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Structural basis for the antipolymer activity of Hb $\zeta_2\beta^s_2$ trapped in a tense conformation

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Abstract

The phenotypical severity of sickle-cell disease (SCD) can be mitigated by modifying mutant hemoglobin S (Hb S, Hb $\alpha_2\beta^s_2$) to contain embryonic ζ -globin in place of adult α -globin subunits (Hb $\zeta_2\beta^s_2$). Crystallographical analyses of liganded Hb $\zeta_2\beta^s_2$, though, demonstrate a tense (T-state) quaternary structure that paradoxically predicts its participation in--rather than its exclusion from--pathological deoxyHb S polymers. We resolved this structure-function conundrum by examining the effects of $\alpha \rightarrow \zeta$ exchange on the characteristics of specific amino acids that mediate sickle polymer assembly. Superposition analyses of the β^s subunits of T-state deoxyHb $\alpha_2\beta^s_2$ and T-state CO-liganded Hb $\zeta_2\beta^s_2$ reveal significant displacements of both mutant $\beta^s\text{Val6}$ and conserved β -chain contact residues, predicting weakening of corresponding polymer-stabilizing interactions. Similar comparisons of the α - and ζ -globin subunits implicate four amino acids that are either repositioned or undergo non-conservative substitution, abrogating critical polymer contacts. CO-Hb $\zeta_2\beta^s_2$ additionally exhibits a unique trimer-of-heterotetramers crystal packing that is sustained by novel intermolecular interactions involving the pathological $\beta^s\text{Val6}$, contrasting sharply with the classical double-stranded packing of deoxyHb S. Finally, the unusually large buried solvent-accessible surface area for CO-Hb $\zeta_2\beta^s_2$ suggests that it does not co-assemble with deoxyHb S *in vivo*. In sum, the antipolymer activities of Hb $\zeta_2\beta^s_2$ appear to arise from both repositioning and replacement of specific α - and β^s -chain residues, favoring an alternate T-state solution structure that is excluded from pathological deoxyHb S polymers. These data account for the antipolymer activity of Hb $\zeta_2\beta^s_2$, and recommend the utility of SCD therapeutics that capitalize on α -globin exchange strategies.

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Graphical abstract

Keywords

Hemoglobin; Sickle Cell Disease; Tense State; Mutation; Structure; Polymerization

INTRODUCTION

Sickle cell disease (SCD) is a recessive monogenic disorder that is characterized by high-level expression of a variant hemoglobin (Hb S; Hb $\alpha_2\beta^S_2$) assembled from β -globin subunits that contain a Glu→Val substitution at codon 6 [1–4]. While Hb S displays O₂-binding properties similar to those of normal adult hemoglobin (Hb A, Hb $\alpha_2\beta_2$), its deoxygenated form (deoxyHb S) assembles into long, rigid, insoluble fibers that distort erythrocyte structure and promote pathological changes to cell function [5–7]. The structure of the deoxyHb S fiber has been detailed using X-ray crystallographic, fiber diffraction, microscopic, and biochemical approaches that largely agree on a fundamental arrangement comprising seven double-stranded fibrils of deoxyHb S organized into a 14-stranded fiber [5,6,8–14]. Individual double-stranded fibrils are stabilized by a principal interaction between the pathological β^S2 Val6 from one molecule and a hydrophobic acceptor region comprising $\beta1$ Ala70, $\beta1$ Phe85, and $\beta1$ Leu88 (hereafter the *Phe85-Leu88 pocket*) from an adjacent heterotetramer [9,13].

The precedent analyses that concur on the molecular pathobiology of β^S2 Val6 additionally agree that secondary interactions between adjacent heterotetramers--involving a large number of non-pathological amino acids--are also critical to the stability of the deoxyHb S polymer. These interactions are commonly categorized either as intra-strand (axial) or as inter-strand (lateral). Axial interactions are primarily mediated by amino acids that are positioned in specific regions of the index heterotetramer (the A helix, AB corner, and GH corner of the α_2 globin; and the G helix and GH corner of the β^S2 globin) and that contact defined elements of a heterotetramer in the same strand (A helix, AB corner, G helix, and GH corner of the β^S1 globin; and GH corner of the α_1 globin). Lateral interactions are effected through different regions of the index heterotetramer (E helix, EF corner, and F helix of the β^S1 globin) that contact specific residues of a heterotetramer positioned in a second, parallel strand (A helix and H helix of the β^S2 globin; and CD corner of the α_2 globin). The deoxyHb S fiber is also stabilized by secondary interactions between adjacent double-stranded (DS) fibrils that are aligned in both parallel and antiparallel orientations

