sample to achieve the desired final concentration.

Measurement of gelation concentration

Two methods have been used to measure the gelation concentration of recombinant HbS mutants. During most of our studies, the gelation concentration of these mutants was determined by the 'Dextran-C_{sat}' micromethod at physiological ionic strength and pH [22,23]. This method is based on the marked decrease in the solubility of deoxy-HbS in the presence of high-molecular-mass dextran and measures the true gelation of deoxy-HbS and HbS mutants. In our earlier studies [13–15], gelation experiments were performed by measuring the effects of Hb concentration on the oxygen affinity by the method of Benesch et al. [24]. This 'oxygen-affinity' method depends on the sudden decrease in oxygen affinity when the sickle Hb mutant concentration reaches the critical value at which gelation begins. However, the comparison of the Dextran-C_{sat} micromethod with the oxygen-affinity method used for the direct measurement of gelation indicates similar the effectiveness of each mutation on the inhibition of polymerization, even though the absolute values differ.

Dextran-C_{sat} micromethod The concentrated Hb sample in the oxy form in 50 mM potassium phosphate buffer, pH 7.5, was mixed with dextran in the same buffer to give a final dextran concentration of 120 mg/ml. Mineral oil was layered on top and fresh sodium dithionite solution (50 mM final concentration) was added anaerobically below the Hb/dextran mixture by using a gas-tight syringe. After stirring and incubation for 30 min in a 37 °C water bath, the resulting gel under the oil layer was disrupted with a narrow wire loop; the tubes were then centrifuged in a microcentrifuge for 30 min. The clear supernatant was carefully separated from the aggregated Hb; its Hb concentration was measured spectrophotometrically and verified by amino acid analysis after acid hydrolysis of an aliquot on a Beckman 6300 analyser. Each determination of the gelation concentration was performed three to four times with an S.D. of 10% or less.

Oxygen-affinity method The concentrated Hb sample in the oxy form in 100 mM potassium phosphate buffer, pH 6.8, was used for measurement of the oxygen binding curve to obtain the oxygen affinity (P_{50}) value as described above for functional studies. Physiological concentrations of haemoglobins between 5 and 48 g/dl, such as those that occur in red cells, were used. The P_{50} values were then plotted as at different Hb concentrations. When the concentration of the

HbS mutant reached the critical value, there was a rapid decrease in oxygen affinity. The junction of the biphasic lines was taken as the gelation concentration (C^*) [24]. Most determinations were performed in duplicate with a precision (S.D.) of ±1 g/dl. For normal HbA there was no abrupt rise in P_{50} as a function of Hb concentration up to a concentration of 50 g/dl, indicating a lack of gelation.

Results and discussion

Polymerization-enhancing sickle Hb mutants

To provide quantitative information on the sites that promote the polymerization of sickle Hb after formation of the initial hydrophobic bond involving Val-6(β) [E6V(β)] and also to provide haemoglobins with an enhanced polymerization that could be used in a mouse model for sickle-cell anaemia, we expressed recombinant double, triple and quadruple HbS mutants with substitutions on both the α- and β-chains: E6V(β)/E121R(β), D75Y(α)/E6V(β)/E121R(β) and D6A(α)/D75Y(α)/E6V(β)/E121R(β) [25]. These recombinant haemoglobins retained the functional properties of the Hb tetramer and polymerized in a linear manner at progressively lower Hb concentrations as a function of the degree of substitution, suggesting that these remote sites (αD6A, αD75Y and βE121R) on the α- and β-chains exhibited additive, enhanced polymerization properties. The general approach of using recombinant haemoglobins as described here should prove useful in elucidating the quantitative aspects of the mechanism of HbS polymerization and in identifying the contribution of individual sites to the overall process. The strategy also demonstrates the feasibility of a systematic approach in achieving future recombinant HbS mutants that could provide a new generation of the transgenic mouse model for sickle-cell anaemia.

Site-directed mutagenesis

Double HbS mutant E6V(β)/E121R(β) An *Xho*I fragment of pGS189 containing the sickle β-globin cDNA was inserted into the *Xho*I site of Bluescript II SK (+) to produce a 4.2 kb plasmid, BSK-Beta$^{E6V(\beta)}$. This plasmid was used as a template in the PCR reactions [26]. The two synthetic oligonucleotides: 5′-AC TTT GGC AAA MGR TTC ACC CCA CCA GTG C-3′ and 5′-G CAC TGG TGG GGT GAA YCK TTT GCC AAA GT-3′ were used for the PCR amplifications; the underlined bases were those used to create the desired mutation. These two primers were used in two PCR reactions to generate two PCR fragments. PCR amplifications were performed on a GeneAmp PCR system 2400 (Perkin–Elmer, Norwalk, CT, U.S.A.) with 25 cycles of denaturation at 97 °C for 30 s, primer/template annealing at 55 °C for 1 min and extension at 72 °C for 1 min. These two fragments were then recombined in a separate PCR reaction by using primer/template annealing at 45 °C to produce recombinant sickle β-globin gene containing the E121R(β) mutation. From this recombinant DNA fragment, the *Bam*HI fragment was excised and used to replace the *Bam*HI fragment of BSK-Beta$^{E6V(\beta)}$. The correct insertional direction of the mutated *Bam*HI fragment in BSK-Beta$^{E6V(\beta)}$ was verified by restriction enzyme analysis. The recombinant plasmid with the desired mutation was identified directly by DNA sequence analysis and the base changes encoding Glu-121(β) → Arg was the only mutation of the sickle β-globin gene. The β-globin cDNA with the base change encoding Glu-6(β) → Val and Glu-121(β) → Arg was isolated as a *Xho*I fragment and used to replace the *Xho*I fragment of pGS189. The resulting plasmid, pGS189$^{E6V(\beta)/E121R(\beta)}$, was digested with *Not*I and *Bgl*I to release the α- and β-gene cassette. The cassette was purified from agarose gel by using a Geneclean kit and inserted into pGS389 previously digested with *Not*I to give the recombinant plasmid pGS389$^{E6V(\beta)/E121R(\beta)}$.

Triple HbS mutant D75Y(α)/E6V(β)/E121R(β) The plasmid pAD190, which was constructed by Dr. A. Dumoulin in our laboratory as a derivative of pGS189, was used as a template in the PCR reactions to create the recombinant α-globin gene. This plasmid contains the full-length α-globin cDNA under transcriptional control of a pGGAP promoter. The two synthetic oligonucleotides 5′-CAC GTG GAC TAC ATG CCC AAC G-3′ and 5′-C GTT GGG CAT GTA GTC CAC GTG-3′ were used for the PCR amplifications; the underlined bases were those used to create the desired mutation. The PCR amplifications were performed under conditions similar to those described above. From the recombinant α-globin gene containing the mutation, the *Nco*I and *Sal*I fragment was excised and used to replace the *Nco*I and *Sal*I fragment of pAD190. The recombinant plasmid, pAD190$^{D75Y(\alpha)}$, was identified directly by DNA sequence analysis and the base change encoding Asp-75(α) → Tyr was the only mutation in the α-globin gene. The *Sph*I fragment of plasmid pGS189$^{E6V(\beta)/E121R(\beta)}$ was excised and inserted into the *Sph*I site of pAD190$^{D75Y(\alpha)}$ to produce a plasmid with mutated α- and β-globin genes, pAD190$^{D75Y(\alpha)/E6V(\beta)/E121R(\beta)}$. This α- and β-gene cassette was excised as a *Not*I fragment and similarly inserted into the *Not*I site of pGS389 to give the recombinant plasmid pGS389$^{D75Y(\alpha)/E6V(\beta)/E121R(\beta)}$.

Quadruple HbS mutant D6A(α)D75Y(α)/E6V(β)/E121R(β) Two overlapping PCR products were synthesized by using 5′-ATA AAC CAT GGT GCT GTC TCC TGC CGC CAA GAC A-3′ and 5′-C GTT GGG CAT GTA GTC CAC GTG-3′, and 5′-CAC GTG GAC TAC ATG CCC AAC G-3′ and 5′-GAA CAA AGT CGA CTT AAC GGT A-3′ respectively. The underlined bases were those used to create the desired

Table 1 Amino acid analysis of the α- and β-globin chains of HbS double mutant E6V(β)/E121R(β)

The amount found for Leu was set at the theoretical value of 18.0 and used to normalize the values for the other amino acids. The amounts for Thr, Cys, Met, Tyr and Trp are very low or absent because they were partly or completely destroyed under the conditions of acid hydrolysis in the presence of oxygen. For the residues shown in **bold** there are significant differences between the amounts in the α- and β-chains.

	Frequency (residues per polypeptide chain)			
	Found		Theoretical	
Amino acid	α-chain	β-chain	α-chain	β-chain
Asx	12.0	11.8	12	13
Thr	7.5	5.9	9	7
Ser	10.7	4.8	11	5
Glx	5.7	9.5	5	9
Gly	7.7	14.1	7	13
Ala	21.2	16.4	21	15
Cys	1.4	0.3	1	2
Val	10.4	14.4	13	19
Met	0	0	2	1
Ile	0	0	0	0
Leu	18.0	18.0	18	18
Tyr	1.3	0.3	3	3
Phe	6.8	7.5	7	8
His	9.9	8.6	10	9
Lys	10.5	10.6	10	10
Trp	0	0	1	2
Arg	2.4	3.5	3	4
Pro	6.1	6.4	7	7

Table 2 Amino acid analysis of the α- and β-globin chains of HbS triple mutant D75Y(α)/E6V(β)/E121R(β)

The amount found for Leu was set at the theoretical value of 18.0 and used to normalize the values for the other amino acids. The amounts for Thr, Cys, Met, Tyr and Trp are very low or absent because they were partly or completely destroyed under the conditions of acid hydrolysis in the presence of oxygen. For the residues shown in **bold** there are significant differences between the amounts in the α- and β-chains.

