Sickle cell anemia is the consequence of a point mutation (Glu6→Val) at the sixth position in the β-chain of the hemoglobin (Hb) molecule. The replacement of a charged residue with a hydrophobic one causes the deoxygenated protein to polymerize into long and multi-stranded helical fibers that are believed to be the principle cause of the sickle cell disease. Biophysical studies have revealed that the basic unit of the sickle hemoglobin (HbS) fiber consists of 14 filaments made out of a double-stranded arrangement of Hb molecules similar to those found in the crystals of HbS. The fiber architecture is stabilized by two kinds of interactions, namely, intra-double strand (axial and lateral) and inter-double strand. While intradouble strand contacts of the crystal are preserved in the fiber, the inter-double strand contacts are unique to the fiber. The identity of many of these intermolecular contact residues are known from solution polymerization experiments of natural variants or mutant hemoglobins and HbS crystal structure. However, our present knowledge of intermolecular interaction in the HbS fiber is largely limited to the identity of the contact residues with little information on the interaction strength of individual contact residues. The effect of simultaneous perturbation of contact residues have been experimentally demonstrated for some sites but the mechanistic basis of additive or non-additive coupling between these interacting sites is far from clear.

HbS fiber formation is a complex process that requires specific contacts, and presumably intricate interplay between several residues from both, α and β, chains of the tetramer. Besides, the polymerization occurs only on in the deoxy state ("T" state) of the HbS suggesting that quaternary conformational events are detrimental to the polymerization process. However studies of quaternary structure vis a vis HbS polymerization has not been carried out though the impact of quaternary structural constrains has been extensively analysed in the context of deciphering the mechanistic imperatives of hemoglobin allostery. In as much as the HbS polymerization is triggered by the acquisition of T structure, it is legitimate to conceive that perturbation of this quaternary state may have perceptible influence on kinetics and/or equilibrium of the polymerization reaction.

The work described in this thesis concerns with the above aspects of HbS polymerization namely, (a) delineation of interaction-linkage (coupled interactions) between selected fibre contact residues, and (b) probing of the role, if any, of the
Summary

quaternary structural features of the HbS tetramer on the polymerization reaction. These are summarized below:

A. Coupling of interactions between pairs of contact residues: The interaction due to a contact residue may simply be independent of all residues (contact site or otherwise) or might be influenced by coupled interaction with other residues. The coupling of interactions might arise from local perturbation of the microenvironment of the contact residue, through space-electrostatic interactions or long-range cooperativity between remote sites. Previous work from this laboratory had shown that mutations in the AB region (corner of the A-and B-helix) of the α-chain that do not occur in contact sites nevertheless affect fiber formation by altering the dynamics of the AB and GH region axial contacts of the Wishner-Love double strands. The existence of long-range and non-additive coupling between AB and EF regions of the α-chain was also demonstrated.

The focus of the present study was to delineate the molecular basis of the coupling of intermolecular interactions, if any, between pairs of charged residues comprising Lys-16, His-20 and Glu-23 of the α-chain on the polymerization of HbS. In the Wishner-Love double strand model, Lys-16 (A14) and Glu-23 (B4) occur in axial contacts and are located in the vicinity of other charged residues of the AB (His-20) and GH region (Glu-116) of the α-chain. Besides, His-20 is located in close proximity to the Glu-22 residue of the β-chain. Together, these residues are capable of generating a large spatial distribution of surface electrostatic charges at the axial interaction interface with possible ramifications on HbS polymerization. Accordingly, a double-mutant cycle among the aforementioned residues were carried out and three double mutants [HbS (K16Q/E23Q), (K16Q/H20Q) and (H20Q/E23Q)] were constructed.

The above α-globin mutants were constructed employing a chemo-enzymatic strategy, namely V8 protease catalysed ligation of complementary fragments, α 1-30 and α 31-141 to generate a full length α- globin. The sequence comprising residues α 1-30 with Gln substituted at 16, 20 and 23 positions were assembled by solid phase peptide synthesis. The respective purified peptides were ligated with the complementary fragment (α31-141). Semi-synthetic α globins were purified by
cation exchange chromatography and characterized by mass spectrometry. The measured mass of the globin was in agreement with the calculated mass.

