Material and Methods

Materials

Fmoc protected amino acids and other chemicals used in peptide synthesis were obtained from Novabiochem (Switzerland). V8 protease and TFA were procured from Pierce Chemical Company (USA), while 1-propanol, PMB, Hemin, Dithiothreitol, EDTA were obtained from Sigma Chemical Company (USA). DE52 and CM52 ion exchange resins were purchased from Whatman (UK). Sodium diothinite was procured from Fluka (Switzerland) and Catalase from Boehringer Mannheim (Germany). Carboxypeptidase was obtained from Worthington Biochemical Corporation (USA).

Methods

Purification of hemoglobin from blood

Blood was drawn from appropriate source into heparinised tubes. The blood sample was centrifuged at 4000 rpm for 15 min (4°C). The supernatant was discarded, and the erythrocytes (pellet) were subsequently washed thrice with chilled isotonic buffer [0.01 M PBS (pH 7.4)] by centrifugation at 4000 rpm and 4°C for 15 min. The washed erythrocytes were lysed in water. The resultant red cell lysate was then dialyzed extensively against PBS (pH 7.4) at 4°C to obtain stripped hemoglobin (hemoglobin devoid of bound allosteric modulators like BPG). The stripped hemoglobin was then loaded onto a pre-equilibrated DE52 column (30cm x 15cm) after extensive dialysis against 0.05 M tris acetate buffer (pH 8.5). The protein was eluted from the column employing a linear gradient of 500 ml each of 0.05 M tris acetate (pH 8.5) and 0.05 M tris acetate (pH 7) at a flow rate of 50 ml/hour. The purified hemoglobin was estimated spectrophotometrically at 540 nm (molar extinction coefficient = 53236 cm^{-1}/M) and stored at -70°C till further use.

Separation of the α and β subunits of hemoglobin

The heme bound α and β subunits were obtained as described by Bucci (1981). Briefly, hemoglobin was reacted with PMB in an eight fold molar excess (8 moles of PMB per mole of hemoglobin). The reaction mixture was dialyzed extensively against 0.01 M potassium phosphate buffer (pH 6.5) and then loaded onto a CM52 column (30cm x 15cm) that was pre-equilibrated with the same buffer.

The β-PMB and α-PMB chains were eluted with a linear gradient of 500 ml each of 0.01 M potassium phosphate buffer (pH 6.5) and 0.015 M potassium phosphate buffer (pH 8.5) at a flow rate of 50 ml/hour. The chains were separately concentrated using Centriprep concentrators (Amicon) and stored in liquid nitrogen till further use.

Preparation of heme-free α chain

The α-PMB chain was subjected to acid-acetone treatment to separate the heme from the α globin. Briefly, a solution of concentrated α-PMB chain (5 ml; 30 mg/ml) was added dropwise to 100 ml of thoroughly chilled acid-acetone solution (0.5% v/v HCl in acetone) with constant shaking, and then incubated at -20°C for 30 min to allow complete precipitation of the globin. The precipitated globin was isolated by centrifugation at 7000 rpm (4°C) for 15 min and the supernatant containing soluble heme was discarded.

Generation of complementary fragments, α1-30 and α31-141, from heme-free α globin

The complementary segments of α globin needed for the semisynthesis of mutant chains were prepared by V8 protease digestion (Sivaram *et al*, 2001). The α globin was dissolved in 0.01 M ammonium acetate buffer (pH 4) at a concentration of 1.0 mg/ml and digested at 37°C with V8 protease (1: 200, w/w) for 3 hours. The completion of digestion was ascertained by RPHPLC, after which the reaction was quenched by addition of neat TFA to a final concentration of 0.1%. The complementary segments, α1-30 and α31-141, from the digestion mixture were isolated in pure form by size-exclusion chromatography on a Sephadex G50 column (98cm x 2.8cm). The column was equilibrated and run in 0.1% TFA. The lyophilized sample of the digest was dissolved in the above solvent and loaded on to the column. The column was run at a flow rate of 30 ml/hour and the elution profile monitored at 280 nm. The individual chromatographic profile of α globin digest showed only two peaks, α31-141 and α1-30 respectively, as expected from a single cleavage at the 30-31 peptide bond. The peak fractions were pooled separately and lyophilized.

