Inhibition of Sickle β-Chain (βS)-dependent Polymerization by Nonhuman α-Chains
A SUPERINHIBITORY MOUSE-HORSE CHIMERIC α-CHAIN

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Horse α-chain inhibits sickle β-chain-dependent polymerization; however, its inhibitory potential is not as high as that of mouse α-chain. Horse α-(1–30) and α-(31–141) segments make, respectively, minor and major contributions to the inhibitory potential of horse α-chain. The sum of the inhibitory potential of the two segments does not account for the inhibitory potential of the full-length horse α-chain.

Although the polymerization inhibitory potential of horse α-chain is lower than mouse α-chain, the inhibitory potential of horse α-(31–141) is comparable to that of mouse α-(31–141). When mouse α-(1–30) is stitched to horse α-(31–141), the product is a chimeric α-chain with an inhibitory potential greater than mouse α-chain. In contrast, the stitching of horse α-(1–30) with mouse α-(31–141) had no additional inhibitory potential.

Molecular modeling studies of HbS containing the mouse-horse chimeric α-chain indicate altered side-chain interactions at the αβ interface when compared with HbS. In addition, the AB/GH corner perturbations facilitate a different stereochemistry for the interaction of the ε-amino group of Lys-16(α) with the β-carboxyl group of Asp-116(α), resulting in a decrease in the accessibility of the side chain of Lys-16(α) to the solvent. Based on molecular modeling, we speculate that these perturbations by themselves, or in synergy with the altered conformational aspects of the αβ interactions, represent the molecular basis of the superinhibitory potential of the mouse-horse chimeric α-chains.

Transgenic mice expressing human sickle β-chains (βS) do not fully exhibit the sickling phenotype, in part due to the inhibition of deoxyHbS polymerization by mouse α-chains (1, 2). The mouse α-chain exhibits a very high inhibitory potential against βS-dependent polymerization compared with any other single point mutations of the contact sites of deoxyHbS (3–5).

Mouse α-chain has 19 sequence differences compared with human α-chain (6–8). Of these, 8 differences are located in α-(1–30) segment and 11 in the α-(31–141) segment. By changing these segments with complementary segments from human α-chain to generate the chimeric α-chains, we have demonstrated that the mouse α-(1–30) segment has a very limited polymerization inhibitory potential (5). This result is consistent with the lack of sequence differences in this segment located at contact sites implicated in the HbS polymer. In contrast, the α-(31–141) segment of mouse α-chain with three sequence differences at the implicated contact sites (α48, α78, and α116; see Ref. 9) inhibited the βS-dependent polymerization significantly (5). However, the inhibition was considerably lower than that of mouse α-chain. Hence, the inhibitory potential of the two segments of mouse α-chain, taken individually, did not account for the inhibitory potential of intact mouse α-chain. Therefore, the complementarity of the sequence differences of the two segments of mouse α-chain is indispensable to inhibit maximally the βS-dependent polymerization reaction. In addition, conformational complementarity of the sequence differences of the two segments seems to facilitate the preservation of a human-like oxygen affinity in the mouse αSβ2 chimeric Hb (8). Such complementarity is apparently lost in the human-mouse chimeric α-chain, in which the α-(1–30) segment of the mouse is replaced by that of human α-chain (8).

One approach to dissect the molecular basis of the inhibition of polymerization by mouse α-chain is to introduce the individual sequence differences of the mouse α-chain into human α-chain by site directed mutagenesis. However, this approach might not provide information on the synergistic influence of the sequence differences of the mouse α-chain. An alternative approach is to establish the synergy of the sequence differences in terms of their inhibitory potential, in a structural linkage map.