	Frequency (residues per polypeptide chain)			
	Found		Theoretical	
Amino acid	α-chain	β-chain	α-chain	β-chain
Asx	11.6	12.8	11	13
Thr	9.0	6.6	9	7
Ser	9.3	5.3	11	5
Glx	6.1	9.7	5	9
Gly	8.0	13.2	7	13
Ala	22.6	16.7	21	15
Cys	0	0	1	2
Val	15.7	18.3	13	19
Met	1.0	0.4	2	1
Ile	0.1	0.1	0	0
Leu	18.0	18.0	18	18
Tyr	2.6	1.8	4	3
Phe	7.1	7.7	7	8
His	10.5	9.0	10	9
Lys	10.8	10.6	10	10
Trp	0	0	1	2
Arg	2.2	2.8	3	4
Pro	6.9	6.7	7	7

mutations Asp-6(α) → Ala and Asp-75(α) → Tyr. PCR amplifications were performed as described above to produce the recombinant α-globin gene containing the D6A(α) and D75Y(α) mutations. The recombinant α-globin gene was excised as the *Nco*I and *Sal*I fragment and used to replace the *Nco*I and *Sal*I fragment of pAD190. The recombinant plasmid, pAD190$^{D6A(\alpha)/D75Y(\alpha)}$, was identified directly by DNA sequence analysis; the base changes encoding Asp-6(α) → Ala and Asp-75(α) → Tyr were the only mutations of the α-globin gene. The *Sph*I fragment of plasmid pGS189$^{E6V(\beta)/E121R(\beta)}$ was excised and inserted into the *Sph*I site of pAD190$^{D6A(\alpha)/D75Y(\alpha)}$ to produce a plasmid with α- and β-genes, pAD190$^{D6A(\alpha)/D75Y(\alpha)/E6V(\beta)/E121R(\beta)}$. This α- and β-gene cassette was excised as a *Not*I fragment and similarly inserted into the *Not*I site of pGS389 to give the recombinant plasmid pGS389$^{D6A(\alpha)/D75Y(\alpha)/E6V(\beta)/E121R(\beta)}$.

Purification of the recombinant HbS mutants

The purification of the recombinant HbS mutants was achieved by CM-cellulose 52 chromatography as described previously [13,14]. However, Because these HbS mutants were relatively basic as compared to with HbS, a gradient of high pH and increased ionic strength was required to elute them from the cation-exchange column. The purification of the double-mutant Hb was accomplished by using a gradient consisting of 10 mM potassium phosphate, pH 6.0, and 25 mM potassium phosphate, pH 8.0 (150 ml of each). The purification of the triple mutant was accomplished by using a gradient of 10 mM potassium phosphate, pH 5.8, and 50 mM potassium phosphate, pH 8.5 (150 ml of each). The quadruple mutant was initially chromatographed on CM-cellulose 52 by using a gradient of 10 mM potassium phosphate, pH 5.8, and 50 mM potassium phosphate, pH 8.5 (150 ml of each). The main fraction containing Hb was then further purified on a Superose-12 HR 10/30 column on a Pharmacia FPLC system and eluted with 120 mM Tris/acetate buffer, pH 7.5, at a flow rate of 0.4 ml/min. The concentrated Hb sample was applied in 100 μl; A_{280} was measured with the Pharmacia on-line mercury lamp detection system with a 5 mm flow cell. The Hb quadruple mutant was eluted at 13.6 ml as a tetramer and was collected for structural and functional study. The purity of each mutant was verified by SDS/PAGE, isoelectric focusing, globin chain analysis and mass spectrometric analysis, as described below.

Isoelectric focusing

Analysis of the purified recombinant mutants by isoelectric focusing (IEF) was performed on the pH 7–10 Hb-Resolve system from Isolab because of the basic nature of these mutants [25]. For the double mutant E6V(β)/E121R(β), the loss of one negative charge (Glu → Val) and a substitution

Table 3 Amino acid analysis of the α- and β-globin chains of HbS quadruple mutant D6A(α)/D75Y(α)/E6V(β)/E121R(β)

The amount found for Leu was set at the theoretical value of 18.0 and used to normalize the values for the other amino acids. The amounts for Cys, Met, Tyr and Trp are very low or absent because they were partly or completely destroyed under the conditions of acid hydrolysis in the presence of oxygen. For the residues shown in **bold** there are significant differences between the amounts in the α- and β-chains.

	Frequency (residues per polypeptide chain)			
	Found		Theoretical	
Amino acid	α-chain	β-chain	α-chain	β-chain
Asx	10.7	13.8	10	13
Thr	8.7	6.9	9	7
Ser	9.9	5.3	11	5
Glx	5.9	10.0	5	9
Gly	7.3	12.9	7	13
Ala	21.4	15.1	22	15
Cys	0	0	1	2
Val	12.0	16.4	13	19
Met	0.2	0	2	1
Ile	0	0	0	0
Leu	18.0	18.0	18	18
Tyr	2.9	0.9	4	3
Phe	6.7	7.7	7	8
His	9.5	9.1	10	9
Lys	10.5	10.5	10	10
Trp	0	0	1	2
Arg	2.6	3.2	3	4
Pro	6.9	7.5	7	7

equivalent to the loss of two negative charges (Glu → Arg) per subunit was confirmed by the IEF migrations, i.e. there was one charge difference between HbA and HbS and a two charge difference between HbS and the double mutant. The triple mutant D75Y(α)/E6V(β)/E121R(β) has a mutation site in which an Asp residue in its α subunit has been replaced by Tyr. Its migration on IEF was consistent with a loss of a negative charge between the double mutant and the triple mutant. Compared with the triple mutant, IEF of the quadruple mutant D6A(α)/D75Y(α)/E6V(β)/E121R(β) indicated an additional loss of a negative charge, which was consistent with an additional mutation site in which an Asp residue of its α subunit had been replaced by Ala. Because all four mutations were on the exterior of the Hb molecule, the full effect of these pK_a changes was reflected in their electrophoretic behaviour.

HPLC analysis of globin chains and amino acid analysis

The purified recombinant HbS mutants were analysed by reverse-phase HPLC with a denaturing solvent to separate the globin chains [25]. With an acetonitrile gradient of 20–60% (v/v), the β-chain of the double mutant E6V(β)/E121R(β) was eluted at 42.9 min, much faster than the normal β-chain from HbS (47.6 min), indicating that the replacement of Glu-121 by Arg significantly changed the chromatographic behaviour of the β chain. For the triple mutant D75Y(α)/E6V(β)/E121R(β) and the quadruple mutant D6A(α)/D75Y(α)/E6V(β)/E121R(β), the elution times of their β-chains at 42.8 min were the same as that of the double mutant, which was consistent with the absence of further mutations of the doubly mutated β-chain. The elution position of the α-chains from these recombinant mutants coincided with that of the α-chain from natural HbS (compare 51.8 min with 52.6, 53.1 and 51.9 min for HbS double, triple and quadruple mutants respectively), indicating that the replacement of the hydrophilic residue Asp in the α-chain by the non-polar amino acids Ala and Tyr did not alter their chromatographic behaviour significantly on the reverse-phase C_4 column.

The α- and β-chains of the recombinant mutants isolated by HPLC as described above were subjected to amino acid analysis, which permitted their assignments (Tables 1, 2 and 3). Each globin chain was collected and hydrolysed in evacuated tubes by using 6 M HCl at 110 °C for 20 h. For the mutated β-chain of the double mutant (Table 1), the mutated α-chain of the triple mutant (Table 2) and the doubly mutated α-chain of the quadruple mutant

Table 4 Mass spectrometric analysis of recombinant Hb mutants

There is an error of 3 or 4 mass units in the measured mass of both the α-chain and the β-chain of some Hb mutants. It is a systematic error; we can therefore safely conclude that mutations were successful and the Hbs were expressed properly.

	Mass of polypeptide chain (mass units)				
	Theoretical		Measured		
Hb variant	α-chain	β-chain	α-chain	β-chain	Reference
E6V(β)/E121R(β)	15126.4	15864.2	15130	15868	[25]
D75Y(α)/E6V(β)/E121R(β)	15174.0	15864.2	15178	15868	[25]
D6A(α)/D75Y(α)/E6V(β)/E121R(β)	15130.4	15864.2	15133	15867	[25]
E6V(β)/L88A(β)	15126.4	15796.0	15126	15794	[34]
E6V(β)/K95I(β)	15126.4	15823.3	15128	15824	[38]
E6V(β)/L88A(β)/K95I(β)	15126.4	15781.2	15124	15779	[23]
D85K(α)/E6V(β)	15139.5	15838.2	15140	15835	[42]

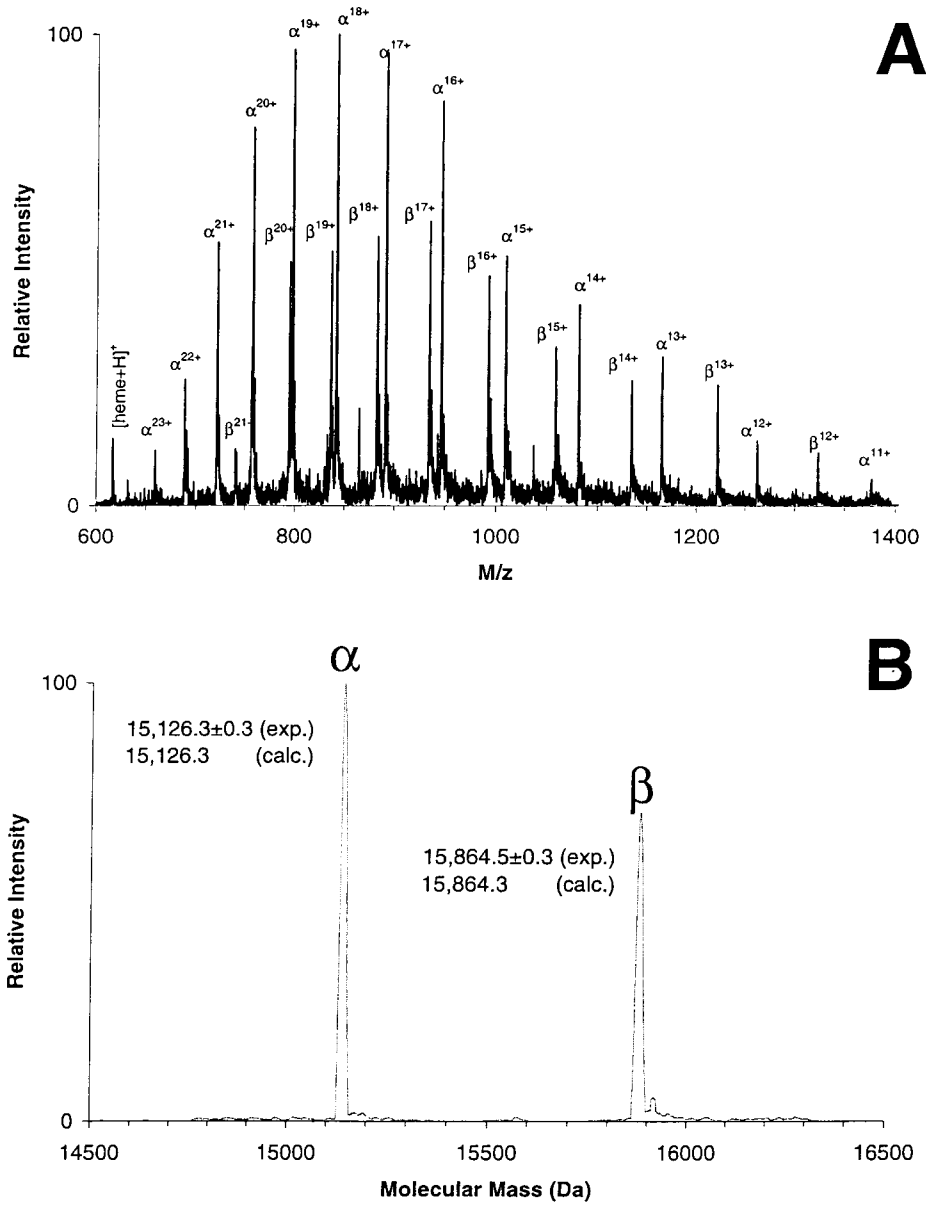

Figure 1 ESI–MS analysis of intact subunits of E6V(*β*)/E121R(*β*) double-mutant Hb

(**A**) Raw ESI–MS spectrum; (**B**) deconvoluted mass spectrum. Deconvolution was performed on charged states ranging from 11+ to 20+. Masses shown in (**B**) are the averages for five independent measurements.

