Linkage of Interactions in Sickle Hemoglobin Fiber Assembly

INHIBITORY EFFECT EMANATING FROM MUTATIONS IN THE AB REGION OF THE α-CHAIN IS ANNULLED BY A MUTATION AT ITS EF CORNER

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The AB and GH regions of the α-chain are located in spatial proximity and contain a cluster of intermolecular contact residues of the sickle hemoglobin (HbS) fiber. We have examined the role of dynamics of AB/GH region on HbS polymerization through simultaneous replacement of non-contact Ala19 and Ala21 of the AB corner with more flexible Gly or rigid α-aminoisobutyric acid (Aib) residues. The polymerization behavior of Hbs with Aib substitutions was similar to the native HbS. In contrast, Gly substitutions inhibited HbS polymerization. Molecular dynamics simulation studies of α-chains indicated that coordinated motion of AB and GH region residues present in native (Ala) as well as in Aib mutant was disrupted in the Gly mutant. The inhibitory effect due to Gly substitutions was further explored in triple mutants that included mutation of an inter-double-strand contact (αAsn78→His or Gln78) at the EF corner. Although the inhibitory effect of Gly substitutions in the triple mutant was unaffected in the presence of αGln78 His at this site almost abrogated its inhibitory potential. The polymerization studies of point mutants (αGln78→His) indicated that the inhibitory effect due to Gly substitutions in the triple mutant was synergistically compensated for by the polymerization-enhancing activity of His78. Similar synergistic coupling, between αHis78 and an intra-double-strand contact point (α16) mutation located in the AB region, was also observed. Thus, two conclusions are made: (i) Gly mutations at the AB corner inhibit HbS polymerization by perturbing the dynamics of the AB/GH region, and (ii) perturbations of AB region (through changes in dynamics of the AB/GH region or abolition of a specific fiber contact site) that influence HbS polymerization do so in concert with α78 site at the EF corner. The overall results provide insights about the interaction-linkage between distant regions of the HbS tetramer in fiber assembly.

Sickle cell anemia arises because of a point mutation at the sixth position of the β-chain (βGlu6→Val) in the hemoglobin (Hb)3 molecule (1). Deoxygenated sickle hemoglobin (HbS) polymerizes into long helical fibers that are believed to be responsible for the pathophysiology of the sickle cell disease. The knowledge gleaned so far from structural analysis of HbS crystals (2–4), solution polymerization studies of natural variants or engineered mutant hemoglobins (5–24), and electron microscopic studies (25) have led to a 14-stranded model of the fiber (26, 27). These strands appear as seven double strands of the type found in HbS crystals, albeit with a slight twist caused by fiber packing. The models specify several amino acid residues from both α- and β-chains that participate in inter- or intra-double-strand contacts stabilizing the fiber structure. By and large, the fiber models agree with the solution polymerization experiments of mutant hemoglobins in that polymerization-sensitive mutations are usually located in fiber contact regions (27). However, the models present an approximate description of the HbS fiber that are limited to the identity of the contact residues and totally lack information on the hierarchy of interactions or additive or synergistic effect of two or more contact residues (4, 26, 27). Furthermore, the models project a static view of the contact sites and do not provide any clues about the influence of flexibility or dynamics of a given contact region on fiber assembly. Here we have examined the consequence of flexibility changes at the boundary of the A- and B-helix (αB region) of the α-chain on the polymerization of HbS. The linkage of interaction in the polymerization process, between the AB region and a remote site located at the EF corner of the α-chain, has also been explored.