The location of the mouse α-chain sequence differences in the three dimensional structure of HbS is such that 7 of the 19 sequence differences are located in the area of AB/GH corner (Table I). Inasmuch as solution studies have identified Wishner-Love double strand as the assembly unit of the HbS polymer (3), it is legitimate to use the crystal structure of deoxyHbS to locate intra-double strand contact sites. Sites α16, α20, α23, and α116 comply with this characteristic and, in addition, are in spatial proximity despite the fact they are widely separated in the primary sequence (10). One of the sequence differences (α78), involved in an inter-double strand contact site (supported by high resolution electron microscopy and image reconstruction is located in the EF corner and is in close proximity to the other contact sites. Thus, 12 out of the 19 sequence differences of the mouse α-chain are concentrated in two domains of α-chain that are involved in the intermolecular interactions of deoxyHbS fiber.

To dissect the inhibitory potential of the partial murinization of human α-chain and construct a structural linkage map, we

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propose to screen the amino acid sequence of available vertebrate α-chains and select those that contain identical or nearly identical sequence as in mouse α-chain. Such α-chains could be hybridized with βS to generate a chimeric HbS having a set of overlapping sequence differences. Moreover, in an added step, by preparing chimeric α-chains, the sequence differences between the parent α-chain and the test α-chain could be further reduced. We have chosen horse α-chain for such an study.

Horse α-chain has 18 sequence differences as compared with human α-chain (11). Most of the sequence differences (15 out of 18) of horse α-chain are located in the α-(31–141) region of the chain (Table I). Of these 18 sequence differences, 8 are located at the same sites of the chain as in mouse α-chain. Therefore, a comparison of the inhibitory potential of horse α-chain with that of mouse α-chain will provide information about the contribution of these common sequence differences in the inhibition.

Of the eight common site-sequence differences between mouse and horse α-chains, six are located in the α-(31–141) segment of the chain (Table I). Four of these are located in the EF corner, an area implicated in the inter-double strand interactions of the deoxyHbS fiber. Therefore, by segregating the sequence differences of horse α-chain into α-(1–30) and α-(31–141) segments, the inhibitory potential of sequence differences of these two segments and possibly the contribution of the sequence differences common between the mouse and horse α-chains could be evaluated.

Accordingly, the α-(1–30) and α-(31–141) segments of horse α-chain have been exchanged with that of human α-chain by the V₈ protease-catalyzed semisynthetic approach to generate horse-human chimeric α-chains (5, 12).

MATERIALS AND METHODS

Horse and mouse (C57BL/10) α-globins were separated from their respective purified hemoglobins by CM 52-urea chromatography (13) and were hybridized with βS by alloplex intermediate pathway into the respective interspecies hybrids (12, 14). Briefly, α-globin was mixed with βS after generating -SH groups on the chain, to form half-filled tetramers. Then hemin cyanide was added to generate the tetrameric form of the tetramer. The Met form is reduced with dithionite to generate oxyHbA.

The chimeric α-chains of horse (α-(1–30)) and human (α-(31–141)), human (α-(1–30)) and horse (α-(31–141)), horse (α-(1–30)) and mouse (α-(31–141)), and mouse (α-(1–30)) and horse (α-(31–141)) were generated by the V₈ protease-mediated semisynthesis of α-globin at pH 6.0 in 30% n-propanol (12). These chimeric α-chains were reconstituted with βS by the same Alloplex intermediate pathway to generate the tetramer. All the hybrids were purified on CM-cellulose columns as described by Roy and Acharya (12).

The oxygen affinity of all hemoglobin hybrids was determined at a protein concentration of 0.5 mM, in 50 mM Bis-Tris acetate, pH 7.4 at 37 °C, using a Hem-O-Scan (Aminco).

The polymerization of the hemoglobin hybrids was studied in 2.0 mM phosphate buffer, pH 7.4 at 30 °C. The kinetics of polymerization has been followed using the method described by Adachi and Asakura (15). Briefly, an aliquot of hemoglobin hybrid was added to the deoxygenated 2.0 mM phosphate buffer, pH 7.4, containing dithionite at 0 °C and the concentration of deoxyhemoglobin was determined spectrophotometrically at 555 nm. The polymerization was initiated by a temperature jump from 0 °C to 30 °C. The kinetics of the formation of turbidity was monitored at 700 nm. At the end of the polymerization, the sample was spun at 9000 rpm for 20 min at 30 °C. The solubility of the deoxyhemoglobin hybrid was determined from the concentration of the hybrid in the supernatant at the same temperature.