Synthesis of α1-30 analogs

Peptides were synthesized by standard solid phase synthesis protocols using Fmoc chemistry on a semi-automated peptide synthesizer (Model 90, Advanced Chemtech). For this, Wang resin pre-loaded with N-α-Fmoc-Glu was used as the starting material. The stepwise coupling of Fmoc amino acids was performed with DIPCDI/HOBT activation procedure. The coupling of each step was monitored by Kaiser test for free amine and wherever necessary, a double coupling was used to increase the yield. Before each coupling step and on completion of the synthesis, the N-terminal Fmoc group was removed using 20% piperidine (v/v in DMF). The peptides were cleaved from the resin and the side chains deprotected with appropriate volume of a mixture containing TFA, ethanedithiol, phenol, thioanisole and water (80:5:5:5:5, v/v). The resin was removed by filtration and the crude cleaved peptides were precipitated using cold diethyl ether and extracted in water. The peptides were purified by RPHPLC and their chemical identity was checked by mass spectrometry.

Construction of mutant α globins

V8 protease-mediated semisynthesis of α globin was carried out at 4°C in 0.05 M ammonium acetate buffer (pH 6) containing 30% 1-propanol. For this, the lyophilized samples of natural or synthetic analogs of α1-30 and respective α31-141 were individually prepared in water. Suitable volumes of the complementary fragments were mixed to obtain a 1:1 molar ratio and lyophilized. The lyophilized material was dissolved in appropriate amount of ammonium acetate buffer (pH 6). To this solution, a suitable volume of 1-propanol was added to a final concentration of 30% 1-propanol and 20 mg/ml substrate. The mixture was cooled on ice subsequent to which suitable volume of V8 protease solution prepared in water (1% w/w of substrate) was added. The ligation reaction mixture was incubated at 4°C for 24 hours. The extent of synthesis was monitored on RPHPLC by loading an aliquot of the reaction mixture on an analytical reverse phase column. The reaction was stopped by addition of chilled 5% TFA solution (0.2 fold v/v) and lyophilized.

Purification of the semi-synthetic α globin

The semisynthetic α globin was purified from the mixture by CM52 urea chromatography as explained below. The lyophilized sample was dissolved in 0.005 M phosphate buffer (pH 6.9) containing 8 M urea and 0.05 M 2-mercaptoethanol at a concentration of 10-15 mg/ml and loaded onto a CM52 column (16 cm x 1.5 cm) equilibrated with the same buffer. After an initial wash with the same buffer, two linear gradients of (a) 100 ml each of 0.005 M to 0.03 M and (b) 100 ml each of 0.02 M to 0.05 M phosphate buffer (all buffers contained 8 M urea and 0.05 M 2-mercaptoethanol, and were adjusted to pH 6.9) were employed at a flow rate of 45 ml/h to elute the semisynthetic α globin. The column was finally washed with 0.05 M buffer to elute unreacted α31-141 fragment from the column. The elution profile was monitored at 280 nm. The fractions for semi-synthetic α globin were pooled, extensively dialyzed against 0.1% TFA and lyophilized. The semisynthetic yield of the protein varied between 35% to 45%.