(Table 3), the values for the mutated amino acids Arg, Glu, Asp and Ala were in reasonable accord with their theoretical values. The values for the other amino acids were also in good agreement with the known composition of the recombinant mutants and confirmed their purity, as a basis for the polymerization studies below.

MS

The results of the molecular mass measurements of the purified recombinant Hb mutants are shown in Table 4. These measurements were obtained by using the 'simple strategy' described in the Materials and methods section, which is simple and fast, although we now advise the use of the 'improved strategy' also described in the Materials and methods section. This latter strategy provides a 10-fold higher accuracy (S.D. ±0.3 compared with ±3.0), 100-fold higher sensitivity (0.1 μM compared with 10 μM) and a higher confidence (direct determination of individual mutated amino acids compared with single molecular mass determination of the intact polypeptide chains). Table 4

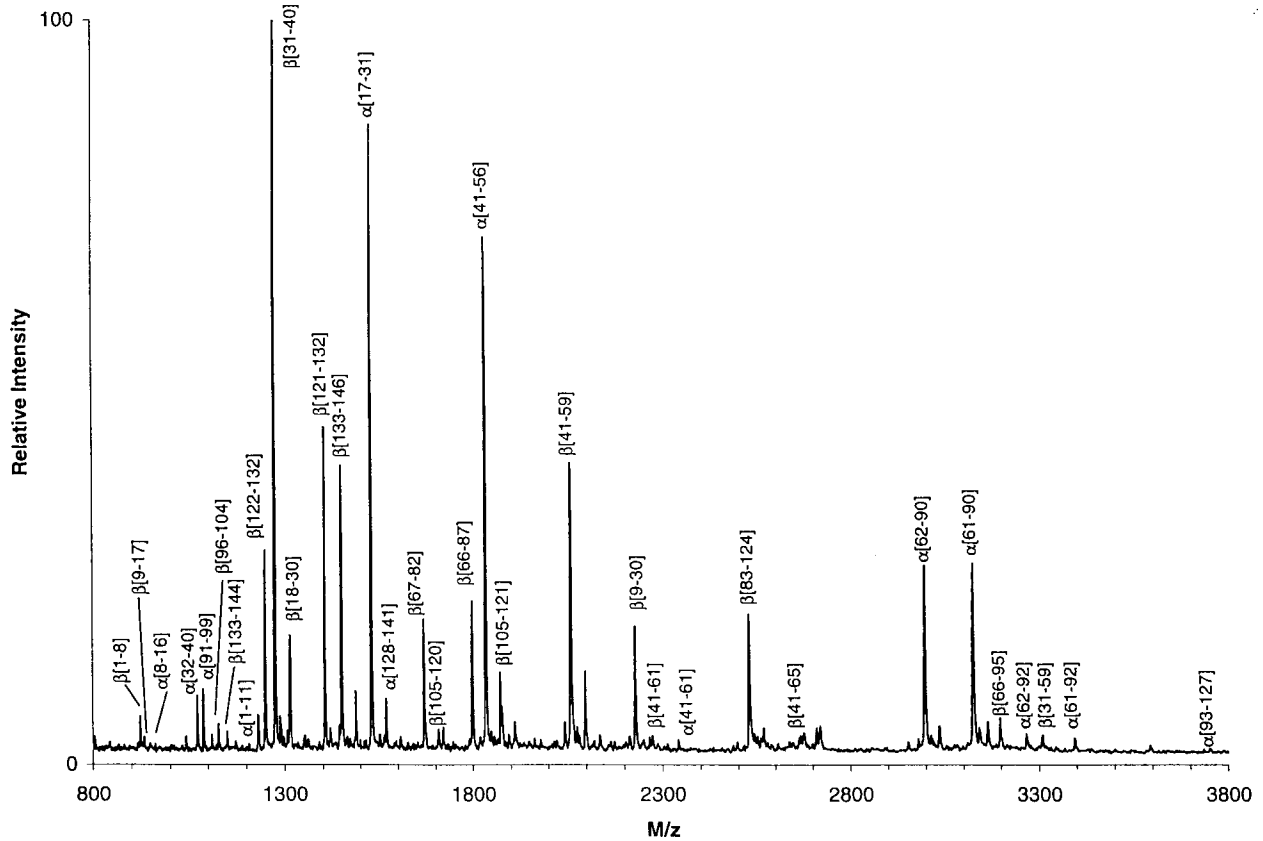

Figure 2 MALDI–TOF-MS spectrum of a tryptic digest of E6V(β)/E121R(β) double-mutant Hb

Peaks are labelled as α- or β-chain peptides followed by the residue number of the first and last residue in the peptide in accordance with the numbering system for the primary structure of the entire polypeptide chain. The MALDI–TOF-MS peptide map yielded information covering 100% of both the α-chain and the β-chain.

presents the molecular masses reported in previous publications. In each case the measured molecular mass corresponds to that of the designed mutant within the error of the mass determination.

As a detailed illustration of the use of the 'improved strategy', we provide data obtained on the E6V(β)/E121R(β) double-mutant Hb. The molecular masses of the intact α-chain and β-chain of the E6V(β)/E121R(β) double mutant were determined by ESI–MS (Figure 1A). The average molecular mass obtained from five independent measurements for the α-chain was 15126.3±0.3 Da ($D = 0$; calculated value of 15126.3 Da) and for the β-chain was 15864.5±0.3 Da ($D = +0.2$; calculated value of 15864.3 Da) (Figure 1B). These values were in excellent agreement with the expected masses for the unmodified α-chain and the mutated β-chain. It is important to mention here that the molecular mass of the unmodified β-chain (absence of both mutated residues) is 15867.2 Da, which is very close to the molecular mass of the mutated β-chain.

Although the accuracy of the molecular mass determination was enough to discriminate the E6V(β)/E121R(β) double-mutant β-chain from the unmodified β-chain, it was not sufficient. Further characterization by MALDI–TOF-MS of the peptides generated by tryptic digestion of the E6V(β)/E121R(β) double-mutant Hb was performed and a complete coverage (100%) of the primary structure of both chains α and β was obtained. By using the information obtained from the MALDI–TOF-MS proteolytic peptide map (Figure 2), two peptides were selected for MS/MS experiments on the basis of their molecular masses, which accounted for the expected mutations at positions Glu-6 → Val (peptide β[1–8], 921.55, expected 921.53) and Glu-121 → Arg (peptide β[121–132], 1404.81, expected 1404.75).

Mass spectrometric fragmentation of these two peptides was obtained as a third step in verifying the correctness of the expressed E6V(β)/E121R(β) double-mutant Hb. The fragmentation pattern of peptide β[1–8], which contained the mutated site Glu-6 → Val, showed basically two series of ions: *b* ions and *y″* ions (Figure 3). The presence of the ions $y''_3{}^+$ and $b_6{}^+$ provided positive identification of the mutated amino acid residue in this peptide. The fragmentation spectrum of peptide β[121–132] was also dominated by *b* ions and *y″* ions (Figure 4). Although ions $y_{12}{}^+$

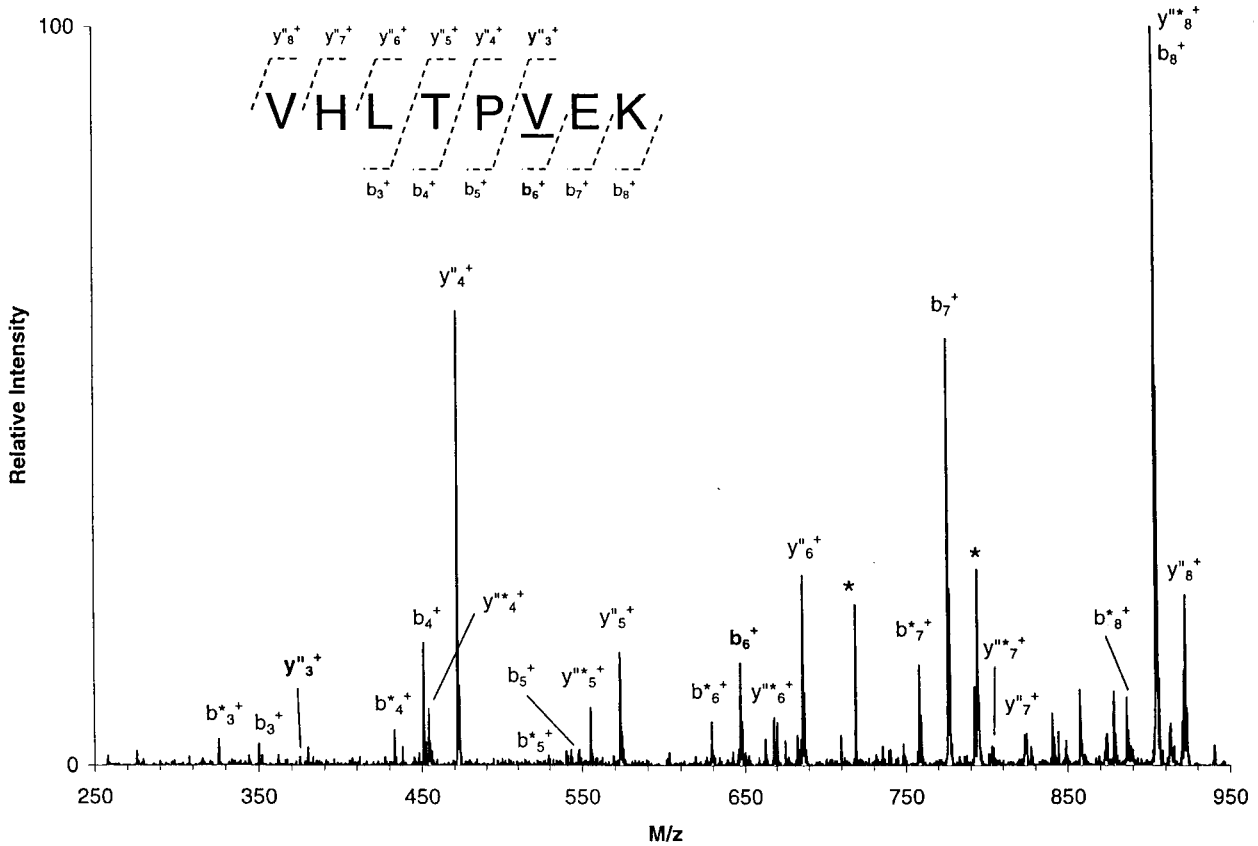

Figure 3 MS/MS analysis of the singly charged peak of the β-chain N-terminal eight-residue tryptic peptide from the E6V(β)/E121R(β) double-mutant Hb