The molecular modeling was carried out on an Indigo II workstation from Silicon Graphics using the software package Insight II from Molecular Simulations, Inc. The amino acid replacements were done using the Biopolymer Module in Insight II. Side-chain torsions were adjusted to explore the possibilities of hydrogen bonds or ionic interactions as well as to enhance the presentation of the figure.

RESULTS AND DISCUSSION

**Purification of Chimeric HbS Containing Either Horse α-Chain or Chimeric α-Chain and Their Functional Properties—**The homogeneity of the hybrid species of Hb containing βS and either horse α-chain or its chimeric form has been established by their chromatographic behavior on CM-cellulose columns (Fig. 1), isoelectric focusing (Fig. 1, inset), and the reverse phase high performance liquid chromatography analysis of their globin chains. The isoelectric focusing patterns of the tetramers are consistent with the results of CM-cellulose chromatography.

The oxygen affinity of the hybrids has been investigated to ascertain the intactness of basic Hb fold in the βS hybrids containing either horse α-chain or the chimeric chains derived from human and horse α-chain. The P₅₀ values of βS hybrid of horse α-chain are comparable to that of HbS (Table II). The cooperativity of the tetramers with the chimeric α-chains is also comparable to that of HbS. Horse α-chain has 18 sequence differences as compared with human α-chain (Table I). Despite these many sequence differences, horse α-chain hybridizes with βS to generate a tetramer that has ligand binding properties equivalent to that of HbS (Table II). Therefore, the horse α-chain appears to be functionally equivalent to human α-chain.

**Polymerization Inhibitory Potential of Horse α-Chain and Horse-Human Chimeric α-Chains—**The hybrid containing horse α-chain and βS (αHbS) polymerized with a delay time greater than HbS (Fig. 2), demonstrating the propensity of the horse α-chain to inhibit the βS-dependent polymerization. The solubility of the hybrid (an equilibrium measurement) was about 5-fold higher than HbS (Fig. 3).

The hybrid containing horse α-(1–30)-human α-(31–141) chimeric α-chain (αHbS) exhibited a delay time of polymerization very close to that of HbS. Therefore, the intrinsic inhibitory potential of horse α-(1–30) is very low. The inhibitory
major contribution to the inhibitory potential of horse
inhibitory effect.

tact sites of HbS fiber either by solution studies or by molecular mod-
elling studies.

However, the solubility of
\( a \)-chain) contributed very little to the
polymerization potential of this segment could be seen only when the kinetics
of polymerization was carried out at a very low protein concen-
tration (Fig. 2, inset). The solubility of \( a_2^HbP_2^S \) was only slightly higher than HbS (Fig. 3). The three sequence differ-
ences present in \( \alpha-(1-30) \) segment of horse \( \alpha \)-chain (as com-
pared with the human \( \alpha \)-chain) contributed very little to the
inhibitory effect.

In contrast, the solubility of \( a_2^HbP_2^S \) was considerably
higher than \( a_2^HbP_2^S \), demonstrating that the sequence differ-
ences of \( \alpha-(31-141) \) segment of horse \( \alpha \)-chain represent the
major contribution to the inhibitory potential of horse \( \alpha \)-chain.
However, the solubility of \( a_2^HbP_2^S \) was only about 60% of
\( a_2^HbP_2^S \). Thus, although the horse \( \alpha-(1-30) \) segment by itself
exhibited very little inhibitory potential, the complementarity of the sequence differences of this region with those of the horse
\( \alpha-(31-141) \) segment, imparted additional polymerization potential
to the tetramer.