with topologies that are less certain [5,15–17]. The critical importance of secondary intra- and inter-DS interactions is illustrated by the large number of naturally occurring mutations--within both the α -globin and β -globin subunits--that reduce experimental and clinical measures of SCD severity by attenuating or ablating these polymer-stabilizing contacts [7,13,14,18–22]. In short, the β^S Val6 is necessary, but is not sufficient, to promote pathological polymerization of deoxyHb S.

We recently reported that both the severity of the SCD phenotype, as well as the underlying polymerization of deoxyHb S, could be mitigated by exchanging its normal adult α globin for the related embryonic ζ globin [23]. These two ontogenically related globins differ at 57 of 141 amino acids (Fig. 1A), but assemble structurally similar heme-bearing subunits (Fig. 1B) that bind any of several β -like subunits into functional Hb heterotetramers [23,24]. Studies conducted *in vivo* in a humanized mouse model of SCD demonstrate that human ζ globin can revert characteristic biochemical, hematological, and physiological metrics for disease severity to near-normal or even normal values [23]. Corresponding *in vitro* analyses confirm that $\alpha \rightarrow \zeta$ exchange significantly slows the assembly of deoxyHb S polymers, accounting for the profound antisickling effects observed *in vivo* in intact animals [23]. Crystallographical studies of the COliganded Hb $\zeta_2\beta^S_2$, however, reveal an unexpected structure that is characterized by quaternary features typifying T-state deoxyHbs and--paradoxically--predicting its inclusion (rather than its exclusion) from the deoxyHb S polymer [25]. This consideration suggests that the antipolymer characteristics of Hb $\zeta_2\beta^S_2$ arise through a different mechanism; specifically, from defined changes in primary or secondary structure that alter critical polymer-stabilizing interactions. The results of these studies validate the importance of specific α - and β^S -globin amino-acid residues to deoxyHb S self-assembly, account for the profound antipolymer activity of ζ -substituted Hb S, discover fundamental differences in intermolecular interactions supported by T-state liganded Hb $\zeta_2\beta^S_2$ and deoxyHb $\alpha_2\beta^S_2$, and specify amino-acid residues that can be targeted for substitution in α -like (or ζ -like) transgenes designed for therapeutic applications.

The present study defines key structural features of ζ -substituted Hb S that account for its antipolymer effect *in vivo*. Our analyses compare the crystal structures of the two related T-state heterotetramers--CO-Hb $\zeta_2\beta^S_2$ and deoxyHb $\alpha_2\beta^S_2$ --to resolve the basis for the paradoxical exclusion of ζ -substituted heterotetramer from the classical deoxyHb S polymer, despite a quaternary structure that would predict otherwise. Specifically, we examine the effects of $\alpha \rightarrow \zeta$ substitution on the positions of residues within neighboring β -globin subunits that are critical to polymer assembly, with particular attention to the potential displacement of the pathological β^S Val6. We then consider the impact of nonconservative substitutions affecting α -globin residues that are critical to polymer-stabilizing interactions, as well as the effects of changes in the positions of polymer-stabilizing amino acids that are conserved between the α - and ζ -globin subunits. Finally, we describe previously unknown dissimilarities in the crystal packing of CO-Hb $\zeta_2\beta^S_2$ and deoxyHb $\alpha_2\beta^S_2$ that predict differences in their intermolecular interactions in solution. The results of these studies validate the importance of specific α - and β^S -globin amino-acid residues to deoxyHb S self-assembly, account for the profound antipolymer activity of ζ -substituted Hb S, discover fundamental differences in intermolecular interactions supported by T-state liganded Hb

$\zeta_2\beta^s_2$ and deoxyHb $\alpha_2\beta^s_2$, and suggest specific amino-acid residues that can be targeted for substitution in α -like (or ζ -like) transgenes designed for therapeutic applications.