HbS mutants reconstituted from the respective mutant α-chain [K16Q/E23Q, K16Q/H20Q, and H20Q/E23Q] and β^8-chain eluted as a single peak when chromatographed on a FPLC anion-exchange column. RPHPLC of each mutant yielded two peaks (corresponding to the α and β^8-chains) in the correct stoichiometry. The tetrameric nature of the reconstituted protein was corroborated by sedimentation velocity experiments; sedimentation coefficients of mutants were found to be similar to native HbS. Far-UV CD spectrum of the mutants in the region of 200-250 nm exhibited double minima at 222 and 208 nm respectively that are characteristic of helical conformation. The spectra of the mutants were comparable to that of the native hemoglobin purified in a similar fashion from human red cell lysate indicating native-like secondary structure in the mutant proteins. Similarly CD spectra of each mutant protein in the soret region were similar to the native HbS, with a maximum at 420 nm, suggesting the retention of native-like conformation of the heme pocket. Derivative UV spectra of the liganded (oxy) and unliganded (deoxy) forms of the double mutants, considered as diagnostic of the conformational aspects of the α1β2 interface, were also very similar to native HbS suggesting the retention of native-like quaternary structure in the mutant proteins.

The polymerization behaviour of the mutants was ascertained by measuring the concentration of hemoglobin in equilibrium with the polymer (C_{sat}). The polymer solubility for HbS was found to be 30 mg/ml. Under identical conditions, HbS (K16Q/E23Q) yielded a C_{sat} (31 mg/ml) that was very similar to native HbS suggesting that this double mutant behaved much like the native HbS. This was an interesting result considering that the associated single mutants [HbS (K16/Q) and HbS (E23/Q) respectively] were previously shown to inhibit HbS polymerization; C_{sat} values of the above single mutants were 39 and 44 mg/ml respectively. However, the double mutants involving sites 20/23 [HbS(H20Q/E23Q)], and 16/20 [HbS(K16Q/H20Q)] were found to be inhibitory. The C_{sat} of HbS(H20Q/E23Q) mutant was determined as 42 mg/ml which was similar to that of the HbS(E23Q) mutant (C_{sat} ~ 44.1 mg/ml) suggesting that the inhibitory effect of the single mutants was non-additive in this double mutant. In contrast, HbS (K16Q/H20Q)
yielded a C_{sat} of about 45 mg/ml that was close to the value of 47 mg/ml expected for an additive effect of the individual C_{sat} of the associated single mutants.

As a beginning to probe how electrostatics might influence the polymerization behaviour of the single and double mutants, the surface electrostatic potential of the mutants was investigated. However the correlation of electrostatic potential changes with polymerization of HbS was not readily discernible. In fact the electrostatic potential around the axial contact surface of the α-chain of native HbS was closer to that of the E23Q mutant than K16Q/E23Q double mutant despite the fact that polymerization of HbS was inhibited in the former and restored in the latter mutant. Thus the effect of the aforementioned mutations on HbS fiber assembly may not be due to electrostatics alone but could involve a complex interplay of both steric and electrostatic factors. Indeed, structural analyses of MD trajectories of isolated mutant α-chains as well as a complex of two HbS tetramers bordering the axial contact site revealed qualitative correlation between dynamics and polymerization behaviour of the mutants. Molecular dynamics (MD) simulations studies of the double mutants and associated single mutants have lead to the excavation of a coupled interaction network involving K16α, H20α, E23α, E116α and E22β residues at the fiber axial interface. Taken together, the results of polymerization experiments and dynamics investigation suggest that mutation of the above axial contact residues exert their influence on HbS polymerization by perturbing this network.

B. Impact of quaternary structural constraints on HbS polymerization: The other objective of the present study was to probe the role of quaternary structural features on the polymerization of HbS. When normal Hb is fully deoxygenated, most of the molecules assume T structure, which has a relatively low affinity for oxygen and other heme ligands. Conversely, normal oxyhemoglobin exists almost exclusively in the R conformation and has a relatively high-affinity for heme ligands. Crystal structures of the T and R states were first determined from (high salt-crystals) by Perutz and colleagues some thirty years ago. For many years, it was thought that the α2β2 mammalian tetramer has only two quaternary structures i.e., R and T. More recently, however, a second, distinctly different crystal structure for
liganded HbA was solved from (low-salt crystals) and designated R2. There are also reports of three different crystal structures of bovine carbonmonoxyhemoglobin, one of which has a structure almost identical to the R2 structure of liganded human Hb and the other two have quaternary structures that are positioned between the R and R2 structures. These findings suggest that the dimer-dimer interface of the liganded hemoglobin has a wide range of energetically accessible structures that are related to each other by a simple sliding motion.