Reconstitution of α globin and β^S chain into HbS tetramers

The HbS tetramers were assembled from semisynthetic α globins and β^S chain through the 'Alloplex pathway' (Roy and Acharya, 1994). Briefly, α globin was dissolved at a concentration of 5 mg/ml in 0.05 M tris HCl buffer (pH 7.4) containing 8 M urea, 2 mM DTT and 1 mM EDTA and incubated at room temperature for 30 min, followed by incubation on ice (4°C) for 30 min. The β-PMB chain (β^S/β^A) in the oxy form was taken at a final concentration of 7.5 mg/ml in 0.05 M tris HCl (pH 7.4) containing 2 mM DTT, 1 mM EDTA and 5 μg/ml catalase and incubated on ice (4°C) for 1h. The two solutions were mixed and diluted with 0.05 M tris HCl (pH 7.4) to a final total protein concentration of 1 mg/ml and incubated on ice for 1h to allow the formation of the 'half-filled' (with respect to amount of heme per tetramer) alloplex intermediate. An appropriate volume of freshly prepared hemin dicyanide solution (1 mg/ml in 0.004 N NaOH) was added dropwise (with constant mixing) and the solution was incubated on ice for 1h for formation of the 'fully-filled' cyanometHb tetramer. This reconstituted tetrameric Hb was extensively dialyzed against 0.05 M Tris-HCl (pH 7.4), after which the sample was centrifuged. The dialyzed protein was concentrated to 30-40 mg/ml using Centriprep

concentrators (Amicon), and subjected to reduction with 0.05 M sodium dithionite. For this an appropriate amount of anaerobically prepared dithionite solution was added to the reconstituted Hb and the reaction mixture was quickly passed through a Sephadex G25 gel filtration column (30 cm x 1.5 cm) equilibrated with 0.05 M Tris HCl (pH 7.4), in order to minimize the duration of contact of dithionite with the protein. The reduced Hb was dialyzed extensively against 0.01 M potassium phosphate buffer (pH 6.5) and loaded onto a CM52 column (10cm x 1.5cm) equilibrated with the same buffer. A linear gradient of 150 ml each of 0.01 M potassium phosphate buffer (pH 6.5) and 0.015 M potassium phosphate buffer (pH 8.5) was employed to elute the protein from the column.

Preparation of HbS[des arg 141α]

Purified HbS was digested with carboxypeptidase B (200 mg hemoglobin to 1 mg of the enzyme) for 3 hours in freshly prepared 0.05 M Tris-acetate buffer pH 7.1) at 25^0C , followed by passage through a cation-exchange column using Whatman CM52.

Analytical procedures

The synthetic peptides were purified by RPHPLC on an Aquapore RP300 column (250 x 7 mm) using a 4–72% linear gradient of solvent B (acetonitrile containing 0.1% TFA) in 130 min at a flow rate of 2 ml/min. Globin chains from respective hemoglobins were separated on a similar column of a smaller dimension (250mm x 4.6 mm) under identical conditions but at a flow rate of 0.7 ml/min.

Electro spray mass spectrometric analysis was carried out on a VG Platform (Fisons) mass spectrometer. The instrument was usually calibrated with standard horse heart myoglobin or gramicidin S solution. Appropriate amount of each sample was taken in 50% acetonitrile containing 1% formic acid and analyzed under the positive ion mode.

Spectroscopic studies

Circular dichroism (CD) spectra were recorded on a J710 Spectropolarimeter (Jasco, Japan) fitted with a Peltier type constant temperature cell holder (PTC-348W). The calibration of the equipment was done with (+)-10-camphorsulfonic

acid. The respective buffer baselines were subtracted from the sample CD data. The ellipticity of the protein samples is reported as mean residue ellipticity (MRE) in deg/cm^2/dmol units. The first derivative UV spectra of the oxy and deoxy- HbS were recorded on a Lambda Bio20 spectrophotometer (Perkin Elmer Life Sciences). The hemoglobin concentration used for the spectral measurements was approximately 50 μM on heme basis.The spectra of unliganded proteins was recorded subsequent to deoxygenating the hemoglobin samples by passing moist gaseous nitrogen extensively over the sample in an airtight cuvette. Completion of deoxygenation was ascertained by recording the visible spectrum of the deoxygenated Hb sample.

Analytical Ultracentrifugation experiments

All experiments were carried out on a Beckman XL-A analytical ultracentrifuge, equipped with absorbance optics, and an An60-Ti rotor, at 20 °C. Sedimentation velocity experiments were performed at 40,000 rpm. Data were collected at 540 nm and at a spacing of 0.005 cm with three averages in a continuous scan mode. The protein concentration varied in the range 4-40 μM (heme) in 50 mM phosphate buffer, pH 7.2.