The AB region of the α-chain contains several contact sites of the fiber (α16, α20, and α23) and lies in spatial proximity to a cluster of GH corner residues that are implicated in fiber interactions (residues α113–α116). Besides, AB-GH corners of the α-chain engage in extensive inter-tetrameric interactions with AB-GH corners of the β-chain, yielding physiologically important intra-double-strand axial contacts of the fiber. Therefore, it is conceivable that even subtle alterations of the conformational dynamics generated through rational amino acid changes at non-contact sites at the AB or GH corners of α/β chains might influence the HbS polymerization by perturbing the inter-tetrameric interaction interface of the fiber. We have tested this surmise by replacing two Ala residues of the AB corner of the α-chain, Ala19 (AB1) and Ala21 (B2), with the

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The abbreviations used are: Hb, hemoglobin; HbS, sickle Hb; Aib, α-aminoisobutyric acid; HL, Hanuman langur; RP-HPLC, reverse-phase high-performance liquid chromatography; ESMS, electrospray mass spectrometry; FPLC, fast protein liquid chromatography; MD, molecular dynamics; RMSF, root-mean-squared fluctuations; BPG, bisphosphoglycerate.
relatively more flexible Gly residues or less flexible α-aminoisobutyric acid (Aib) residues (Fig. 1A).

Aib (also called α-methylalanine), an analog of alanine, is a non-protein amino acid found in microbial peptides that form membrane channels. Aib exhibits higher helix-forming ability compared with Ala residues, which has the highest helical propensity among the 20 coded amino acids. Model studies on short peptides have shown that incorporation of one or more Aib residues significantly enhance the helical backbone structure (28, 29). The stabilizing effect of Aib emanates from the presence of an extra methyl group (relative to Ala) that restricts the peptide backbone torsional angles ($\phi$, $\psi$) to helical conformation. In fact, Gly → Ala substitutions also introduce a methyl group on Cα atom, and similar effects, albeit to a lesser extent than Aib, are realized due to Ala. Therefore, a hierarchy of conformational constraint is expected to be generated at the $\alpha$-helix region while traversing from Gly to Ala to Aib; the ensuing sequence of residues 18–22 at the AB corner (Fig. 1B) with Gly substitution (GGHGG) is likely to be potentially more flexible than the native sequence (GAHAG), which in turn, would be more flexible than the sequence generated by Aib replacements (GAibHAG).

We have earlier successfully employed a semi-synthetic reaction, namely, V8 protease-mediated coupling of $\alpha$1–30 and $\alpha$31–141 fragments to produce $\alpha$141 (α-globin), for the construction of mutant $\alpha$-subunits (23, 24). This strategy, although restrictive in nature, is especially suited for the incorporation of non-coded Aib residues and, in appropriate situations, could be effectively utilized for the introduction of 20 standard amino acids. Accordingly, we have constructed the aforementioned α-globin mutants containing Gly or Aib residues at 19 and 21 positions through the $\alpha$-globin semisynthetic reaction. We have also taken advantage of the fortuitous co-presence of Gly residues at the 19/21 positions and a contact point mutation at position 78 (EF7, Asn → His or Gln) in the $\alpha$-chain of the Hanuman langur (Presbytus entellus) hemoglobin (30) to authenticate the role of conformational flexibility of the AB region as well as to explore linkage of interactions, if any, between these two remote regions of the tetramer.

MATERIALS AND METHODS

The ion-exchange resins (CM52 and DE52) were obtained from Whatman. The chemicals used in peptide synthesis were obtained from Nova Biochem. The hemoglobins from homozygous (SS) sickle cell patients and Hanuman langur (HL) were purified from their red cell lysates by DE52 and CM52 chromatography according to standard procedures. Heme-bound individual subunits of hemoglobins were obtained by acid-acetone precipitation.

Isolation and Characterization of HL $\alpha$-Chain—Hemoglobin from the HL erythrocyte lysate was subjected to CM52 cation-exchange chromatography, and the elution profile was monitored at 540 nm. Cooperative Interactions in the HbS Fiber Assembly