The underlined residue was mutated. Unlabelled peaks with more than 5% relative intensities can be attributed to water losses from *b* and *y″* ions. Peaks labelled with an asterisk were not identified.

and b_1^+ were not present in the fragmentation pattern, which would have yielded a direct identification of the mutation Glu-121 → Arg, the identification of the mutated arginine residue was accomplished by using two pieces of information: (1) the presence of the y_{12}^{2+} ion and (2) the mass difference of 156.2 observed between the m/z of the intact peptide (1405.9, parent ion) and the m/z of ion $y''_{11}{}^+$ (1249.7), which was in good agreement with the expected value for the release of an arginine residue (amino acid residue mass of 156.1 Da) on fragmentation.

The combination of the three steps of the 'improved strategy' thus yielded unambiguous identification of the two sites of mutations, Glu-6 → Val and Glu-121 → Arg, in the recombinant E6V(β)/E121R(β) double-mutant Hb.

Functional studies

The oxygen-binding properties of the recombinant mutants were determined at a Hb concentration of 0.6 mM (Table 5). The oxygen affinity of the double and triple mutants showed an average P_{50} of 9 mmHg (1 mmHg ≈ 133.32 Pa) with Hill coefficients of 3.0 and 3.1 respectively, indicating that they retained full co-operativity. The quadruple mutant showed a high oxygen affinity with a P_{50} of 5 mmHg, which was consistent with the reported value of natural Hb Sawara [27]; this mutant was co-operative, with a Hill coefficient of 2.8.

The effects of DPG (at a DPG-to-Hb ratio of 2:1) on both double and triple mutants (P_{50} values of 22 and 21 mmHg respectively) were similar to that on natural HbS (P_{50} = 22 mmHg) [15]. The effect of DPG (at a DPG-to-Hb ratio of 2:1) on the quadruple mutant was also comparable to its effect on natural HbS under the same conditions, with P_{50} approx. 2-fold higher. In the presence of 500 mM chloride, the oxygen affinity of the double and triple mutants was one-half to one-third of those in its absence, which was similar to the effect observed for HbS. However, the quadruple mutant showed a somewhat smaller response to chloride. The reasons for this behaviour are under investigation.

Tetramer–dimer dissociation

The tetramer–dimer K_d values of these recombinant Hbs directly reflect the integrity of subunit interactions. If the $\alpha_1\beta_2$ interface of the mutant is not as stable as in HbA,

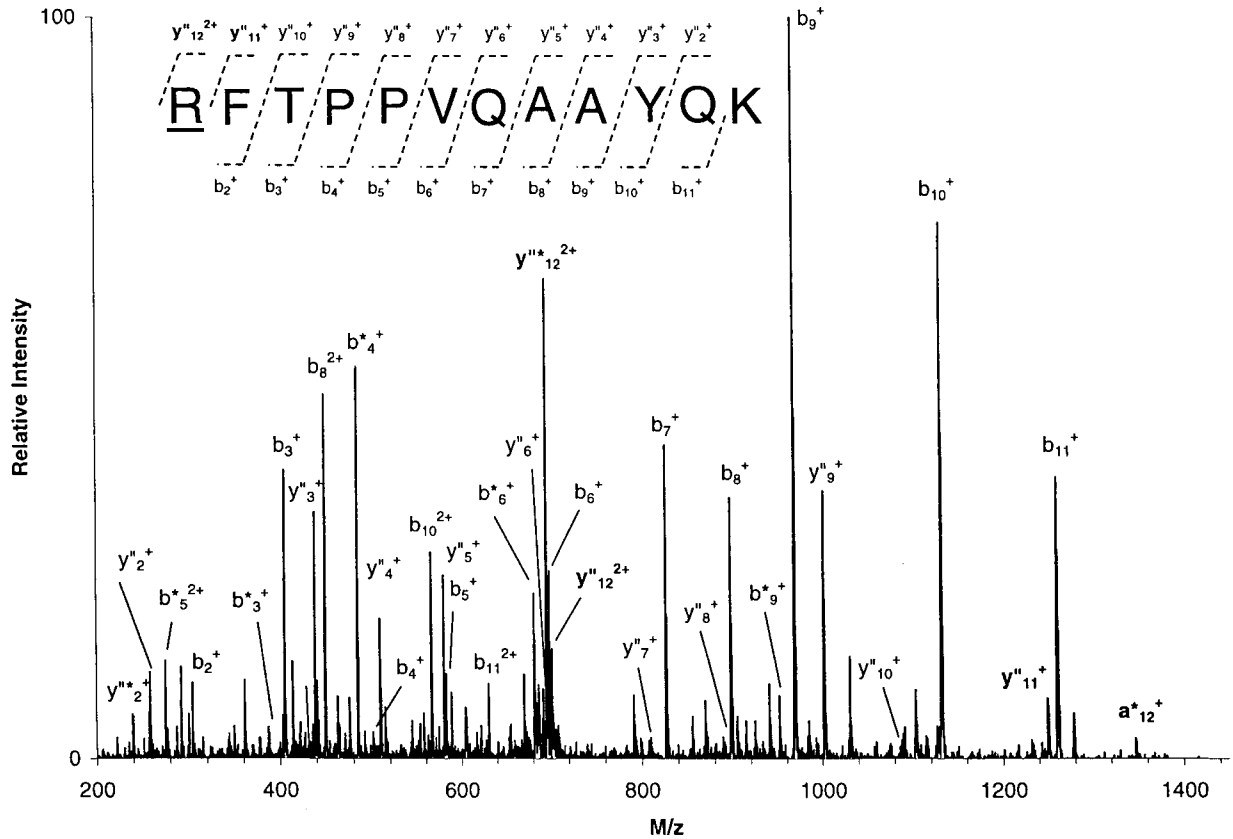

Figure 4 MS/MS analysis of the doubly charged peak of the β-chain 12-residue tryptic peptide from the E6V(β)/E121R(β) double-mutant Hb

The underlined residue was mutated. Unlabelled peaks with more than 5% relative intensities can be attributed to water losses from *b* and *y″* ions and *a*-series ions. The mutated residue was assigned on the basis of the occurrence of the ion $y''_{12}{}^{2+}$ and also the difference observed between the parent ion (*m/z* 1405.8) and the ion $y''_{11}{}^{+}$ (*m/z* 1249.7), which in accordance with only an Arg residue (156.1).

significant dissociation of the tetramer into $\alpha\beta$ dimers could take place under physiological conditions. As shown in Table 6, the tetramer–dimer dissociation constants for the liganded recombinants were between 0.3 and 1.0 μM, which is within the same range as those for native HbS (K_d = 0.4 μM) and recombinant HbS (K_d = 0.7 μM) (Table 6) [21]. These results show that the amino acid replacements in all these recombinant proteins had no significant effect on the dissociation behaviours at the $\alpha_1\beta_2$ interfaces in the liganded conformation of Hb, even though these substitutions contained progressively more positive charges on the surface of Hb. These results therefore confirm that once Hb has assembled into the tetramer, the presence of progressively increased surface charges does not affect its tetramer–dimer equilibrium.

Gelation of the recombinant HbS mutants

Gelation experiments of these HbS mutants were performed by the dextran-C_{sat} micromethod: gelation concentration C_{sat} values are shown in Table 7. The C_{sat} of HbS was 35 mg/ml. Under the same conditions, the double mutant E6V(β)/E121R(β) polymerized at a concentration of 24 mg/ml, indicating a 32% decrease in gelation concentration as compared to with that of HbS. The triple mutant D75Y(α)/E6V(β)/E121R(β) showed a gelation concentration 66% lower than that of HbS: it gelled at 12 mg/ml. The average C_{sat} of the quadruple mutant D6A(α)/D75Y(α)/E6V(β)/E121R(β) was 7.0 mg/ml, which was 20% of the polymerization concentration of HbS itself. These results show that polymerization can be significantly enhanced by selected amino acid substitution on various regions of the HbS tetramer. Substitution at these widely separated sites (αD6A, αD75Y and βE121R) on the α- and β-chains gives rise to an additive effect on the overall process of HbS polymerization [48].

Comments on strategy

In choosing the replacement sites for the double, triple and quadruple mutants of HbS described in this study, we used the naturally occurring single and double mutants that lead to increased polymerization as a guide to select the mutation sites and to achieve the additive effect. Hb D-Los Angeles

Table 5 Functional properties of natural HbS and recombinant HbS mutants

The procedures used to calculate P_{50} and h are described in the text; the Hb concentration for oxygen dissociation curves was 0.5–0.7 mM in 50 mM Bis-Tris, pH 7.5.