The logarithmic plot of reciprocal delay time of polymeriza-
tion versus Hb concentration was linear for all these hybrids
(Fig. 4). The slopes (n) of these plots were 2.1, 2.2, 3.2, and 3.2
for \( a_2^HbP_2^S \), \( a_2^HbP_2^S \), \( a_2^HbP_2^S \), and \( a_2^HbP_2^S \), respectively. The
difference in nucleation number (n) for these hybrids may
reflect the difference in the mechanism of polymerization (16).
Thus, the sequence differences present in the \( \alpha-(31-141) \) segment
of horse \( \alpha \)-chain contribute toward an increase in the
nucleation number.

This result is similar to that obtained with mouse \( \alpha \)-chain,
with the exception that the sequence differences present in
mouse \( \alpha-(1-30) \) segment also resulted in a noticeable increase
in the nucleation number. Such changes in the nucleation
number can be considered as a reflection of the flexibility in the
pathway of the assembly of the polymer from the tetramers containing the $\beta^S$.

What is the molecular basis of the inhibitory potential of the horse $\alpha$-chain? Out of 18 sequence differences of horse $\alpha$-chain, 3 are located in the 1–30 region: $\alpha_4$, $\alpha_15$, and $\alpha_{19}$. Sites $\alpha_4$ and $\alpha_{15}$ are implicated as contact sites, respectively, in the Edelstein model (17) and by solution studies (reviewed in Ref. 10). The substitution of Ala for Pro at $\alpha_4$ could alter the flexibility of A-helix, but the substitutions at $\alpha_{15}$ and $\alpha_{19}$ are conservative (Table I). Therefore, the horse $\alpha$-(1–30) should exhibit very little inhibitory potential and our results are consistent with this analysis.

Horse $\alpha$-(31–141) has 15 sequence differences compared with human; six of these are located at $\alpha_{57}$, $\alpha_{68}$, $\alpha_{78}$, $\alpha_{82}$, $\alpha_{115}$, and $\alpha_{116}$, the sites that have been implicated as contact residues by solution or molecular modeling studies (Table I). The Ala $\rightarrow$ Asp at $\alpha_{82}$ clearly could alter the contact site, but all other sites have conservative substitutions in terms of charge. Nonetheless, the bulkiness of the side chains of these substitutions could perturb the interactions involved in the polymerization. Thus, the horse $\alpha$-(31–141) segment is likely to make a major contribution to the inhibitory potential of horse $\alpha$-chain.

The inhibitory potential of horse $\alpha$-(31–141) accounts for about 60% of that of the full-length horse $\alpha$-chain. Intriguingly, the remaining 40% of the inhibitory potential is not associated with the $\alpha$-(1–30) segment of the horse $\alpha$-chain. Hence, the complementation of the sequence differences of the two segments of the horse $\alpha$-chain endows a higher polymerization inhibitory potential to the full-length $\alpha$-chain. This represents a synergy of the sequence differences of the complementary segments of horse $\alpha$-chain to generate additional polymerization inhibitory potential to the full-length $\alpha$-chain, and this phenomenon is reminiscent of the results obtained previously on the generation of chimeric $\alpha$-chains from mouse $\alpha$-chain (5).

Comparison of Molecular Aspects of the Inhibitory Potential of Horse and Mouse $\alpha$-Chains—The inhibitory potential of the horse $\alpha$-chain is considerably lower than that of mouse $\alpha$-chain. The hybrids of mouse $\alpha$-chain and $\beta^S$ exhibit a solubility that is 8 times higher than Hbs (5). Mouse $\alpha$-chain has 19 sequence differences as compared with human $\alpha$-chain (Table I). Eight of these sequence differences are located at the same sites as in horse $\alpha$-chain ($\alpha_4$, $\alpha_{19}$, $\alpha_{71}$, $\alpha_{73}$, $\alpha_{76}$, $\alpha_{78}$, $\alpha_{111}$, and $\alpha_{116}$). Six of these sites have the same amino acid substitutions in both the chains ($\alpha_{19}$, $\alpha_{71}$, $\alpha_{73}$, $\alpha_{76}$, $\alpha_{78}$, and $\alpha_{116}$).