The $\alpha$-globe mutants containing Gly or Aib residues at 19 and 21 positions through the $\alpha$-globin semisynthetic reaction. We have also taken advantage of the fortuitous co-presence of Gly residues at the 19/21 positions and a contact point mutation at position 78 (EF7, Asn → His or Gln) in the $\alpha$-chain of the Hanuman langur (Presbytus entellus) hemoglobin (30) to authenticate the role of conformational flexibility of the AB region as well as to explore linkage of interactions, if any, between these two remote regions of the tetramer.
mental mass of the first peak in each case (15,896.4 Da and 15,896.39 Da, respectively) was similar and in agreement with the reported sequence (calculated mass, 15,895.24 Da) of langur β-globin. In contrast, the mass of the second peak of RP-HPLC, corresponding to the α-chain from the two samples, was different. Interestingly, the mass of this chain from HL2, 15,121.36 Da, fitted well to the calculated mass (15,121.36 Da) of the documented sequence of the HL α-chain (30) that differs from human at only three sites (Gly19, Gly21, His78). The experiment-inal mass of the α-chain from the HL1 sample was, however, less by about 10 Da (15,113.81). Thus the heterogeneity in the HL hemoglobin was localized in the α-chain.

To define the region of sequence change(s), the two α-chains of HL were subjected to tryptic peptide mapping (Fig. 2). A comparison of the peptide maps of the two HL chains revealed altered retention times for T8⁺₉ (residues 61–90) and T9 (62–90). The retention time of T4 peptide (residues 17–31) of the two langur chains was identical to but distinct from the human T4 peptide.

The hemoglobin molecules in the crystal pack as two strands of HbS molecules. Hemoglobin tetramers in each strand are connected through a network of axial contacts (4). These contacts involve AB and GH regions of both the α- and β-chains that include αLys16, αHis20, αPro114, αAla115, αGlu116, βGly16, βLys17, βGlu22, βHis17, βPhe118, βGly119, and βGlu121. The presence of a large number of contacts at the interface between the two HbS molecules in each strand of the fiber raises the intriguing possibility that even subtle changes in the conformational dynamics generated because of the perturbation of non-contact residues around AB or GH regions of the chains might influence the polymerization process by interfering with inter-tetrameric interaction interface of the fiber.

We chose sites Ala116 and Ala221 at the AB region of the α-chain to address the above issue based on the fact that these are not contact points themselves, but their replacements with
Gly residues generate a sequence (18GGHGG22) that has the propensity to enhance the conformational flexibility of the AB region. Secondly, the readily available V8 protease-mediated α-globin semisynthetic procedure permits us to incorporate non-coded Aib residues that are expected to contrast the effects because of Gly residues and confer conformational rigidity to the AB region.

**Molecular Dynamics Simulations of the Mutant α-Chains**—We carried out MD simulations studies on native (Ala) and mutant (Gly or Aib) α-chains to investigate the extent of dynamic perturbations in the mutant chains. The MD simulations for each mutant α-chain were checked for stability by monitoring several global properties like the radius of gyration, root mean squared deviation from the initial structure as well as the kinetic and potential energies. These parameters were found to stabilize after about 0.1 ns and, hence, data from 0.1–2 ns were used for all subsequent analyses. The root-mean-squared fluctuations (RMSF) of the Ca carbon atoms were calculated for the native (αA19-A21) and mutant chains (αA19G/A21G and αA19Aib/A21Aib) and is shown in Fig. 3A. The crystallographic temperature factors for the starting structure are also shown for comparison (Fig. 3B). Structural fluctuations in the mutant α-chains are, overall, similar to those of the native chain and follow the same trend as that seen in the experimental structure, suggesting that the introduction of mutations did not cause any large-scale deviation of the dynamical properties of the α-chain.

The regions around residues 42 and 93 within the CD and FG corner, respectively, show the most fluctuations and a large difference between the native α-chain and its mutants. Both of these regions are particularly close to the highly mobile propionate side chains of the heme moiety. The RMSF difference in these regions also varies when different time windows of the simulation were used for calculation. This result indicates that, perhaps because of strong interaction with the highly mobile propionate side chains, the dynamics in these regions becomes much more complicated, and it was not possible to sample fully the total conformational space even during 2 ns of MD simulation, resulting in the observed difference in conformational fluctuation between the native α-chain and its mutants. The RMSF values in the AB corner regions rise to a local maximum in all three trajectories (Ala, Gly, Aib), indicating that this region is relatively mobile. Introduction of Ala → Gly and Ala → Aib mutations do cause small differences in RMSF values in this region. The GH corner region also lies in a local maximum of the RMSF plot. Although the average fluctuation in the GH region is comparable between the three trajectories, a significant difference between the fluctuation profile of the AB and the GH region is observed. This is clearly apparent in Fig. 4, where the frequency distribution of Ca-Ca distance between pairs of AB and GH corner residues is shown. The distribution of Ca-Ca distance between Ala13 and Glu116 is more variable in the Gly mutant and tends toward larger values. A similar trend is also apparent in the Ca-Ca distance distribution calculated for the Ala13-Leu113 pair. In the case of the Lys16-Glu116 and Lys16-Leu113 pairs, the effect is less pronounced for the Ca-Ca distances, but is clearly visible when one considers the side chain atoms NZ of Lys16 and CD of Glu116 (data not shown).