MATERIALS AND METHODS

Protein production and structure determination of CO-liganded Hb $\zeta_2\beta^s_2$

The experimental procedures used to prepare human Hb $\zeta_2\beta^s_2$ and solve the crystal structure of its CO-liganded form are described in detail elsewhere [23,25,26]. The refined structure for CO-liganded Hb $\zeta_2\beta^s_2$ is deposited in the PDB with ID code 3W4U.

Comparison of CO-Hb $\zeta_2\beta^s_2$ and deoxyHb $\alpha_2\beta^s_2$

Local structural differences between CO-Hb $\zeta_2\beta^s_2$ and deoxy-Hb $\alpha_2\beta^s_2$ (PDB accession 2HBS) were determined using the program LSQKAB as incorporated into the CCP4 suite [27]. Superpositions were conducted using the corresponding α - and ζ -globin subunits (C α atoms from residues 3–138) or the corresponding β^s -globin subunits (C α atoms from residues 3–144). Root mean square deviation (rmsd) values were calculated for each superposition, to permit comparison to the positional displacements of single informative amino acids that participate in polymer contacts [7,9] or that neighbor known polymer contact sites but that have not been formally implicated in this process [7,14,22,28,29].

Solvent accessible surface area

The buried solvent accessible surface area (SASA) was calculated for deoxyHb $\alpha_2\beta^s_2$ and CO-Hb $\zeta_2\beta^s_2$ using the web-based PDBePISA as described [30,31].

RESULTS

Exclusion of CO-Hb $\zeta_2\beta^s_2$ from deoxyHb S fibers

Polymeric deoxyHb S readily incorporates a number of heterotetrameric hemoglobins that contain β^s subunits, and that characteristically exist in T-like quaternary states [7,13,32–35]. In contrast, polymeric deoxyHb S typically excludes hemoglobins—including those that contain β^s subunits—that revert to R-like quaternary states following O₂ liganding [7,13,33,34,36,37]. Our previous crystallographic studies demonstrate that Hb $\zeta_2\beta^s_2$ is unique among β^s -containing heterotetramers in maintaining a T-like quaternary structure, even when fully liganded [25]. This unusual characteristic predicts that O₂-liganded Hb $\zeta_2\beta^s_2$ will co-assemble with deoxyHb S, an expectation that is inconsistent with the robust antipolymer properties of Hb $\zeta_2\beta^s_2$ that are observed *in vitro*, and also with corresponding antisickling effects that are demonstrated *in vivo*. These analyses suggest that other, unrecognized structural features of liganded Hb $\zeta_2\beta^s_2$ are likely to preclude its polymeric activities in intact erythrocytes [23].

Based on these and other considerations, we posited that $\alpha \rightarrow \zeta$ subunit exchange in Hb S either weakens the primary interaction between the pathological $\beta^s_2\text{Val6}$ and the Phe85- βLeu88 acceptor pocket or, alternately, reduces the strengths of one or more polymer-stabilizing secondary interactions between non-pathological amino-acid residues. To address these possibilities, we compared both the identities and the relative positions of

corresponding amino acids in the two T-state heterotetramers, with particular focus on residues that are known to stabilize the deoxyHb S polymer *in vitro* or to mediate the severity of the sickle phenotype *in vivo*. Structural comparisons of the two hemoglobins were conducted by least-squares superposition of each corresponding α -like subunit (α globin and ζ globin), and of each corresponding β -like subunit (β^S globin). The positional displacement of specific, informative amino acids was then determined, and assigned significance by comparison to the rmsd value calculated for the cognate globin subunit as a whole. As both deoxyHb $\alpha_2\beta^S_2$ and CO-Hb $\zeta_2\beta^S_2$ exist as heterotetramers in their respective crystal asymmetric units, it was necessary to calculate displacement values for amino acids in each of two analyses (i.e., both $\beta^S1 \nu \beta^S1$ and $\beta^S2 \nu \beta^S2$; or both $\alpha 1 \nu \zeta 1$ and $\alpha 2 \nu \zeta 2$), although in many cases only one of the two corresponding subunits is pathologically relevant. For example, since the β Val6 from the $\beta 2$ subunit (but not the $\beta 1$ subunit) participates in a critical polymer contact, its displacement was calculated for superposed β^S2 subunits, and not for superposed β^S1 subunits.