Hb variant	[DPG] (mM)	[Cl^-] (mM)	P_{50}	n	Reference
Natural HbS	0	0	9	3.1	[15]
	1.2	–	22	2.8	
	–	10	10	2.8	
	–	20	10	2.8	
	–	100	12	2.8	
	–	200	17	2.8	
	–	500	23	2.8	
	–	1000	28	2.8	
Recombinant HbS	0	0	9	2.9	[15]
	1.2	–	22	2.9	
	10	11	2.9		
	20	10	2.9		
	100	15	2.9		
	200	16	2.9		
	500	21	2.9		
	1000	25	2.9		
E6V(β)/E121R(β)	0	0	9	3.1	[25]
	1.2	–	22	3.1	
	–	500	21	2.9	
D75Y(α)/E6V(β)/E121R(β)	0	0	9	3.0	[25]
	1.2	–	21	2.8	
	–	500	19	2.8	
D6A(α)/D75Y(α)/E6V(β)/E121R(β)	0	0	5	2.8	[25]
	1.2	–	9	2.8	
	–	500	7	2.8	
E6V(β)/L88A(β)	0	0	–	3.3	[34]
E6V(β)/K95I(β)	0	0	10	–	[38]
	0.3	–	11	–	
	0.6	–	15	–	
	1.1	–	17	–	
	1.3	–	20	–	
	2.9	–	22	–	
	3.9	–	21	–	
	–	20	10	–	
	–	50	14	–	
	–	100	17	–	
	–	200	19	–	
	–	500	22	–	
	–	1000	23	–	
	–	1250	22	–	
D85K(α)/E6V(β)	0	0	10	2.8	[42]
	0.4	–	17	–	
	0.6	–	23	–	
	–	100	13	–	
	–	500	17	–	
Natural HbA	0	0	8	2.1	[38]

(Hb D-Punjab), which is part of the SAD mouse construct [28], has a Gln substitution for Glu at β121. Its presence in the red cell together with HbS leads to a more severe clinical state than homozygous sickle-cell disease [29]. Hb O-Arab with a Lys substitution at the same site promotes the gelation of HbS to an even greater extent [30]. A natural double-mutant HbS-Oman (Glu-6 → Val, Glu-121 → Lys) gels at approx. 30% lower Hb concentration than that of HbS [31]. We reasoned that the substitution for Glu at β121 by an even larger basic amino acid could stabilize the polymer structure to an even greater extent. Therefore Arg was selected to achieve this effect. Our results show that the double mutant E6V(β)/E121R(β) polymerized at an approx. one-third lower Hb concentration than that of HbS. This value is comparable to that from the natural double mutant HbS-Oman, suggesting that the enhancing effect is retained by the introduction of a more basic amino acid residue and the size of the residue at this site does not have a significant role in the polymerization process of HbS.

In choosing the third and fourth replacement sites for the triple and quadruple mutants, we selected the sites on the α-chain distant from the substitution in the β-chain, expecting an additive effect on the enhanced polymerization. We therefore selected Hb Winnipeg (Asp-75 → Tyr) [32]

Table 6 Tetramer–dimer dissociation constants of recombinant mutants

Most results were calculated by curve-fitting with the GRAFIT program with dimeric Hb Rothschild and the tetrameric cross-linked Hb as authentic standards (V_d and V_t fixed); the results for D75Y(α)/E6V(β)/E121R(β) and D6A(α)/D75Y(α)/E6V(β)/E121R(β) were calculated with the GRAFIT program with V_d and V_t floating.

Hb variant	K_d	Reference
Natural HbA	0.68	48
Natural HbS	0.42	21
Recombinant HbS	0.75	21
E6V(β)/E121R(β)	1.04	X. Li and J. M. Manning, unpublished work
D75Y(α)/E6V(β)/E121R(β)	0.31	X. Li and J. M. Manning, unpublished work
D6A(α)/D75Y(α)/E6V(β)/E121R(β)	0.39	X. Li and J. M. Manning, unpublished work
E6V(β)/K95I(β)/L88A(β)	0.4	21
D85K(α)/E6V(β)	2.1	21

Table 7 Gelation concentrations of natural HbS and recombinant HbS mutants measured with the dextran-C_{sat} micromethod

All measurements were performed in the presence of dextran in 50 mM potassium phosphate buffer, pH 7.5, at 37 °C, as described in the text. Values of C_{sat} are averages from at least three determinations.

Hb variant	C_{sat} (mg/ml)	Reference
Natural HbS	34	[23]
E6V(β)/E121R(β)	24	[25]
D75Y(α)/E6V(β)/E121R(β)	12	[25]
D6A(α)/D75Y(α)/E6V(β)/E121R(β)	7	[25]
E6V(β)/L88A(β)	67	[23]
E6V(β)/K95I(β)	90	[23]
E6V(β)/K95I(β)/L88A(β)	91	[23]
D85K(α)/E6V(β)	53	[42]
HbA	> 149	[23]

and Hb Sawara (Asp-6 → Ala) as examples [27]. The two HbS double mutants with α-chains from Hb Winnipeg and Hb Sawara, prepared from hybridization of the Hb single mutant and the sickle β-chain, were found to polymerize at about 50% and 20% lower Hb concentrations respectively [33]. In this instance we saw no advantage in the substitution of an amino acid different from that occurring naturally. This strategy has been borne out because the triple mutant D75Y(α)/E6V(β)/E121R(β) twice the enhanced polymerization of the mutant E6V(β)/E121R(β) and the substitution of an alanine residue for Asp at α6 decreased further the solubility of the HbS triple mutant by approx. 40%. These results show not only the overall enhanced effects but also the individual contributions of different sites on HbS polymerization.

An important conclusion of these studies is that recombinant higher-order sickle Hb mutants can be selected beginning with a fundamental knowledge of the gelation concentration of the natural HbS double mutants as a guide. These results also suggest that some sites are more effective than others in promoting polymerization. The correlation of the extent of polymerization enhancement with increasing order of mutation, i.e. the linearity which has been shown previously [25], represents a concept that we plan to extend. The absence of any saturation effect from this profile suggests that recombinant mutants with even lower polymerization concentrations could be designed.

Polymerization-inhibiting sickle Hb mutants

Double mutant E6V(β)/L88A(β) A recombinant double mutant of sickle Hb, E6V(β)/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 [34]. Thus a construct encoding the donor Val-6(β) of the expressed recombinant HbS and a second mutation encoding an alanine residue in place of Leu-88(β) was assembled. The gelation concentration of E6V(β)/L88A(β) (31.2 g/dl), measured by the oxygen-affinity method, was significantly higher than that of either recombinant or natural sickle Hb (23.7 g/dl). These results indicate that the strength of the interaction in this important donor–acceptor region in sickle Hb was considerably decreased even with such a conservative hydrophobic mutation.

Site-directed mutagenesis. The construction of an M13mp18 recombinant phage containing the β-globin cDNA has been described previously [12–14]. 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 5′-AGT GCA GCT CAC TAG CTG TGG CAA AGG TG-3′ was used to create the new mutation Leu-88(β) → Ala by the procedure described by Kunkel [17]. The underlined bases were used to create the mutation. The new recombinant phage therefore contained the double mutation Glu-6(β) → Val and Leu-88(β) → Ala. The replicative form of the mutant phage was digested with *Sph*I and the fragment containing the mutated β-globin gene was recombined with the large *Sph*I fragment of pGS189, creating $pGS189^{E6A(\beta)/L88A(\beta)}$. This α- and β-gene cassette was excised as a *Not*I fragment and similarly inserted into the *Not*I site of pGS389 to give the recombinant plasmid, $pGS389^{E6A(\beta)/L88A(\beta)}$.

Purification and characterization. Purification was achieved on the CM-cellulose 52 and Synchropak CM-300 HPLC columns as described by Martin de Llano et al. [13–15]. The mutant E6V(β)/L88A(β) was subjected to isoelectric focusing by using the pH 6–8 Hb-Resolve system; it behaved as a single band with no detectable impurities. There was no separation between recombinant HbS and E6V(β)/

L88A(β), which was consistent with the lack of a charge difference in the substitution. The purified Hb double mutant was subjected to HPLC on a Vydac C_4 column to separate the α- and β-subunits. The mutant β-chain was eluted earlier from this column than the β-chain of the recombinant HbS, which was consistent with the less hydrophobic nature of the second mutation, i.e. Ala for Leu. As shown in Table 4, the measured mass of the α-chain was 15126 (calculated value 15126). The mass of the β-chain from the double mutant was 15794 (calculated value 15796) as compared to with a mass of 15838 for the natural sickle β-chain. This difference of 42 mass units is consistent with the substitution of an alanine residue for a Leu on the sickle β-chain. The results from tryptic digestion of the mutant β-chain are also consistent with the desired mutation [34].

CD studies. The CD spectra of the recombinant HbS and recombinant double mutant E6V(β)/L88A(β) were as compared to with those of natural HbA and HbS isolated from human red cells. The spectra of liganded Hb in three different spectral regions have been shown previously [34]. The regions from 200 nm to approx. 290 nm are due mainly to the globin portions of Hb and the regions from 290 to 650 nm are a reflection of the haem contribution to the CD properties as it is bound to the globin. In general, the results showed 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 UV region of the spectra with a minimum at 222 nm, which is a measure of the overall protein conformation [35]. The ellipticity values at 222 nm are independent of the presence of haem on the protein except to the extent that haem contributes to correct folding. Comparison of all four spectra indicated that some minor differences existed between the individual Hbs [34]. However, even for natural HbS and HbA, there are small differences at 222 nm that are probably intrinsic to these two 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. For unliganded Hb samples, the CD spectra showed just minor differences for all four samples (results not shown).

Functional studies. The shape of the oxygen equilibrium curve of this double mutant was the same as that found for natural HbS and recombinant HbS [15]. The degree of co-operativity ($h = 3$) was the same as that found for recombinant sickle Hb (Table 5) [15]. These results are also consistent with the conclusion that the recombinant double mutant L88A(β) expressed by the yeast system is fully functional.

Gelation. Gelation experiments of this double mutant were performed by the oxygen-affinity method. The gelation concentration of both natural and recombinant HbS was 23.7 g/dl; that for the E6V(β)/L88A(β) double-mutant gels was at a 30% higher concentration, i.e. 31.2 g/dl. This rather conservative mutation Leu-88(β) $\rightarrow$ Ala, which maintained the hydrophobic nature of the acceptor site, has a quite significant effect on the gelation of sickle Hb. The data also show that HbA does not gel at protein concentrations up to 50 g/dl.

Comments. A comprehensive study employing CD was undertaken to investigate the overall conformation of the recombinant Hb expressed in the yeast system. Because few studies on the overall structure of recombinant Hb have been performed so far, 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 has an important role in the aggregation process. The CD results indicate that the overall structures of both recombinant HbS and the recombinant βL88A 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 the conformation of the folding of the protein backbone [35]. The data for these ellipticity values indicated that the two natural Hbs and the two recombinant Hbs had the same degrees of secondary structure. The small differences in the regions of the haem absorbances for the recombinant Hb did indicate some minor differences, but these were insufficient to affect the functional properties of the recombinant Hb. There were also some minor differences between the natural HbA and HbS in various parts of the CD spectra, which were of the same order of magnitude as the differences between the recombinant and natural Hbs. These differences are probably intrinsic to HbA and HbS. In general, these results agree with previous reports on the CD properties of Hb [35] and indicate that the yeast expression system produces recombinant Hbs that are folded correctly.