We used dynamical cross-correlation maps to identify corre-
lated movements of residues from MD simulations (36, 37). Fig. 5 shows the dynamical cross-correlation maps calculated from the simulations by averaging across the entire length of the simulations, except for the first 0.1 ns. Averages calculated with smaller time windows (between 50 ps to 0.1 ns) showed essentially similar patterns (data not shown). Positive peaks in the maps indicate that the movements of an atom pair are correlated, and negative peaks indicate that they are anti-correlated. There exist both positive and negative correlations between the AB and GH region residues in the Ala trajectory (native α-chain), as indicated by peaks in the appropriate regions. One finds a strong peak of positively correlated fluctuations (correlation coefficient 0.6–0.8) between the AB and the GH regions in the Ala simulation. However, in the same region, the correlation coefficient reduces to around 0.2 in the Gly mutation at the AB corner of the native HbS.—Cooperative Interactions in the HbS Fiber Assembly

The mutant α-chains obtained by RP-HPLC runs of each semi-synthetic construct was further analyzed by ESMS and tryptic peptide mapping to rule out any possible chemical modifications during the process of chemo-enzymic assembly of these chains. The experimental mass of α(A19G/A21G) and α(A19Aib/A21Aib) mutant chains were found to be 15,908.93 and 15,154.76 Da, respectively (Fig. 7). These values were in excellent agreement with their theoretical masses (15,908.32 and 15,154.98 Da, respectively). That the sequence of the polypeptide chain was intact and the sequence alteration was specific to sites 19 and 21 was confirmed by tryptic peptide mapping. The tryptic peptide profiles of the mutant α-chains were compared with the α-chain that was isolated from native HbS. RP-HPLC profiles of the tryptic digest of the respective mutant globins showed altered retention time (relative to that of the native globin) for only one peptide namely, αT4. This result is consistent with the fact that sites 19 and 21 are contained in the tryptic fragment αT4 (residues 17–31). The order of elution of αT4 peptide (Gly substitutes first followed by Ala and Aib) from the reverse-phase column was also found to be in accord with the hierarchical hydrophobicity of Gly, Ala, and Aib residues. Taken together, the results unequivocally establish the chemical composition and sequence integrity of the α-chains in the mutant tetramers.

\[ \text{Structural Characterization of the Gly/Aib Mutant HbS—} \]

Circular dichroism spectra of the hemoglobins in the far UV region (200–250 nm) were recorded to assess the secondary structures of the polypeptide chains in the tetramer. The spectra (Fig. 8A) of both the mutant tetramers α(A19G/A21G) and α(A19Aib/A21Aib) were similar to those of the native HbS (α(A19–A21)β ), suggesting that Gly or Aib substitutions at the AB corner of the α-chains in the tetramer did not cause any global changes in the polypeptide helical fold and that the effects on conformation, if any, were subtle and localized to the AB-corner residues.