Measurement of gelation concentration, C_{sat}

The gelation concentrations of HbS constructs were determined by the dextran-C_{sat} method of Bookchin *et al* (Bookchin *et al*, 1999). This method allows measurement of C_{sat} under near-physiological conditions and at much lower concentration of HbS (about 5-fold or less) than that required in standard C_{sat} assays, but essentially provides the same information. Briefly, a suitable aliquot of a concentrated solution of hemoglobin in potassium phosphate buffer (0.05 M, pH 7.50) was taken in a 1.5 ml micro-centrifuge tube. A concentrated dextran (70 kDa) solution prepared in the same buffer was added to it and mixed well. This mixture was overlaid with 0.5 ml of mineral oil, chilled on ice bath and deoxygenated with an anaerobically prepared dithionite solution through an airtight Hamilton syringe. The final concentrations of dextran and dithionite in the mixture were 120 mg/ml and 0.05 M respectively. The deoxygenated sample above was allowed to polymerize at 37°C for 30 min after which the gel under the oil layer was disrupted

with the plunger of a Hamilton syringe. The tube was centrifuged at room temperature at 14,000 rpm for 30 min. The above process of gel-disruption and centrifugation was repeated twice subsequent to which the oil layer was aspirated and suitable aliquots from the supernatant were taken for estimation of C_{sat} by Drabkin's reagent (Goldberg *et al*, 1977).

Kinetics of polymerization

The time kinetics of deoxyhemoglobin polymerization were studied in 1.8M, 1.5 and 1 M potassium phosphate buffer (pH 7.25) respectively as described by Adachi and Asakura (1979a, b) using a Cary 400 spectrophotometer equipped with a Peltier temperature controller. Deoxygenation of the hemoglobin sample was ensured by passing moist gaseous nitrogen over the sample in an airtight cuvette and by addition of sodium dithionite. The polymerization of the resultant deoxyhemoglobin samples was initiated by a temperature jump from 4 to 30 °C within 10 sec and the progress of the reaction was followed by monitoring turbidity changes at 700 nm. The delay time was calculated from the kinetic traces.

Electrostatic Potential

Electrostatic potentials were calculated by the Finite Difference Poisson-Boltzmann (FDPB) method using the program MEAD running within the PCE web server (http://bioserv.rpbs.jussieu.fr/PCE) (Miteva *et al*, 2005; Bashford *et al*, 1992). Additions of hydrogen atoms as well as assigning of atomic radii and charges were performed automatically within the server. MEAD numerically solves the Poisson-Boltzmann equation to yield the distribution of electrostatic potential on the protein surface. Calculations were performed on one of the native α-chains of the 2HbS crystal structure (Harrington *et al*, 1997) as well as its SCWRL (Dunbrack *et al*,1993) generated mutants. All calculations were performed by setting the internal protein dielectric constant to 4 and the external solvent dielectric constant to 80. The ionic strength parameter was held at 0.1.