One 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 haem but the extent of interaction, if any, between them and any possible role of Leu-88 in stabilizing the haem moiety were not known. Therefore a hydrophobic substitution was chosen especially because the two known natural Hb mutants, which have an Arg or Pro residue at this position, are unstable. In none of our studies did we find any evidence for haem loss even after the recombinant mutant had been subjected to a number of procedures including cation-exchange chromatography in several systems, dialysis and ultrafiltration. Adachi et al. [36] have also addressed this initial hydrophobic interaction by making substitutions at both the donor Val-6(β) and the part of the acceptor region involving Leu-88(β).

The results 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 to 31.2 g/dl

brought about by the substitution of the isobutyl chain of a leucine residue by the methyl group of an alanine residue 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. Replacement 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 is considerably weakened. In accord with this concept are the results of Baudin-Chich et al. [37], who found that replacement of the hydrophobic side chain of Val-6(β) by an Ile residue led to an enhancement in gelation.

Double mutant E6V(β)/K95I(β) The role of Lys-95(β), which is on the exterior of the Hb (HbS) tetramer, in the aggregation process has been addressed because there is a lack of agreement on its importance [38]. The early studies on the aggregation of HbS in the presence of other mutant haemoglobins are consistent with the subsequent electron microscopic studies in demonstrating the participation of Lys-95(β) in gelation; the results of the crystal structure do not agree with this conclusion. Therefore, with the objective of clarifying its role, we performed site-directed replacement of Lys-95(β) by an isoleucine residue. Of particular interest was the finding that this Hb mutant aggregated at a concentration of approx. 40 g/dl, measured by the oxygen-affinity method, nearly twice that at which HbS itself aggregated (24 g/dl). Therefore Lys-95(β) has a very important role in the aggregation process and is a good candidate site for the design of a therapeutic agent for sickle-cell anaemia.

Site-directed mutagenesis. To prepare the E6V(β)/K95I(β) double mutant, an *Xho*I fragment of pGS189 containing the sickle β-globin cDNA was first inserted in the *Xho*I site of Bluescript SK(+). This plasmid was used as a template in the PCR reactions [26]. Two overlapping PCR products were synthesized by a PTC-100-60 instrument (MJ Research, Watertown, MA, U.S.A.) by using separately the 5′-ATC CAC GTG CAG GAT GTC ACA GTG CAG-3′ and pUC/M13 Forward and the 5′-CTG CAC TGT GAC ATC CTG CAC GTG GAT-3′ and M13 Reverse primers. The underlined bases were those used to bring about the desired mutation. These products were recombined in a separate PCR reaction by using the pUC/M13 forward and M13 reverse primers. The final amplified DNA was digested by *Xho*I; the 1280 bp fragment was purified from agarose gel. This fragment was subcloned to the 4130 bp *Xho*I fragment of pGS189. This α- and β-gene cassette was excised as a *Not*I fragment and similarly inserted into the *Not*I site of pGS389 to give the recombinant plasmid pGS389$^{E6V(\beta)/K95I(\beta)}$.

Purification and characterization. Purification of the double-mutant Hb was accomplished by chromatography on CM-cellulose 52 and Synchropak CM-300 HPLC columns (Linden, IN, U.S.A.) [13–15]. Its purity was verified by isoelectric focusing with the pH 6–8 Hb-Resolve system; its migration was consistent with the charge differences at the two mutation sites, i.e. the removal of one negative charge in sickle Hb (Glu → Val) was compensated for by the removal of one positive charge in the double mutation (Lys → Ile). Thus the double mutant migrated near HbA on isoelectric focusing. Both mutations were on the exterior of the protein, so that the full effect of these pK_a changes was reflected in its electrophoretic behaviour. A molecular mass of 15823.9 Da of the β-chain agrees well with the calculated molecular mass of 15823.3 Da (Table 4). The difference of 14.4 Da from the mass of sickle Hb (15838.3 Da) is within experimental error of the expected difference of 15 Da between a Lys (146.2 Da) and an Ile (131.2 Da) residue. The molecular mass obtained for the α-chain (15128.4 Da) was consistent with the calculated value (15126.4 Da) within the error of the measurement.

When the α- and β-chains were separated by an HPLC Vydac C_4 column equilibrated with 38% (v/v) acetonitrile in 0.1% (v/v) TFA, the double-mutant β-chain was eluted after the α-chain with an elution time of 56.2 min compared with an elution time of 36.6 min for a normal β-chain and 38.7 min for a sickle β-chain. The order of elution of α- and β-globin chains was the reverse of that usually observed. Thus the double-mutant β-chain showed a marked difference in its elution behaviour as compared to with both HbS and HbA. The choice of Ile as the substitution also facilitated the identification of the mutation site, because natural HbS does not contain this residue. The results from tryptic digestion of the mutant β-chain are also consistent with the desired mutation [38]. The amino acid composition of the isolated β-chain showed the theoretical value of 1 mol of Ile per Hb chain.

Functional studies. This recombinant mutant retained the functional properties of Hb, with a typical sigmoidal oxygen binding curve. The P_{50} at high Hb concentration (4.1 mM) of E6V(β)/K95I(β) was 33.5 mmHg; it was fully co-operative with a Hill coefficient of 3.1. It also responded normally to the allosteric regulators chloride and 2,3-diphosphoglycerate (Table 5).

Gelation. Gelation experiments of this double mutant were performed by using the oxygen-affinity method. The P_{50} of different concentrations of the double mutant was determined in 100 mM potassium phosphate buffer, pH 6.8. The P_{50} values ranged from 23.5 to 58.5 mmHg between Hb concentrations of 5 and 48 g/dl. There was a rapid decrease in the oxygen affinity once the concentration of the E6V(β)/K95I(β) double mutant reached 39.5 g/dl. When compared with the value of 23.7 g/dl for sickle Hb, it is obvious that the substitution for Lys-95(β) had a major effect on the process of HbS aggregation, in agreement with the

conclusions of Bookchin and Nagel [4], of Josephs and co-workers [6] and of Edelstein and Crepeau [7].

Comments. If a therapeutic intervention for sickle-cell disease is directed at the sickle Hb molecule itself, it would be advantageous to know the strengths of the various side-chain interactions between the deoxy-HbS tetramers to be able to choose the most effective target site. The solution of the structure of sickle Hb aggregate revealed many contact sites for the aggregate but did not provide an indication of their relative strengths. Our objective was to measure such a value for Lys-95(β), a prominent site on the exterior of the molecule. From our results, the contribution of this site to the aggregation process seems to be even greater than that of the initial hydrophobic interaction involving Val-6(β) and the Phe-85(β)/Leu-88(β) region, because the Hb concentration for the E6V(β)/K95I(β) double mutant at the onset of gelation increased much more (39.5 g/dl) than that for the E6V(β)/L88A(β) double mutant (31.2 g/dl) compared with 23.7 g/dl for HbS [24]. Considering that the E6V(β)/K95I(β) double mutant retained the original Leu-88(β), this effect on gelation is especially marked.

In considering the earlier approach of using mixtures of different mutant haemoglobins with substitutions at various positions to assess possible points of interaction in the sickle Hb aggregate, the argument could be raised that, because the haemoglobins had the substitutions on different tetramers, it was not an accurate representation of the aggregation process in red cells. However, our conclusions do not support such an argument because our results on the recombinant double-mutant tetramer of HbS with the substitutions on the same β-chain agree with those of Bookchin and Nagel [4] on the importance of Lys-95(β).

In general, in choosing the amino acid to be introduced by site-directed mutagenesis, it has been our experience that it is useful to evaluate first the properties of the natural Hb mutants at the same position [26,39]. In this instance the effects of the natural mutants Hb Detroit [Lys-95(β) → Asn] and HbN Baltimore [Lys-95(β) → Glu] on the aggregation of HbS [4] were evaluated. The latter Hb impeded the gelation of HbS substantially more than did the former, which is consistent with the idea that an ionic bond between Lys-95(β) and some negative side chain on an adjacent tetramer was involved as a contact site. Therefore the acidic substitution in HbN Baltimore would be expected to generate charge repulsion at this site and decrease gelation. The Asn substitution in Hb Detroit would not be expected to have such an effect. It was expected that if this rationale were correct, the Ile side chain in the recombinant Hb might seek an interaction elsewhere rather than with the putative negatively charged acceptor and hence lower the gelation concentration. However, if the Ile substitution were without effect on gelation, such a movement would not have been necessary because no such interaction existed at this site. The findings of a large inhibitory effect for the Ile-95(β) substitution are consistent with the possibility that an ionic contact involving Lys-95(β) was prevented by the replacement. Eaton and Hofrichter [8] point out that Lys-95(β) is near the dimer interface, so its replacement by a non-polar side chain could have a large effect on the intratetrameric contacts.

In addition to the very large effect of the Ile-95(β) substitution on the overall process of aggregation as measured by the decrease in oxygen affinity with increasing HbS concentration, there are some other interesting differences with the profile of the aggregation of HbS alone. Hence, at Hb concentrations lower than at the onset of gelation, the slope of the line for the initial increase in P_{50} is less than that for HbS itself. However, once aggregation commences (above 39.5 g/dl), the rates of the decrease in oxygen affinity (P_{50} increase) are parallel for both HbS and the K95I(β) double mutants. This behaviour is probably due to the decreased ability of the double mutant to aggregate during the initial phase, leading to an increased concentration requirement for the onset of gelation.

The oxygen-affinity method used for this mutant measures the overall extent of polymerization but does not provide information on the details of the process [24]. With the use of this same procedure, it has been reported that HbF participates very little in the process of polymerization (i.e. an 80% increase in the gelling concentration). Our results indicate an increase in the gelling concentration for K95I(β) of approx. 70% (i.e. of the same order of magnitude as the inhibitory effect of HbF). It will be interesting to compare the relative strength of the interaction at Lys-95(β) with those of other sites in the aggregate for which there is currently no quantitative information. The results of the present study clearly indicate the importance of Lys-95(β) in the process of sickle Hb aggregation and reveal this site to be a candidate as a target for the development of a therapeutic agent for sickle-cell anaemia directed at the HbS molecule itself.

Triple mutant E6V(β)/L88A(β)/K95I(β) As part of a comprehensive effort to map the most important regions of sickle Hb that are involved in polymerization, we have determined whether two sites, Leu-88(β) and Lys-95 (β), had additive effects when mutated. A sickle Hb triple mutant with three amino acid substitutions on the β-chain, E6V(β)/L88A(β)/K95I(β), has been expressed in yeast [23]. The polymerization concentration was identical with that of the E6V(β)/K95I(β), i.e. when the K95I(β) substitution was present on the same tetramer together with the naturally occurring E6V(β) substitution, the L88A(β) replacement had no additive effect on polymer inhibition. The results suggest that Lys-95(β) on the surface of the tetramer and its

complementary binding region on the adjoining tetramer are potential targets for the design of an effective anti-sickling agent.