β^S -globin residues

The T-like quaternary structure of CO-Hb $\zeta_2\beta^S_2$ wrongly predicts that this potentially therapeutic heterotetramer will incorporate into the deoxyHb S fiber. To investigate a potential structural basis for this paradox, we superposed the crystal structures of CO-Hb $\zeta_2\beta^S_2$ and deoxyHb $\alpha_2\beta^S_2$, permitting the identification of specific amino acids within either the β^S1 or β^S2 subunits that are repositioned in a manner that would likely destabilize the deoxyHb S polymer (Table 1). The pathological β^S2 Val6 exhibits a displacement of 1.9Å--more than double the rmsd value of 0.8Å calculated for all β^S2 -globin residues--while the positions of the three amino acids that comprise the hydrophobic acceptor pocket ($\beta 1$ Ala70, $\beta 1$ Phe85, and $\beta 1$ Leu88) are largely preserved, displaying displacements of 0.5Å–0.9Å. These measurements suggest that the repositioned β^S2 Val6 will align inefficiently with the Phe85-Leu88 pocket (whose location is unaffected by $\alpha \rightarrow \zeta$ exchange), weakening this polymer-stabilizing interaction and accounting for the exclusion of CO-Hb $\zeta_2\beta^S_2$ from the deoxyHb S polymer (Fig. 1C).

The antipolymer effect of β^S2 Val6 repositioning appears to be separately augmented by structural displacements of both $\beta 2$ Thr4 (2.2Å) and $\beta 1$ Asn19 (1.4Å). The repositioned $\beta 2$ Thr4 is likely to misalign with $\beta 1$ Asp73 (which maintains a similar position in both α - and ζ -containing heterotetramers), weakening a hydrogen-bond interaction that stabilizes the deoxyHb S polymer (Fig 1C) [9]. This model for antipolymer activity is consistent with the physical properties of Hb Korle Bu (Hb $\alpha_2\beta^{\text{Asp73} \rightarrow \text{Asn}2}_2$), a naturally occurring variant heterotetramer that both increases the solubility and slows the polymerization of deoxyHb S mixtures by eliminating the critical $\beta 2$ Thr4- $\beta 1$ Asp73 hydrogen-bond contact [38,39], and is associated with a benign phenotype when co-expressed with β^S -globin in man [40]. The antipolymer significance of $\beta 2$ Thr4- $\beta 1$ Asp73 hydrogen-bond disruption is additionally validated by a second naturally occurring mutation at the same position (β^{Mobile} , $\beta^{\text{Asp} \rightarrow \text{Val}}$) that retards gelation of Hb S mixtures *in vitro* [41]. Similarly, the positional importance of $\beta 1$ Asn19 is consistent with biochemical analyses of Hb D^{Ouled Rabah}--containing a natural β Asn19 $\rightarrow$ Lys substitution--which significantly increases the minimal gelling concentration of deoxyHb S in solution [13]. The observed changes in the positions of β^S2 Val6, $\beta 2$ Thr4,

and $\beta 1\text{Asn}19$ establish the principle that α -like subunit exchange in general--and $\alpha \rightarrow \zeta$ subunit substitution in particular--can act at a distance to alter features of β^s -subunit structure that are integral to sickle pathobiology.

In contrast to the three β^s -chain residues discussed above, approximately two dozen non-pathological β^s -globin residues that participate in deoxyHb S axial, lateral, and inter-DS contacts do not appear to be significantly displaced by $\alpha \rightarrow \zeta$ substitution, implicating polymer effects that are likely to be preserved in T-state Hb $\zeta_2\beta^s_2$. For example, the importance of $\beta 1\text{Gly}16$ and $\beta 1\text{Gly}83$ to sickle polymer stability has been clearly established both by gelation studies of deoxyHb S mixtures [13], as well as by observations that co-expression of variant $\beta^{\text{Gly}16 \rightarrow \text{Asn}}$ globin ($\beta^{\text{J-Baltimore}}$) or $\beta^{\text{Gly}16 \rightarrow \text{Asn}}$ globin (β^{Pyrgos}) does not exacerbate the benign phenotype of β^s heterozygotes [42–44]. Both $\beta 1\text{Gly}16$ and $\beta 1\text{Gly}83$ display relatively small displacements following $\alpha \rightarrow \zeta$ exchange ($<0.2\text{\AA}$ and $0.7\text{\AA}$, respectively) suggesting that the intermolecular contacts that they mediate in deoxyHb $\alpha_2\beta^s_2$ are likely to be preserved in CO-Hb $\zeta_2\beta^s_2$. These considerations indicate that the antipolymer consequences of $\alpha \rightarrow \zeta$ substitution result from the loss of a small number of intermolecular contacts, rather than from a global restructuring of the full β^s subunit, consistent with a general hypothesis for sickle pathobiology that acknowledges the critical importance of secondary interactions to Hb S polymer stability.