Molecular dynamics (MD) simulation

MD simulations on the α-chain of HbS and the mutants were carried out using the GROMACS suite of programs (Lindahl *et al*, 2001). The initial structure of the native protein was taken from the high-resolution x-ray crystal structure (Harrington *et al*, 1997) of HbS (PDB entry: 2HBS). The structures of the mutants were generated interactively using INSIGHT-II. The initial model structures were placed in a simulation box of size 42.8 x 31.5 x 42.7 Å. The closest distance from any protein atom to the walls of the box was not less than 9 Å. The system was then solvated by adding a bath of SPC (Berendson *et al*, 1981) waters in such a way that the density of the system was as close to 1 as possible. The overall charge of the system was neutralized by placing suitable counter ions wherever necessary (Chapter 1, *Table 4)*. The resulting system was then energy minimized for 1000 steps using the steepest descent algorithm. This was followed by 0.3ns of position restrained MD during which the solvent and counter ions were allowed to move freely but the protein atoms were harmonically restrained to their initial positions. Finally, normal MD was run for 3ns using the default GROMACS force field. Bond lengths were restrained to their equilibrium values using the LINCS (Hess *et al*, 1997) algorithm and a cut-off radius of 0.9nm was used for non-bonded interaction calculation. The temperature of the system was maintained close to 300K by weak coupling to an external temperature bath with a coupling constant of 0.1ps. The integration time step used throughout the simulation was 1fs. MD simulations of single mutant α-chains (K16Q, E23Q and H20Q) as well as the double mutants (K16Q/H20Q, K16Q/E23Q and H20Q/E23Q) were carried out in a similar fashion for 3ns. In order to directly analyze the effects of the mutations on the contact interface, we also carried out MD simulations on a miniature model of the sickle hemoglobin fiber consisting of a complex of two hemoglobin tetramers. In the low salt crystal structure of deoxyhemoglobin S (Harrington *et al,* 1997) the asymmetric unit consists of two HbS tetramers that pack as two strands of HbS molecules running parallel to the crystallographic *a* axis. Axial contacts occur between two tetramers of the same strand and lateral contacts occur between tetramers of different strands. A canonical fiber model was generated by taking one of the two tetramers in the asymmetric unit together with its neighbor translated along the crystallographic *a* axis [Figure 7 (A, B)]. Fiber models incorporating mutant α-

chains were generated in the same way as for the isolated α-chains. Thus the model consisted of eight polypeptide chains (4 α-chains and 4 β-chains) with the central two α and two β chains making up the axial contact interface. The simulations of the above fiber models were carried out without explicit solvent using the GROMOS96 vacuum force field as implemented within the GROMACS suite of programs. Models of HbS mutants were generated using the program SCWRL and the mutant fiber models were generated as for the native HbS model. SCWRL replaces only the side chains of desired residues with the best possible rotamer of the mutated amino acids. Thus the initial backbone conformation of the isolated α-chains and the fiber models remained identical in the native HbS and the mutants. The MD simulation protocol for the fiber models consisted of an initial steepest descent energy minimization for 1000 steps followed by full MD simulation for 1.2ns at 300 K. Essential parameters of the simulation like the radius of gyration, root mean squared deviation from the initial structure as well as the kinetic and potential energies are summarized in Chapter1, Table 4. It was observed that all the global indicators of the simulation stabilized to their average values within 0.2ns. Hence data from 0.2ns till the end of the simulations were used for all subsequent analysis.

Fluctuation Maps:

The fluctuation maps (F_{ij}) were calculated from the MD trajectories of the □-chains as described earlier (Hery *et al*, 1997). The fluctuation value F_{ij} is given by the equation,

$$F_{ij} = \sqrt{\langle d_{ij}^2(t)\rangle - \langle d_{ij}(t)\rangle^2}$$

where $d_{ij}(t)$ is the distance between a pair of designated atoms (C^{α} atoms as used here) at time t and the angle brackets represent time averages. The F_{ij} values are the standard deviation of interatomic distance. The fluctuation maps in Figure 6 has a black dot wherever the F_{ij} value is less than or equal to 0.5Å. Thus dark regions of the map indicate those parts of the molecule which undergo strongly coupled movements.

Distance Frequency Distribution (DFD):

The DFD between an atom pair is the normalized frequency distribution of the interatomic distances sampled from equal time snapshots taken from the MD simulations. DFDs of corresponding atoms taken from the different simulations were used to qualitatively compare the effect of mutational perturbations on the HbS fiber. Considering that the fiber simulations were carried out only for a relatively short time scale of 1.2ns, the calculated DFDs might suffer from errors due to limited sampling. Hence a quantitative comparison of the different DFDs were not attempted, however given that the global parameters monitored during the simulation had already become reasonably stable after 0.2ns (Chapter1, *Table 5*), it is expected that the gross features of the DFDs would remain unaltered even in much longer simulations.