Eight common sequence differences (between the horse and the mouse $\alpha$-chains) are, apparently, not solely responsible for the high inhibitory potential of mouse $\alpha$-chain. Alternately, the $\alpha$-chain has sequence differences that favor the polymerization reaction and thus neutralize in part the polymerization inhibitory potential of the eight common sequence differences between the mouse and the horse $\alpha$-chains.

That the sequence differences of $\alpha$-(31–141) segments common to mouse and horse $\alpha$-chain contribute to the inhibitory potential of the two segments is supported by the following points:

(i) Most of the contact site-sequence differences of horse $\alpha$-chain are also located in the $\alpha$-(31–141) region consistent with the high inhibitory potential of this region. Out of eight common sequence differences between mouse and horse $\alpha$-chains, six are located in the $\alpha$-(31–141) (9, 18, 19).

(ii) The polymerization inhibitory potential of horse $\alpha$-(31–141) is comparable to that of mouse $\alpha$-(31–141) (5). The mouse $\alpha$-(1–30) segment does not have any sequence differences at the inter molecular contact sites of Hbs. However, this segment exhibits a significant amount of polymerization inhibitory potential (5). Moreover, the inhibitory potential of mouse $\alpha$-(1–30) is higher than that of horse $\alpha$-(1–30), as reflected by the solubility of the chimeric Hbs containing the respective chimeric $\alpha$-chains. Mouse $\alpha$-(1–30) has eight sequence differences as compared with human $\alpha$-chain. Of these, two sequence differences are located at the same sites ($\alpha_{4}$ and $\alpha_{19}$) as in horse $\alpha$-(1–30). The higher inhibitory potential of mouse $\alpha$-(1–30) must come from one or more of the sequence differences that are not common between the mouse and horse $\alpha$-(1–30).

(iii) Two of the eight common sequence differences are located at implicated contact sites of the fiber ($\alpha_{78}$ and $\alpha_{116}$). In addition, mouse $\alpha$-chain has two more sequence differences at the implicated contact sites ($\alpha_{34}$ and $\alpha_{48}$) according to one of the fiber models (9, 20).

(iv) The consequence of segregating the horse $\alpha$-chain into $\alpha$-(1–30) and $\alpha$-(31–141) segments, to generate chimeras of human and horse $\alpha$-chain, are reminiscent of the results obtained with mouse $\alpha$-chain (5). However, although horse $\alpha$-chain exhibited a high degree of inhibitory potential, the solubility and delay time of the chimeric Hbs, $\alpha_2^{\text{H}}\beta_2^S$, is much higher (5). Inasmuch as horse $\alpha$-(31–141) is the major contributor of the inhibitory potential of horse $\alpha$-chain, and this segment inhibits similarly to mouse $\alpha$-(31–141) (5), it follows that the six common sequence differences between the horse $\alpha$-(31–141) and mouse $\alpha$-(31–141) may be the primary determinant of the polymerization inhibitory potential of these two fragments. The observations that mouse $\alpha$-(1–30) exhibits an inhibitory potential higher than that of horse $\alpha$-(1–30) and that, when stitched to mouse $\alpha$-(31–141), additional inhibitory potential is observed suggest that a chimeric $\alpha$-chain that can be generated by stitching together mouse $\alpha$-(1–30) and horse $\alpha$-(31–141) could exhibit polymerization properties similar to that of mouse $\alpha$-chain. To test this hypothesis, we have generated the appropriate chimeric $\alpha$-chains.