α -like globin residues

The $\alpha \rightarrow \zeta$ exchange that converts Hb $\alpha_2\beta^s_2$ to Hb $\zeta_2\beta^s_2$ alters the identities of 57 (of 141) amino acids (Fig. 1A), and anticipates a shift in the positions of other, conserved residues. We considered the possibility that a change in either metric could alter specific intra- or inter-DS interactions that stabilize the deoxyHb S polymer. To study these effects, we superposed the α subunits from deoxyHb $\alpha_2\beta^s_2$ and the ζ subunits from CO-liganded Hb $\zeta_2\beta^s_2$ and categorized amino-acid residues--previously implicated in Hb S polymer assembly--into three groups that reflect differences in their chemical identities, their positions, or both features (Table 2).

Nonconserved residues that maintain their positions—A substantial literature illustrates the principle that the self-assembly of deoxyHb S can be inhibited by non-conservative substitutions for amino acids that mediate secondary polymer-stabilizing interactions, even in the presence of the pathological $\beta^s\text{Val}6$ [22,29]. For example, α -globin subunits that contain a His $\rightarrow$ Gln substitution at position 20 (as occurs in the natural variant $\alpha^{\text{Le Lmentin}}$) display a robust antipolymer activity when assembled with β^s subunits into a variant Hb S, ostensibly by disturbing a polymeric axial interaction with $\beta 1\text{Glu}22$ [20]. Our analysis of nonconservative substitutions effected by $\alpha \rightarrow \zeta$ subunit exchange indicates that the antipolymer activity of liganded Hb $\zeta_2\beta^s_2$ may arise from a similar effect (Table 2). Three α -chain residues that mediate polymer-stabilizing interactions are replaced by unlike ζ -chain amino acids, without a significant change in their relative positions: $\alpha 2\text{His}20 \rightarrow \zeta 2\text{Gln}20$ (basic $\rightarrow$ neutral polar; $0.9\text{\AA}$), $\alpha\text{Asn}68 \rightarrow \zeta\text{Asp}68$ (neutral polar $\rightarrow$ acidic; $0.7\text{\AA}$), $\alpha\text{Asn}78 \rightarrow \zeta\text{Gly}78$ (neutral polar $\rightarrow$ neutral; $0.8\text{\AA}$ and $0.6\text{\AA}$ for the $\alpha 1/\zeta 1$ and $\alpha 2/\zeta 2$ superpositions, respectively). The antipolymer effect of the first exchange ($\alpha 2\text{His}20 \rightarrow \zeta 2\text{Gln}20$) is particularly informative as it reproduces the amino-acid substitution

that defines $\alpha^{\text{Le Lamentin}}$, and alone may account for the anti-sickling characteristics of Hb $\zeta_2\beta^{\text{S}}_2$ [20]. The potential antipolymer properties of ζAsp68 and ζGly78 are consistent with the activities of α -globin subunits containing naturally occurring mutations at the corresponding positions-- $\alpha\text{Asn68}\rightarrow\text{Lys}$ (neutral polar $\rightarrow$ basic; $\alpha^{\text{Philadelphia}}$) and $\alpha\text{Asn78}\rightarrow\text{Gly}$ (neutral polar $\rightarrow$ neutral; $\alpha^{\text{Stanleyville}}$)--that increase the solubility of deoxyHb S heterotetramers that incorporate them [21,22,29], maintain the benign phenotype of β^{S} heterozygotes [45,46], and mitigate the severity of the sickle phenotype in β^{S} homozygotes [45,47,48].