Site-directed mutagenesis. To prepare the E6V(β)/L88A(β)/K95I(β) triple mutant, we used the M13mp18 recombinant phage as a template. The construction of this phage containing the β-globin cDNA with the E6V(β) and the L88A(β) coding mutations has been described [13,14]. The oligonucleotide 5′-ATC CAC GTG CAG GAT GTC ACA GTG-3′ CAG was used to create the Lys-95(β) → Ile mutation by the method of Kunkel [17]. The presence of the mutations was screened by partial sequencing of the mutation site. The mutation frequency was increased to 65% by supplementing the reaction mixture with the Gene 32 Protein from Boehringer Mannheim and by prolonging the reaction duration. The mutated β-globin region was subcloned to pGS189^{sickle}, which contains the native α-globin and the Glu-6(β) → Val mutated β-globin cDNA species. This α- and β-gene cassette was excised as a *Not*I fragment and similarly inserted into the *Not*I site of pGS389 to give the recombinant plasmid pGS389$^{E6V(\beta)/L88A(\beta)/K95I(\beta)}$.

Purification and characterization. E6V(β)/L88A(β)/K95I(β) was purified on a CM-cellulose 52 column and its purity was verified by isoelectric focusing with the pH 6–8 Hb-Resolve system. It showed a similar pI to the E6V(β)/K95I(β) mutant [38], in agreement with the expected mutations, because the L88A(β) mutation has been shown not to have an altered pI [34]. The α- and β-globin chains were separated by reverse-phase HPLC on a Vydac C_4 column by using isocratic elution with 38.8% (v/v) acetonitrile in 0.1% TFA. Each had the expected amino acid composition; the results from tryptic digestion of the mutant β-chain were also consistent with the desired mutation [23]. The expected molecular mass (15779.0 Da) of the mutant β-chain agreed well with the calculated value of 15781.2 Da for a β-chain with the three substitutions (Table 4). The measured molecular mass for the α-chain (15124.0 Da) is in accord with the calculated value (15126.4 Da) for the natural α-chain of HbA, within experimental error.

Functional studies. The oxygen affinity of this mutant at a Hb concentration of 0.5 mM showed an average P_{50} of 10 mmHg; the Hill coefficient was 2.7, indicating that the triple mutant retained full co-operativity (Table 5). DPG at a DPG-to-Hb ratio of 1.2:1 showed a significant decrease in oxygen affinity. The effects of chloride were comparable with those measured for HbS.

Tetramer–dimer dissociation. Considerable dimerization might occur in Hb even when the Hill coefficient shows high co-operativity, as discussed by Forsen and Linse [40]. The tetramer–dimer dissociation constant for the triple mutant was 0.4 μM (Table 6), compared with the K_d of 0.7 μM for HbS, indicating that the newly produced mutant Hb did not undergo increased dissociation; in other words, at the Hb concentrations used for the functional studies (0.5–2.2 mM) the Hb was predominantly tetrameric.

Gelation. Gelation concentrations of the mutant E6V(β)/K95I(β)/L88A(β) and other two mutants described above, E6V(β)/L88A(β) and E6V(β)/K95I(β), were measured by using the dextran-C_{sat} method; the results are shown in Table 7. A comparison of the dextran-C_{sat} method used for the direct measurement of gelation with the oxygen-affinity method indicates the similar effectiveness of each mutation on the inhibition of polymerization, even though the absolute values differ. Thus ratios of 1.27 and 1.36 between the gelation concentrations of recombinant haemoglobins E6V(β)/K95I(β)/L88A(β) and E6V(β)/L88A(β) were calculated by the oxygen-affinity method and the present method respectively. At initial concentrations below C_{sat}, the final concentrations of each Hb at equilibrium, when plotted as a function of the initial concentrations, fell on a line with a slope of 1.03, indicating that the procedure itself did not result in precipitation (denaturation) of the haemoglobins. When initial Hb concentrations exceeded C_{sat}, the final supernatant Hb concentrations (C_{sat} values) remained constant, independently of further increases in initial Hb concentrations over the ranges tested. The mean dextran-C_{sat} value of the triple mutant was 91 mg/ml, indicating that it required a considerably higher concentration than that of deoxy-HbS (mean: 34 mg/ml) for polymerization. The C_{sat} of the triple mutant was not significantly different from the value found for the E6V(β)/K95I(β) double mutant (90 mg/ml); the value for E6V(β)/L88A(β) (67 mg/ml) was between that of deoxy-HbS and those containing the Lys-95(β) substitutions. Deoxy-HbA remained soluble at concentrations up to 149 mg/ml with no evidence of precipitation during the procedure.

Comments. The residue Lys-95(β), which is distant from the hydrophobic pocket in the region of Phe-85(β)–Leu-88(β) comprising the acceptor site for Val-6(β), inhibits gelation much more than the replacement of a residue in the pocket itself [34,38]. Our results agreed with some previous reports implicating Lys-95(β) in the gelation process and as an intermolecular contact site in the polymer [4,6–7], although this site was not involved in the Wishner–Love double-strand crystal of deoxy-HbS tetramer [5]. The strong influence of the β-95 site on gelation, which is located on the exterior of the tetramer at the lateral contact site of the HbS tetramer, strongly suggests that the K95I(β) mutant of HbS has different protein self-assembly properties from those of HbS itself [41].

The role of Val-6(β) and its hydrophobic acceptor pocket might be to provide a molecular switch to turn the gelation either on or off. If this position is mutated to Ala (Hb Makassar), no gelation occurs because Ala prevents sufficient stabilization of the primary nuclei. Our results on the gelation of E6V(β)/L88A(β) mutant also suggest that the Leu to Ala

substitution in the acceptor pocket affects mainly the initial nucleation process; however, once nucleation has taken place other residues stabilize the polymer. These findings also emphasize the importance of certain ionizable surface amino acids. Their potential importance, as well as that of their complementary sites on adjacent tetramers, forms the basis for the possible development of clinical intervention against sickle-cell disease. The results presented previously demonstrate that two sites on the HbS tetramer exert significantly different and independent effects on the inhibition of polymerization [23].

Because the polymer solubility of the triple mutant was the same as that of the double mutant without the L88A(β) substitution, i.e. E6V(β)/K95I(β), the present results demonstrate that the inhibitory effects of the two β-chain substitutions [L88A(β) and K95I(β)] on HbS are not additive. Although the L88A(β) mutant, in which the substitution is in the hydrophobic acceptor pocket, has a gelation concentration about midway between the K95I(β) mutant and HbS itself, it does not seem to influence the overall behaviour of the triple mutant.

The results of recent studies on recombinant mutants are consistent with the notion that once the initial contact site has been established by the Glu-6 → Val substitution in the sickle Hb tetramer, additional substitutions might strengthen or weaken the tendency to polymerize. The only previous study involving two β-chain mutations of HbS was by Trudel et al. [28] on a transgenic mouse system, with the purpose of promoting polymerization to obtain a better transgenic mouse model of sickle-cell anaemia. In that study there was no quantification of the individual effects of the substitutions on polymer solubility. The present study was aimed at furthering our understanding of the mechanism of gel formation by inhibiting polymerization and at identifying the most important sites that influence the polymerization process significantly. The results indicate that amino acid replacement at Leu-88(β) and Lys-95(β) act independently in inhibiting polymerization, i.e. certain sites can influence the overall prevention of polymerization to a greater extent than others. Such sites might be potentially accessible to anti-sickling agents that could be designed to fit their particular environment as well as that of their complementary binding site on adjacent tetramers. The Lys-95(β) site and the site to which it binds seem to fulfil such criteria.

Double mutant D85K(α)/E6V(β) Clinical modalities based on the inhibition of gelation of HbS are hindered by the lack of quantitative information on the extent of participation of different amino acid residues in the aggregation process. One such site is Asp-85(α), which is involved in a parallel ionic interaction between double strands with Lys-144(β) according to the crystal structure of HbS, but electron microscopy does not specifically show Asp-85(α) as a contact site for fibre formation. We have replaced this site by Lys to abolish ion pairing and to make a quantitative determination of its participation in aggregation [42]. The gelation concentration of the double mutant D85K(α)/E6V(β) was measured by using the dextran-C_{sat} method with a C_{sat} of 53 mg/ml compared with 34 mg/ml for HbS.

Site-directed mutagenesis. To prepare the D85K(α)/E6V(β) mutant we first inserted the α-globin-coding gene from pGS189 into pBluescript II SK(+) as a *Sal*I fragment. The modified plasmid was transformed in *E. coli* BW313; the uridine-containing single-stranded DNA was isolated from the supernatant of the bacterial culture after infection of the cells with M13KO7 helper phage. The oligonucleotide 5′-GTG CGC GTG CAG CTT GCT CAG GGC CGG A-3′ was used to create the Asp-85(α) → Lys mutation by the method of Kunkel [17]. The presence of the mutations was screened by partial sequencing of the mutation site. The mutated α-globin region was subcloned to pGS189^{sickle}, which contains the native α-globin and the Glu-6(β) → Val mutated β-globin cDNA species, by digestion with *Bss*HII and *Bst*EII to create incompatible cohesive termini and thus to increase the percentage of the insert in correct orientation. Finally, this α- and β-gene cassette was excised as a *Not*I fragment and similarly inserted into the *Not*I site of pGS389 to give the recombinant plasmid pGS389$^{D85K(\alpha)/E6V(\beta)}$.

Purification and characterization. D85K(α)/E6V(β) adhered more avidly than HbS to a CM-cellulose 52 column, as expected for a mutant having an Asp → Lys surface mutation. For elution, a gradient of up to 25.5 mM potassium phosphate was required, in contrast with 15 mM for HbS. The purified Hb was rechromatographed on a Mono Q column from which it was eluted as a single peak [42]. Isoelectric focusing of the purified D85K(α)/E6V(β) indicated a pI close to 8.0. The molecular mass (15140.3 Da) obtained for the α-chain agrees well with the theoretical value of 15139.5 Da for the mutant α-chain (Table 4). The difference of 13.9 Da between the measured value and the calculated value of a wild-type α-chain of HbA (15126.4 Da) is close to the calculated difference (13.1 Da) between the molecular masses of the Asp and Lys residues. The molecular mass obtained for the β-chain (15835.4 Da) agreed with the calculated value for HbS (15838.2 Da) within the error of the measurement. The results from tryptic digestion of the mutant α-chain are also consistent with the desired mutation [42].

Tetramer–dimer dissociation. The tetramer–dimer dissociation constant for the mutant D85K(α)/E6V(β) was found to be 2.1 μM (Table 6). This value is slightly higher than the K_d of 0.7 μM for HbS. Because it is lower than the Hb concentration used for the functional studies such as the oxygen binding curve, the Bohr effect and the gelation studies, this mutant was predominantly tetrameric during these measurements.