Purification and Oxygen Affinity of Chimeric HbS Containing Chimeric $\alpha$-Chains Generated from Mouse and Horse $\alpha$-Chains—The chromatographic behavior of $\beta^S$ hybrids containing mouse-horse or horse-mouse chimeric $\alpha$-chain is shown in Fig. 5. The isoelectric focusing pattern of the species is shown in the inset (Fig. 5, inset). The studies establish the homogene-

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**Fig. 4. The relationship between the logarithm of reciprocal delay time and logarithm of initial Hb concentration.** The initial concentration range that has been employed was 0.3–0.5 mg/ml for $\alpha_2^{\text{H}}\beta_2^S$ and $\alpha_2^{\text{H}}\beta_2^S$ and 0.9–1.5 mg/ml for $\alpha_2^{\text{H}}\beta_2^S$ and $\alpha_2^{\text{H}}\beta_2^S$. 

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Superinhibitory Chimeric $\alpha$-Chains
The inhibitory potential of the chimeric HbS containing the animal-animal chimeric α-chain.

The β^S hybrids containing horse-mouse chimeric α-chains exhibited cooperative oxygen binding as reflected by their Hill coefficient (Table II). However, the oxygen affinity of both of the hybrids is higher than that of HbS. These results demonstrate that chimeric HbS containing nonhuman-nonhuman chimeric α-chains also conserve the basic Hb fold and reflects the high flexibility that exists in the assembly of basic Hb fold from diverse α and β-globin chains.

**Inhibitory Potential of Horse-Mouse Chimeric α-Chains**

The hybrid containing horse α-(1–30)-mouse α-(31–141) chimeric α-chain (α^Hm^S) polymerized with a delay time much longer than that of HbS (Fig. 6), and the solubility is close to that of the β^S hybrid containing human mouse chimeric α-chain (segment 1–30 from human and segment 31–141 from mouse α-chain; Fig. 7). This is consistent with the observation that very little polymerization inhibitory activity is attributed to the α-(1–30) segment of horse α-chain. The α-(1–30) segments of horse and human are indistinguishable from one another in terms of their inhibitory potential when they are stitched to the mouse α-(31–141). However, this hybrid (α^Hm^S) exhibited a solubility lower than that of α^H^M^S (Fig. 7). Therefore, the interaction of the sequence differences of horse α-(1–30) with those of mouse α-(31–141) does not elicit additional inhibitory potential to bring its value to a level comparable to that of horse α-chain. Therefore, the two common sequence differences between the mouse α-(1–30) and horse α-(1–30) are not responsible for the synergy seen between the sequence differences of the two segments of the mouse α-chains, α-(1–30) and α-(31–141) (loss of polymerization inhibitory potential on generation of human-mouse and mouse-human chimeric α-chains).

In contrast, the hybrid with the chimeric α-chain generated from mouse α-(1–30) and horse α-(31–141) (α^H^M^S) did not polymerize under all the experimental conditions attempted. The inhibitory potential of this chimeric α-chain is even higher than that of the most inhibiting parent α-chain, mouse α-chain. This suggests that the sequence differences of mouse α-(1–30) could activate one or more sequence differences of horse α-(31–141) better than that seen with the sequence differences of mouse α-(31–141), which results in an enhanced inhibitory potential. The unique complementation of the 8 sequence differences of the mouse α-(1–30) with 15 sequence differences of the horse α-(31–141) is achieved in the mouse-horse chimeric α-chain, and this results in the generation of a superinhibitory α-chain.

**Molecular Modeling Studies of Mouse-Horse Chimeric α-Chain**

The delineation of the molecular basis of the superinhibitory activity of mouse-horse chimeric α-chain should facilitate the design of novel antisickling hemoglobin for gene therapy of sickle cell disease. Mouse α-chain and the chimeric α-chain generated from mouse α-(1–30) and horse α-(31–141) have one common factor; the AB and GH corners of the two α-chains have a significant number of the sequence differences, and some residues of the AB region are involved in the intradouble strand axial contacts (α16 and α20) of the fiber (9).