Two additional exchanges are likely to contribute to the antipolymer properties of CO-Hb $\zeta_2\beta^{\text{S}}_2$; specifically, $\alpha\text{Lys11}\rightarrow\zeta\text{Val11}$ (basic $\rightarrow$ neutral hydrophobic; $<0.2\text{\AA}$) and $\alpha\text{Leu113}\rightarrow\zeta\text{Phe113}$ ($<0.2\text{\AA}$). Although contacts for αLys11 are not observed in polymeric deoxyHb S, a natural substitution at this site ($\alpha^{\text{Lys11}\rightarrow\text{Glu}}$, $\alpha^{\text{Anantharaj}}$) increases the solubility of the cognate β^{S} -containing heterotetramer *in vitro* in solution [29]. The situation is similar for αLeu113 which, though located at the periphery of the axial interface, does not participate in any pathological polymer contact. While the effect of an αLeu113 substitution on the sickle phenotype has not previously been described *in vivo*, variant α -globin chains that contain an $\alpha\text{Leu113}\rightarrow\text{His}$ exchange ($\alpha^{\text{Twin Peaks}}$) exert significant antipolymer activity when incorporated into Hb S *in vitro* [49], presumably because the large αHis113 side-chain perturbs the positions of neighboring αPro114 and αAla115 and abrogates the strengths of their polymer-stabilizing contacts. This mechanism accords with our observation that $\alpha\text{Leu113}\rightarrow\zeta\text{Phe113}$ exchange (which introduces an enlarged amino-acid side chain) is accompanied by positional displacements of both Pro114 ($1.2\text{\AA}$ and $1.3\text{\AA}$ for the $\alpha1/\zeta1$ and $\alpha2/\zeta2$ superpositions, respectively) and Ala115 ($0.9\text{\AA}$ and $1.1\text{\AA}$). These data, then, suggest that a portion of the antipolymer activity of CO-Hb $\zeta_2\beta^{\text{S}}_2$ arises from nonconservative substitution of one or more α -chain residues that are required to stabilize individual deoxyHb S fibrils, as well as the 14-strand fiber as a whole.

Conserved residues that are repositioned—The deoxyHb S polymer is stabilized by both axial and lateral hydrogen-bond interactions that typically extend from $2.8\text{\AA}$ to $4.0\text{\AA}$ [9]. Six of the 84 amino acids that are conserved between human α and ζ globins have been implicated in such interactions, including $\alpha2\text{Lys16}$, $\alpha1/\alpha2\text{Asp47}$, $\alpha1/\alpha2\text{Gln54}$, $\alpha1/\alpha2\text{Asp75}$, $\alpha1/\alpha2\text{Pro114}$, and $\alpha1/\alpha2\text{Ala115}$ [9,14,22,28,29]. The positional displacements of $0.2\text{\AA}$ – $1.1\text{\AA}$ for five of these residues are not significantly different from the rmsd value of $0.8\text{\AA}$ calculated for both α/ζ -subunit superpositions, indicating modest repositionings that are unlikely to alter critical hydrogen-bond interactions (Table 2). The sixth residue (Pro114) is displaced to a greater extent ($1.2\text{\AA}$ and $1.3\text{\AA}$ for the $\alpha1/\zeta1$ and $\alpha2/\zeta2$ superpositions, respectively), predicting a more meaningful impact on the strengths of corresponding hydrogen-bond interactions. The potential contribution of a repositioned Pro114 to the antipolymer activity of CO-Hb $\zeta_2\beta^{\text{S}}_2$ accords with studies demonstrating that Hb S-variant heterotetramers that incorporate recombinant $\alpha\text{Pro114}\rightarrow\text{Arg}$ substitutions display increased solubility *in vitro* [50], naturally occurring variants at this position are unusual and have not been reported in patients with sickle syndromes [51–53]. Consequently, secondary intermolecular contacts subserved by conserved residues are largely preserved by $\alpha\rightarrow\zeta$

exchange and, with the possible exception of Pro1 14, are likely to promote--rather than to inhibit--inclusion of CO-Hb $\zeta_2\beta^S_2$ in the deoxyHb S polymer.