We also used CD spectroscopy to probe the heme-environment of the mutant proteins. For this, CD spectra of the proteins (Fig. 8B) were recorded in the Soret region. Both the mutants exhibited a CD spectrum that was very similar to that of the native HbS, indicating the conservation of the native-like folding of the heme pocket in the mutants. Thus, simultaneous replacements of Gly or Aib for Ala at positions 19 and 21 of the α-chain do not produce any tertiary structural aberrations in the mutant HbS.

\[ \text{Functional Behavior of Gly/Aib Mutant HbS—The oxygen-binding properties of the mutants were studied to evaluate the functional integrity of the mutant tetramers (Table I). Both of the mutants, α(A19G/A21G)β and α(A19Aib/A21Aib)β , bound oxygen cooperatively, and their Hill coefficients (n) were similar (2.4 and 2.5, respectively) or identical to that of the native HbS (2.5). The oxygen affinity of the mutants, as reflected in their respective P50 value (7.5 or 8 mmHg), was also comparable with native HbS (8.5 mmHg). In both the mutants, 2,3-bisphosphoglycerate (BPG) induced a decrease in oxygen affinity that was comparable with that of native HbS. The high cooperativity of oxygen binding was also maintained in the presence of BPG. The retention of normal oxygen affinity and cooperativity in the mutant HbS suggests the presence of native-like quaternary structure and dimer interactions in the tetramers. This interpretation seems consistent with the results of the CD spectroscopy described earlier.} \]

\[ \text{Influence of the Gly/Aib Residues at the AB Corner on HbS Polymerization—We measured the polymer solubility (C_{pol}) of the mutant tetramers under near-physiological conditions in} \]

\[ \text{Co-Co distance distribution plotted for residue pairs of AB-GH region. The distributions were calculated from Ala (continuous), Aib (dashed), and Gly (dotted) simulations in each case. A, Ala13-Glu116, B, Ala13-Leu113, C, Lys16-Glu116, D, Lys16-Leu113. All distances are in Angstrom units.} \]

\[ \text{Fig. 4. Co-Co distance distribution plotted for residue pairs of AB-GH region. The distributions were calculated from Ala (continuous), Aib (dashed), and Gly (dotted) simulations in each case. A, Ala13-Glu116, B, Ala13-Leu113, C, Lys16-Glu116, D, Lys16-Leu113. All distances are in Angstrom units.} \]
the presence of 70-kDa dextran following the protocol of Bookchin et al. (33). The dextran C_{sat} of native HbS control α_{2}(A19-A21)β_{2}^{S} and the two mutants, α_{2}(A19G/A21G)β_{2}^{S} and α_{2}(A19Aib/A21Aib)β_{2}^{S}, is shown in Fig. 9A. Under identical conditions, the solubility of mutant HbS (C_{sat} = 53 mg/ml) containing Ala → Gly substitutions was found to be considerably higher as compared with the native HbS (C_{sat} = 30 mg/ml). In contrast, HbS mutant with Ala → Aib replacements produced a C_{sat} = 28 mg/ml, which was similar to or slightly less than that of the native control. Importantly, the C_{sat} values of both the native and mutant proteins were independent of the initial hemoglobin concentrations in the tested range of 70–90 mg/ml. Thus, the solubility measurements clearly demonstrate that the HbS tetramers with Ala → Gly substitutions at the AB corner of the α-chain are endowed with significant polymerization-inhibitory potential. However, the Aib residues at the same sites behave in much the same way as Ala residues.

**Coupling of the Gly Mutations of the AB-corner with d78 Mutation (His/Gln) at the EF-corner**—To further substantiate the inhibitory effect of the Gly substitutions at the AB corner on HbS polymerization, we undertook the construction of triple-mutant HbS comprising langur α-chain and β^{S} chain. As described under “Materials and Methods,” the langur α-chain contains an Asn^{78} → His or Gln mutation at the EF-corner, as compared with the human α-chain, besides the Gly^{19} and Gly^{21} residues at the AB-corner. Thus, the studies of polymerization behavior of the above triple-mutant HbS would corroborate the inhibitory effect because of Gly mutations and also reveal the additive or synergistic nature of the interactions between the two distant regions (AB and EF corner) of the tetramer. Be-
sides, the polymerization results of the mutants would provide information on the effects of chemically disparate side chain at the /H9251 78 site (Asn 78 3 His/Gln) on fiber formation; the inhibitory potential of this site has been previously deciphered (38) from the co-polymerization studies of HbS and HbA Stanleyville II (Asn78 3 Lys).