In mouse and mouse-horse chimeric (both have the same α-(1–30) segment) α-chains, four sequence differences are located in the AB region (α17, α19, α21, and α22) (Fig. 8). The three-dimensional structure of Hb brings the AB and GH corners of the α-chain close to each other. Accordingly, the interaction of sequence differences at the AB corner with the sequence differences of horse α-(31–141) located in GH corner represents the main structural difference between horse and mouse-horse chimeric α-chain. This difference could perturb the intrachain interactions of the AB/GH corner of the chimeric α-chain in a fashion distinct compared with that in horse α-chain. The α-(31–141) segment of both mouse and horse α-chains have many sequence differences that are common, including the two contact site sequence differences (α78 and α116). Mouse α-(31–141) has sequence differences at α111, α113, and α116 (as compared with human), and horse α-(31–141) has sequence differences at α107, α111, α115, and α116 in the GH region. The site α115 has been implicated as an axial contact site of Wishner-Love double strand (18, 19) in Josephs’ model (20).

The consequences of the replacements of the amino acid side chains, found in the mouse-horse chimeric α-chain in the AB/GH corner of the HbS molecule, have been examined by molecular modeling (Fig. 9) to test the above hypothesis. The findings are as follows.

(a) The sequence differences of the segment from α107 to α118 of horse α-chain and those in the segment from α15 to α24 of mouse α-chain have been introduced at the respective sites in the structure of human HbA (Fig. 9, A and B). The introduction of side chains of the four sequence differences of the mouse α-chain (those located at α17, α19, α21, and α22) into HbA by themselves does not appear to result in any serious perturbations at the AB/GH corner, i.e., intrachain interactions (data not shown). This result is consistent with the experimental observation that the mouse human chimeric α-chains exhibits very little inhibition.

In contrast, the sequence differences of the GH corner of the horse α-chain at α107 and α111 perturb the α1β1 interface interactions (Fig. 10, A and B). These two residues are at the COOH-terminal end of the G helix and interact along with the side chains of residues of G helix of the β-chains (intradimeric interactions). In HbS, Val-107(a) interacts with Gln-127(b), Ala-115(β), and Cys-112(β) (Fig. 10B).

(b) In the chimeric HbS containing either horse α-chain or mouse-horse chimeric α-chain, Cys-112(b), absent in the α1β1 interface of horse hemoglobin, has been introduced into the α1β1 interface of chimeric HbS, without the other sequence differences of the horse β-chain in this region. Thus, the interactions at the α1β1 interface of the chimeric HbS containing the mouse-horse chimeric α-chain are expected to contain elements of the interactions of both HbS and horse Hb.
The presence of Ser-107(a) in the horse (a(31–141)) (in place of Val in the human (aH)) and mouse (aM)–chain in the chimeric HbS containing the mouse-horse chimeric a-chain (aHrM) and mouse (a(1–30)) plus horse (a(31–141)) chimeric a-chain (aMHr), respectively. The kinetics of polymerization of the hybrids has been carried out as described for Fig. 2. The initial concentration used for these hybrids is 1.5 mg/ml, whereas the results presented in the inset are at an initial concentration of 0.9 mg/ml.

Although the overall Hb fold is expected to be conserved, a considerable degree of dynamic changes are anticipated in the a1b1 interface in tetramers containing either horse a-chain or horse-mouse chimeric a-chain as result of the following. (i) Increased intradimeric interaction between the COOH terminal of the G-helix of the chimeric a-chain and the NH2 terminal of the G-helix of the BH is suggested to be present in the horse Hb. (ii) The presence of Cys at b112 in the chimeric HbS could facilitate normal interaction present between the carboxyl-terminal region of G helix of the B chain and the amino-terminal region of the H-helix of the chimeric a-chain at the a1b1 interface, which is different from that in horse Hb. (iii) The presence of Val at a111 in the chimeric a-chain is also expected to perturb the interactions involving the residues Ala-115(b) and Cys-112(b). Although Val-111(a) could increase the interaction with Ala-115(b), the presence of Cys-112(b) in the chimeric HbS attempts to restore the interaction with the side chain at a111 close to that seen in HbS.