Functional studies. The mutant D85K(α)/E6V(β) showed a typical sigmoidal oxygen equilibrium curve with a P_{50} of 10 mmHg, which is the same as that for HbA and HbS. It was co-operative with an average Hill coefficient of 2.7 (Table 5).

Gelation. The C_{sat} of D85K(α)/E6V(β) at three different initial Hb concentrations of 56, 64 or 127 mg/ml was between 50 and 55 mg/ml. An initial Hb concentration of 31 mg/ml was below the C_{sat}, so no change in the Hb concentration was observed after incubation and centrifugation. The average C_{sat} of 53 mg/ml D85K(α)/E6V(β) is clearly higher than the C_{sat} of 34 mg/ml for HbS but much lower than the values for two other double mutants, namely E6V(β)/L88A(β) (C_{sat} 67 mg/ml) and E6V(β)/K95I(β) (C_{sat} 90 mg/ml).

Comments. The gelation concentration of the D85K(α)/E6V(β) double mutant was elevated to 53 mg/ml, in comparison with 34 mg/ml obtained for HbS. This decreased tendency for gelation is consistent with the X-ray studies [5], i.e. in the HbS crystal the Asp-85(α) residue forms an ion pair with Lys-144(β) of the adjacent Hb tetramer, which is abolished in the D85K(α) mutant; gelation is consequently inhibited. Three natural Asp-85(α) mutants, Hb G-Norfolk (Asp → Asn) [43], Hb Atago (Asp → Tyr) [44] and Hb Inkster (Asp → Val) [45], have been described but no studies have been reported on their participation in gelation. Our conclusion that Asp-85(α) contributes moderately to the strength of the HbS aggregate is also consistent with, but does not distinguish between, the electron microscopic models [6,44] and further establishes its quantitative participation in gelation. The model of Watowich et al. [46] suggests some degree of participation of Asp-85(α) in the gelation, because it is 5–8 Å distant from the adjacent tetramer.

Conclusions

The findings demonstrate the feasibility of expressing multiple HbS mutants in the yeast system to understand the sites that lead to an enhanced polymerization as well as those that give a decreased polymerization [48]. For the polymerization-enhancing HbS mutants we have determined the overall enhanced effects and also the individual contributions of some different sites to HbS polymerization. These mutants polymerize in a linear manner at progressively lower Hb concentrations as a function of the degree of substitution, suggesting that these remote sites [D6A(α), D75Y(α) and E121R(β)] on the α- and β-chains exhibit additive, enhanced polymerization properties. We have also demonstrated that the inhibition of polymerization of HbS was dependent on the site of the amino acid substitution. Furthermore, we found that a substitution at one particular site on the exterior of the HbS tetramer inhibited polymerization more than a substitution at the initial hydrophobic interaction itself between Val-6(β) and Leu-88(β) on the adjacent tetramer and that they acted independently. Those results clearly demonstrate in a quantitative manner that some sites are more important than others in promoting and inhibiting the polymerization of HbS [47].

Acknowledgments

We thank Dr. Antoine Dumoulin and Dr. Vincenzo Nardi-Dei for their continual assistance with the techniques, Dr. Peter W. van Ophem for performing the amino acid analysis, and William Fowle for the photography. This work was supported in part by NIH grant HL-18819 (J.M.M.) and NIH grant RR-00862 (B.T.C.). J.C.P. is the recipient of a postdoctoral fellowship from FAPESP, São Paulo, Brazil.

References

1 Pauling, L., Itano, H., Singer, S. J. and Wells, J. C. (1949) Science **110**, 543–548
2 Ingram, V. M. (1956) Nature (London) **178**, 792–794
3 Bunn, H. F. and Forget, B. (1986) Hemoglobin: Molecular, Genetic and Clinical Aspects, Saunders, Philadelphia
4 Bookchin, R. M. and Nagel, R. L. (1971) J. Mol. Biol. **60**, 263–270
5 Padlan, E. A. and Love, W. E. (1985) J. Biol. Chem. **260**, 8272–8279
6 Watowich, S. J., Gross, L. J. and Josephs, R. (1989) J. Mol. Biol. **209**, 821–828
7 Edelstein, S. J. and Crepeau, R. H. (1979) J. Mol. Biol. **134**, 851
8 Eaton, W. A. and Hofrichter, J. (1990) Adv. Protein. Chem. **40**, 63–279
9 Cerami, A. and Manning, J. M. (1971) Proc. Natl. Acad. Sci. U.S.A. **68**, 1180–1183
10 Dean, J. and Schechter, A. N. (1978) New Engl. J. Med. **299**, 863
11 Manning, J. M. (1991) Adv. Enzymol. Relat. Areas Mol. Biol. **64**, 55–91
12 Wagenbach, M., O'Rourke, K., Vitez, L., Wieczorek, A., Hoffman, S., Durfee, S., Tedesco, J. and Stetler, G. L. (1991) Bio/Technology **9**, 57–61
13 Martin de Llano, J. J., Schneewind, O., Stetler, G. and Manning, J. M. (1993) Proc. Natl. Acad. Sci. U.S.A. **90**, 918–922
14 Martin de Llano, J. J., Schneewind, O., Stetler, G. and Manning, J. M. (1994) Methods Enzymol **231**, 390–403
15 Martin de Llano, J. J., Jones, W., Schneider, K., Chait, B. T., Rodgers, G., Benjamin, L. J., Weksler, B. and Manning, J. M. (1993) J. Biol. Chem. **268**, 27004–27011
16 Silver, J., Limjoco, T. and Feinstone, S. (1995) in PCR Strategies (Innis, M. A., Gelfand, D. H. and Sninsky, J. J., eds.), pp. 179–188, Academic Press, San Diego, CA

17 Kunkel, T. A. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 488–492
18 Chait, B. T. and Kent, S. B. (1992) Science **257**, 1885–1894
19 Chowdhury, S. K., Katta, V. and Chait, B. T. (1991) in Methods and Mechanisms for Producing Ions from Large Molecules (Standing, K. G. and Ens, W., eds.), pp. 201–210, Plenum Press, New York
20 Beavis, R. C. and Chait, B. T. (1996) Methods Enzymol. **270**, 519–551
21 Manning, L. R., Jenkins, W. T., Hess, J. R., Vandegriff, K., Winslow, R. M. and Manning, J. M. (1996) Protein Sci. **5**, 775–781
22 Bookchin, R. M., Balazs, T. and Lew, V. L. (1994) Blood **86**, 473a
23 Himanen, J.-P., Mirza, U. A., Chait, B. T., Bookchin, R. M. and Manning, J. M. (1996) J. Biol. Chem. **271**, 25152–25156
24 Benesch, R. E., Edalji, R., Kwong, S. and Benesch, R. (1978) Anal. Biochem. **89**, 162–173
25 Li, X., Mirza, U. A., Chait, B. T. and Manning, J. M. (1997) Blood **90**, 4620–4627
26 Yanase, H., Manning, L. R., Vandegriff, K. D., Winslow, R. M. and Manning, J. M. (1995) Protein Sci. **4**, 21–28
27 Sumida, I., Ohta, Y., Imamura, T. and Yanase, T. (1973) Biochim. Biophys. Acta **322**, 23–26
28 Trudel, M., Saadane, N., Garel, M.-C., Bardakdjian-Michau, J., Blouquit, Y., Guerquin-Kern, J.-L., Rouyer-Fessard, P., Vidaud, D., Pachnis, A., Romeo, P.-H. et al. (1991) EMBO J. **10**, 3157–3165
29 Charache, S. and Conley, C. L. (1964) Blood **24**, 25–48
30 Milner, P. F., Miller, C., Grey, R., Seakins, M., De-Jong, W. W. and Went, L. N. (1970) New Engl. J. Med. **283**, 1417–1425
31 Langdown, J. V., Williamson, D., Knight, C. B., Rubenstein, D. and Carrell, R. W. (1989) Br. J. Haematol. **71**, 443–444
32 Vella, F., Wiltshire, B., Lehmann, H. and Galbraith, P. (1973) Clin. Biochem. **6**, 66–70
33 Benesch, R. E., Kwong, S., Edalji, R. and Benesch, R. (1979) J. Biol. Chem. **254**, 8169–8172
34 Martin de Llano, J. J. and Manning, J. M. (1994) Protein Sci. **3**, 1206–1212
35 Fasman, G. D., Foster, R. J. and Beychok, S. (1966) J. Mol. Biol. **19**, 240–253
36 Adachi, K., Konitzer, P., Kim, J., Welch, N. and Surrey, S. (1993) J. Biol. Chem. **268**, 21650–21656
37 Baudin-Chich, V., Pagnier, J., Marden, M., Cohn, B., Loraze, N., Kister, J., Schaad, O., Edelstein, S. J. and Poyart, C. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 1845–1849
38 Himanen, J.-P., Schneider, K., Chait, B. T. and Manning, J. M. (1995) J. Biol. Chem. **270**, 13885–13891
39 Yanase, H., Cahill, S., Martin de Llano, J. J., Manning, L. R., Schneider, K., Chait, B. T., Vandegriff, K. D., Winslow, R. M. and Manning, J. M. (1994) Protein Sci. **3**, 1213–1223
40 Forsen, S. and Linse, S. (1995) Trends Biochem. Sci. **20**, 495–497
41 Eaton, W. A. and Hofrichter, J. (1994) in Sickle Cell Disease: Basic Principles and Clinical Practice (Embury, S. H., Hebbel, R. P., Mohandas, N. and Steinberg, M. H., eds.), pp. 57–83, Raven Press, New York
42 Himanen, J.-P., Popowicz, A. M. and Manning, J. M. (1997) Blood **89**, 4196–4203
43 Lorkin, P. A., Huntsman, R. G., Ager, J. A. M., Lehmann, H., Vella, F. and Darbre, P. D. (1975) Biochim. Biophys. Acta **379**, 22–27
44 Fujiwara, N., Maekawa, T. and Matsuda, G. (1971) Int. J. Protein Res. **3**, 35–39
45 Reed, R. E., Winter, W. P. and Rucknagel, D. L. (1974) Br. J. Haematol. **26**, 475–484
46 Watowich, S. J., Gross, L. J. and Josephs, R. (1993) J. Struct. Biol. **111**, 161–179
47 Manning, J. M., Dumoulin, A., Li, X. and Manning, L. R. (1998) J. Biol. Chem. **273**, 19359–19362
48 Dumoulin, A., Manning, L. R., Jenkins, W. T., Winslow, R. M. and Manning, J. M. (1997) J. Biol. Chem. **272**, 31326–31332

Received 7 January 1999; accepted 12 January 1999