HbS tetramers composed of /H9252 S-chain and respective triple-mutant /H9251 -chains, /H9251 (A19G/A21G/N78Q) or /H9251 (A19G/A21G/N78H), were generated. The tetramers were isolated by CM52 cation-exchange chromatography followed by an analytical FPLC using a Mono Q column, as described previously. The heme stoichiometry in the purified tetramers was ascertained by comparing the 280:540 nm absorbance ratio for these proteins with that of the native HbS. These ratios (2.50 and 2.52, respectively) compared well with a value of 2.54 of native HbS, thus confirming the correct stoichiometry of heme in the reconstituted tetramers. RP-HPLC analysis of the respective hemoglobins revealed correct composition of /H9251 and /H9252 chains. The far-UV CD and Soret region CD spectral characteristics of both the triple mutants were comparable with and similar to those of native HbS (data not shown).

The functional integrity of the triple mutants was assessed by oxygen-affinity measurements. The /H50 2 for the /H9251 2(A19G/A21G) /H9252 2 S and /H9251 2(A19G/A21G/N78Q) /H9252 2 S mutants were found to be 8 and 7 mmHg, respectively. Thus, both HbS mutants exhibited /H50 values that were similar to native HbS (/H8.5 2). Besides, the mutants also displayed normal cooperativity of oxygen binding. The results suggest that substitution of His or Gln for Asn at the 78th position in the co-presence of Ala 3 Gly replacements at the AB-corner of the /H9251 -chains do not have any deleterious effect on the function of HbS.

Polymerization Behavior of Triple Mutants—The gelation concentration (C sat) of the above triple mutants was determined to ascertain the combinatorial influence of sequence changes at the AB and EF corners on the polymerization reaction. For this, C sat of the triple mutants (Fig. 9B) was measured by the dextran-C sat method in a manner identical to that used for the double mutants (Fig. 9A). The /H50 (A19G/A21G/ NA8H)β2 S tetramer yielded a C sat of about 28 mg/ml that was
very close to the $C_{\text{sat}}$ of native HbS ($C_{\text{sat}} = 30$ mg/ml). In contrast, the $C_{\text{sat}}$ of $\alpha_d(19G/21G)\beta_d^{5}$ tetramer was considerably higher ($C_{\text{sat}} = 50$ mg/ml), as compared with native HbS, but very similar to that of the $\alpha_d(19G/21G)\beta_d^{5}$ tetramer ($C_{\text{sat}} = 53$ mg/ml).

The $C_{\text{sat}}$ measurements of HbS containing only the point mutation of a Gln or His for Asn at a/78 was determined to assess the individual contribution of these residues in the polymerization of HbS. The $C_{\text{sat}}$ of $\alpha_d(N78Q)\beta_d^{5}$ mutant was found to be similar (31 mg/ml) to that of the native HbS, suggesting that Gln residue behaves in much the same way as Asn. However, the HbS mutant $\alpha_d(N78H)\beta_d^{5}$ containing His at this site yielded a $C_{\text{sat}} = 22$ mg/ml, which was significantly lower than that of native HbS, suggesting that the presence of His residue at the a/78 site confers polymerization-enhancing propensity to the HbS molecule. The potentiating effect of His at this site has been commented upon in a recent publication (39).

Given that the $C_{\text{sat}}$ of $\alpha_d(19G/21G)N78Q\beta_d^{5}$ and $\alpha_d(19G/21G)\beta_d^{5}$ mutants are similar (50 and 53 mg/ml, respectively), and the point mutant, $\alpha_d(N78Q)\beta_d^{5}$, behaves in much the same way as native HbS, it can be concluded that the inhibition of polymerization observed in the triple mutant must emanate from the Gly substitutions at the AB corner. Further, inhibition of HbS polymerization because of Gly substitutions was not noticeable in the His-containing triple mutant, $\alpha_d(19G/21G)N78H\beta_d^{5}$, because of the polymerization-enhancing effect of His$^{78}$. However, the $C_{\text{sat}}$ of the $\alpha_d(19G/21G)N78H\beta_d^{5}$ (28 mg/ml) is far less than that expected from an additive contribution of constituent double-mutant $\alpha_d(19G/21G)\beta_d^{5}$ (53 mg/ml) and single mutant $\alpha_d(N78H)\beta_d^{5}$ (22 mg/ml). Because the $C_{\text{sat}}$ of the Hbs (30 mg/ml) increased by 23 mg/ml because of Gly substitutions and was lowered by 8 mg/ml because of His at a/78, a simple additive effect would have yielded a $C_{\text{sat}}$ of about 45 mg/ml (30 mg/ml + 23 mg/ml) for the triple mutant. The shift in $C_{\text{sat}}$ of the triple mutant by about 17 mg/ml (45 – 28 mg/ml) clearly suggests that the polymerization-enhancing effects due to the presence of His$^{78}$ at the EF corner synergistically compensate for the inhibitory effect emanating from the Gly substitutions at the AB corner.