In contrast, the sequence differences at a115 and a116 are
expected to influence the $\alpha_1\beta_1$ interface interactions, between the AB and GH corners of the chimeric $\alpha$-chain. In horse Hb, the presence of Asn at $\alpha_{115}$ generates an additional interaction between $\alpha_{115}$ and $\beta_{123}$, facilitating the anchoring of the amino terminus of the H-helix of the $\beta$-chain close to the GH corner or vice versa based on the energetics involved in other perturbed interactions. The replacement of Glu-116($\alpha$) with Asp facilitates a better interaction between the amino group of Lys-16($\alpha$) with the $\beta$-carboxyl group of Asp-116($\alpha$). As seen in Fig. 9B, the net effect is that the $\epsilon$-amino group Lys-16($\alpha$) becomes relatively more buried inside the crevice of the AB/GH corner compared with human $\alpha$-chain or mouse-human chimeric $\alpha$-chain.

We have noted above, the perturbations of the $\alpha_1\alpha_1$ interface in the HbS containing either horse $\alpha$-chain or human-horse chimeric $\alpha$-chain are not directly responsible for the superinhibitory activity of the chimeric $\alpha$-chain. Nonetheless, it is still conceivable that the altered interactions of the $\alpha_1\beta_1$ interface of the chimeric HbS may facilitate interaction of Lys-16($\alpha$) with Asp-116($\alpha$) in a way that decreases the accessibility of Lys-16($\alpha$) for intertetrameric interactions during the polymerization of deoxy chimeric HbS containing mouse-horse chimeric $\alpha$-chain. Further studies of superinhibitory $\alpha$-chains using x-ray crystallography or molecular fingerprinting by NMR spectroscopy, should confirm, expand, or correct the molecular basis of the antipolymerization capacity exhibited by these chimeric molecules.

**Fig. 9.** Nonequivalent intrachain interactions at the AB/GH corner of $\alpha$-chain in HbS (A) and in mouse-horse chimeric $\alpha$-chain of chimeric HbS (B). These molecular models were generated on an Indigo II workstation from Silicon Graphics using the software package Insight II from Molecular Simulations, Inc. For generating the molecular models of the chimeric $\alpha$-chain, amino acid replacements were done using the Biopolymer module in Insight II. Side-chain torsions were adjusted using Insight II followed by its default bump check. The torsions were explored for possible new hydrogen or ionic interactions. For presentation, segments A14–A24 of the $\alpha$-chain (backbone shown in black) and A105–A125 of the $\alpha$-chains (backbone shown in red) have been selected. Amino acid replacements are shown in green. Amino acid residues are identified by single-letter code, with superscript numbers identifying their location. Note the altered orientation of the side chain of Lys-16 in the chimeric chain.

**Fig. 10.** Modeling of the side-chain interaction of $\alpha_{107}$ and Gln-127($\beta$) in the chimeric protein. A new hydrogen bond can be formed at the $\alpha_1\beta_1$ interface of chimeric HbS between the side chain of the Ser at 107($\alpha$) of chimeric $\alpha$-chain and the Gln-127($\beta$) as shown in A, and this is absent in HbS. The orientation of Val-107($\alpha$) and Gln-127($\beta$) in HbS (B) is shown for comparison.
The results presented here support the mechanism of synergy between the sequence differences present in the $a-(1–30)$ and $a-(31–141)$ of the nonhuman $a$-chains and the potential use of this phenomena in the construction of $a$-chains with super-inhibitory potential that could serve as a blueprint for the design of therapeutic agents for the gene therapy of sickle cell disease.

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