The key to HbS polymerization is the presence of Val-β6 and the formation of a hydrophobic acceptor pocket between E and F helices that occurs in the T state but is absent in the R state. Since the acquisition of the T state is important in this respect, it is legitimate to assume that quaternary conformational events might play detrimental role in the polymerization process. If a specific amino acid substitution decreases the stability of the T structure, then transition to the R state is favoured. This has been demonstrated for a number of chemically modified hemoglobins as well as for many hemoglobin variants. In the present work, two HbS mutants of HbS, namely HbS[des arg 141α] and HbS[W14Aα], were engineered. The former is a deletion mutant in which Arg 141 is removed from the C-termini of α chains while the latter is a substitution mutant. In liganded Hb the C-terminal residues Arg141α and His146 β do not interact with other groups. However, they are engaged in several interactions in the unliganded hemoglobin. The guanidine of Arg141α forms a salt bridge with the carboxyl of Asp126α in the opposite chain and its C-terminal carboxylate forms salt bridges with the ε-amino group of Lys127α, and indirectly, with the α-amino group also of the opposite α chain. These interactions are partly responsible for the extra stability of the deoxy or T quaternary structure over the oxy or R state. When Hb undergoes R to T transition, it involves displacement of the helices E and F, which sandwich the heme prosthetic group. These displacements weaken the H-bonds connecting the E helix to the A helix on one hand and the F helix with the H helix on the other. These H-bonds are subsequently reformed by following motions of the A and H helices, which serve to move the N and C termini into positions from which they establish the inter-subunit salt bridges that stabilize the T structure. One of the tertiary H-bonds which play a key role in the allosteric reaction path between the R and T state is donated by the indole side chain of
Trp14α which bridges the outer A helix to the inner E helix that line the distal side of the heme pocket. Hence, both des arg141α and W14Aα variants of HbS are likely to have perturbed ‘T’ quaternary state that might have a bearing on the polymerization reaction.

While the HbS[W14Aα] mutant was assembled by semisynthetic procedure alluded to above, HbS[des arg 141α] was prepared by carboxypeptidase digestion of native HbS followed by ion-exchange chromatography. The chemical and structural integrity of the mutants were established and their polymerization behaviour was assessed. Under identical conditions, the HbS[W14Aα] mutant yielded $C_{sat}$ values that were very similar to native HbS. In contrast, the $C_{sat}$ value of HbS[W14Aα] was found to be considerably higher indicating significant polymerization inhibitory propensity of the mutant.

To further investigate, the possible ramifications of these mutations, kinetic assays were carried out. The delay time for HbS[W14Aα] in 1.8 M phosphate buffer was found to be much longer than HbS consistent with the fact that $C_{sat}$ of this mutant was significantly higher than that of native HbS. The kinetic behaviour of HbS[des arg 141α] on the other hand, was unusual. Although the corresponding $C_{sat}$ value was comparable to that of native HbS, this mutant exhibited a much longer delay time. Thus, the polymerization behaviour of this mutant did not appear to follow the notion that equilibrium and kinetics are linked. Therefore, kinetic behaviour of HbS[des arg 141α] needed to be assessed more rigorously to explain the anomaly observed in its kinetic behaviour contrasted with solubility. Although the 1.8 M phosphate buffer system has been widely used for assessing polymerization kinetics, there are concerns that the polymerization behaviour of mutants of HbS in such high ionic strength media might not reflect its properties in physiological conditions in part due to the possible impairment of physiologically relevant electrostatic interactions in the high ionic strength buffer. In order to lend credence to the data emanating from experiments carried out in 1.8 M phosphate buffer, similar kinetic experiments were carried out in 1.5 M and 1 M phosphate buffers. In comparison with HbS, HbS[des arg 141α] maintained the trend of increased delay time as seen earlier in 1.8 M phosphate buffer. Interestingly, the
Csat values of HbS[des arg 141α] was found to be similar to native HbS further authenticating the solubility and kinetics data.

Taken together, the overall data demonstrated that HbS[W14Aα], in accordance with its polymerization-inhibitory propensity exhibited a significant longer delay time kinetics. HbS[des arg 141α] on the other hand, showed a propensity to impede the nucleation step in kinetics of HbS polymerization even though it did not significantly alter the polymer solubility. Thus, this data supports the current notion that kinetics and equilibrium in HbS polymerization need not be always linked.

Mutational effects on HbS polymerization are traditionally interpreted in terms of contact residues directly occurring in the two interfaces in the polymer as defined in the crystal structure or the fiber models. While in accord with this formulation, the work described in here suggest existence of dynamic pathways in HbS that can transfer the effects of mutational perturbations over much longer distances. The consideration of such interacting pathways will be helpful in not only understanding the effects of a larger number of mutations (some of which are not on contact sites) but also the observed additive/synergistic coupling of multiple mutations. In addition, the results also suggest that perturbation of quaternary interactions might influence the polymerization process by affecting either kinetics or equilibrium or both.