**DISCUSSION**

HbS fiber formation is a complex process that requires specific contacts and intricate interplay between several residues from both, $\alpha$ and $\beta$, chains of the tetramer. In the current scenario, the definition of a fiber contact by both theoretical as well as experimental approach is centered on specific amino acid residues. The solution studies consider a given residue as a fiber contact only if the mutation of that site results in an altered polymerization behavior relative to the native HbS fibers. On the other hand, the fiber models define a contact residue based on the distance; residues with contact distances of about 5 Å or less are generally considered as interacting partners (26, 27). Notwithstanding the approximate concordance and inter-dependence between experimental and modeling studies, the above specific amino acid residue-based description of contact points does not shed any light on the influence of regio-specific conformational dynamics of HbS molecule on the polymerization process, as also the short- or long-range cumulative interaction between the contact sites. Further study is required to determine the influence of regio-specific conformational dynamics of HbS molecule on the polymerization process, as also the short- or long-range cumulative interaction between the contact sites. Further study is required to determine the influence of regio-specific conformational dynamics of HbS molecule on the polymerization process, as also the short- or long-range cumulative interaction between the contact sites. Further study is required to determine the influence of regio-specific conformational dynamics of HbS molecule on the polymerization process, as also the short- or long-range cumulative interaction between the contact sites.
Gly or Aib substitutions at the AB corner of the hemoglobin molecule. The choice of residues Ala19 and Ala21, located at the boundary of A- and B-helices of the α-chain is appropriate in this regard, because alterations at the beginning or end of helices, as compared with those located in the middle of helices, were less likely to cause such untoward structural changes. Indeed, CD spectroscopy showed that HbS tetramers with either Ala → Gly or Ala → Aib substitutions exhibited secondary structure and tertiary heme folding that was very similar to the native HbS. Besides, the functional behavior of the mutants, as reflected by the oxygen-affinity measurements of the tetramers, were also similar, suggesting the preservation of native-like quaternary structure and dimer interactions in the mutant proteins. These results confer legitimacy to the solubility data of the HbS mutants carrying Ala to Gly or Aib substitutions at the AB corner of the α-chains, and indicate that the effects on HbS polymerization are primarily related to their intrinsic ability to perturb the conformational dynamics of the polypeptide chain.

MD simulations of mutant α-chains show that both Gly and Aib mutants perturbed the dynamics of the AB and GH region of the chain. However, distribution of Ca-Ca distances between pairs of AB-GH (Fig. 4) residues indicated that Gly mutants exhibit greater fluctuations as compared with Aib and Ala (native chain) mutants. This was further corroborated by the fact that dynamical cross-correlation maps of native (Ala) and Aib-substituted α-chains showed strong motional coupling of AB and GH region residues, whereas that of Gly-substituted chains displayed significantly less coordinated movement. This can be interpreted to mean that AB-GH regions of native or Aib mutant chains may be more rigid than the Gly mutant chain. The shift in the tail of the distance distribution profiles for the Gly mutant simulation toward larger values (shown in Fig. 4), therefore, appear to have arisen because of the relative motional independence that the GH corner residues gain in the presence of Gly residues at the AB corner. The perturbation of Lys816, Leu113, and Glu116 in the Gly mutant is particularly interesting, because mutations of these residues of the GH corner are known to affect the polymerization reaction. Thus, the results of the MD simulation of the α-chains indicate that Gly mutations inhibit HbS polymerization by perturbing the intra-chain interactions of the α-chain that, in turn, influences the overall intra-double-strand axial interactions in the fiber emanating from AB-GH regions of the α/β chains.

The ability of a polymerization-enhancing mutation of an established inter-double-strand contact point (αHis78) at the EF corner of the α-chain to completely neutralize the inhibitory effect of Gly substitutions at the AB corner is an interesting result that suggest long-range and non-additive coupling of mutational effects in HbS polymerization. This view is strengthened with the observation that the coupling of αHis78 at the EF corner to mutation of a specific intra-double-strand fiber contact (α16) in the AB region occurs in a similar synergistic fashion as it happened with Gly mutants. The fact that αLys16 makes axial contacts with the GH corner residues (α114, α115), and Gly substitutions at the AB corner exert their influence on polymerization by inducing dynamic changes in the AB-GH region of the α-chain, raises the intriguing possibility of interaction-linkage between AB, GH, and EF regions of the α-chain in the HbS fiber assembly. Our previous work (24), demonstrating that the inhibitory effect of α16 was additive with α113 (GH1), is consistent with this idea.

The polymerization studies of the mutant HbS molecules described in this report are important in another context. Transgenic mice expressing the human β’-chain do not fully exhibit the sickling phenotype due to inhibition of HbS polymerization by mouse α-chains (40). There are 19 mutations in the mouse α-chain compared with the human sequence. Of these, only three (α78, α113, and α116) are at the known contact points of the fiber. In previous work, the 19 sites of sequence changes were segregated into 8 (located in α1–30 segment) and 11 (located in α31–141 fragment) through the construction of human/mouse chimeric α-chains. Interestingly, along with other sequence changes, the mouse α-chain contains Ala19 → Gly, Ala21 → Gly, and Asn78 → Gly substitutions. HbS constructs composed of either chimeric α-chains were shown to inhibit the polymerization reaction, albeit to different extents. Further, the inhibitory potential of both the constructs was much lower than the intact mouse α-chain. The results lead to the speculation that one or more residues among the eight sites of sequence changes in the α1–30 segment must make intermolecular contacts in the HbS fiber and that the interactions of contact residue(s) in the two complementary fragments are synergistic. The results of the present work, demonstrating inhibitory effects due to residues 19 and 21 at the AB corner and interaction-linkage with α78 located at the EF corner, provide a rational explanation for the earlier observations. However, it must be reiterated here that α19 and α21 are not fiber contact sites in the conventional sense, because they are not implicated in any interactions by either solution experiments or model-building studies. It turns out that modification of fiber interactions through perturbation of conformational dynamics of a contact-rich region of the HbS may be an effec-
tive mechanism to inhibit HbS polymerization.

In summary, the results show that Ala\(^{19/21} \rightarrow\) Gly mutations of the non-contact residues at the AB corner of the \(\alpha\)-chain inhibit HbS polymerization by enhancing the dynamics of the AB-GH region that contains numerous intra-double-strand contact residues of the HbS fiber. The synergistic coupling of Ala\(^{19/21} \rightarrow\) Gly or Lys\(^{16} \rightarrow\) Gln mutations of the AB region with a mutation located in the EF corner (Asn\(^{78} \rightarrow\) His) of the \(\alpha\)-chain suggest some sort of linkage between fiber interactions that emanate from AB, GH, and EF regions of the \(\alpha\)-chains. Further studies involving NMR and x-ray crystallography of a mutation located in the EF corner (Asn\(^{78} \rightarrow\) His) of the HbS \(\alpha\)-chains suggest some sort of linkage between fiber interactions that emanate from AB, GH, and EF regions of the \(\alpha\)-chains.

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Linkage of Interactions in Sickle Hemoglobin Fiber Assembly: INHIBITORY EFFECT EMANATING FROM MUTATIONS IN THE AB REGION OF THE \( \alpha \) -CHAIN IS ANNULLED BY A MUTATION AT ITS EF CORNER

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