Nonconserved residues that are repositioned—The $\alpha \rightarrow \zeta$ substitution that creates Hb $\zeta_2\beta^S_2$ alters both the position as well as the biochemical identities of two residues that have been implicated in deoxyHb S polymer structure: $\alpha 1/\alpha 2\text{Glu}23 \rightarrow \zeta 1/\zeta 2\text{Thr}23$ (acidic $\rightarrow$ neutral polar; 1.3Å) and $\alpha 2\text{Ser}49 \rightarrow \zeta 2\text{His}49$ (neutral $\rightarrow$ basic; 1.7Å) [9,19,54,55]. The data anticipate that the polymer-stabilizing interactions mediated by amino acids in these two positions will be fully ablated by $\alpha \rightarrow \zeta$ subunit exchange (Table 2). These predictions are consistent with the properties of the naturally occurring variant $\alpha^{\text{Memphis}} (\alpha^{\text{Glu}} \rightarrow \text{Glu}$; acidic $\rightarrow$ neutral polar), which significantly mitigates the phenotype of patients with SCD, lowers their whole-blood viscosity, and reduces gelling of hemoglobin purified from their red-blood cells [54,55]; and also with the properties of $\alpha^{\text{Savaria}} (\alpha^{\text{Ser}49} \rightarrow \text{Arg}$; neutral $\rightarrow$ basic), which nearly doubles the saturation concentration (C_{Sat}) for Hb S in solution [19]. While the antipolymer activities of both the $\alpha 23$ and $\alpha 49$ substitutions might be more substantial if they were *not* repositioned (juxtaposing hydrophobic and hydrophilic side chains), it is clear that these two amino-acid changes ablate separate intermolecular contacts that are critical to deoxyHb S polymer stability.

Nonconserved residues located near the polymer contact interface, not previously implicated in polymer contact

Amino-acid residues positioned at the polymer interface are critical to deoxyHb S solubility [56], through contacts that may not be fully described by existing X-ray crystallographic studies or fiber models [8,15,49,57]. The α -globin subunits of the high-resolution crystal structure of deoxyHb S display 10 surface residues that neighbor known polymeric contact sites, but that have not previously been implicated in polymer stability [9]. Strikingly, none of these residues are conserved in the ζ -globin subunit (Table 3), while four exhibit positional displacements that significantly exceed the full α/ζ -subunit rmsd value of 0.8Å ($\zeta 2\text{Ser}18$, 1.7Å; $\zeta 2\text{Thr}19$, 1.9Å; $\zeta 2\text{Asp}22$, 1.9Å; and $\zeta 2\text{Pro}50$, 2.1Å). A fifth exchange ($\alpha\text{Tyr}24 \rightarrow \zeta\text{Ile}24$) does not alter residue position (displacement <0.2Å) but is expected to mitigate polymer assembly by disrupting an inter-DS hydrogen-bond interaction with the heme propionate of the $\beta 1$ -chain of an adjacent Hb $\zeta_2\beta^S_2$ molecule. While there is no precedent indicating the possibility that these residues participate in polymer stability, the aggregate affect of 10 nonconserved residues in a region that is critical to deoxyHb S solubility is highly suggestive.

CO-Hb $\zeta_2\beta^S_2$ does not form a double-strand fibril in the crystal

The previous analysis suggests that T-state Hb $\zeta_2\beta^S_2$ may compromise the integrity of the deoxyHb S polymer by incorporating into fibrils that participate suboptimally in critical intra- or inter-DS interactions. We have also considered that the antipolymer activity of ζ -substituted Hb S may result from its full exclusion from deoxyHb S fibrils, lowering the effective concentration of polymer-compatible heterotetramers and therefore negatively impacting the kinetics of polymer assembly. A close examination of the crystal packing of deoxyHb $\alpha_2\beta^S_2$ and CO-Hb $\zeta_2\beta^S_2$ reveals two structures that are striking in their differences. T-state CO-Hb $\zeta_2\beta^S_2$ assembles into a trimer comprising three lateral heterotetramers, each

of which interacts with an axial heterotetramer that is constituent to a separate trimer assembly (Tables 4, 5; Fig. 2A, B). T-state deoxyHb $\alpha_2\beta^s_2$, in contrast, packs as two parallel strands, with a building block comprising two axial and one lateral heterotetramers (Fig. 2C, D) [5,6,8–14]. As might be anticipated, the unique crystal arrangement in CO-Hb $\zeta_2\beta^s_2$ is sustained by intermolecular interactions that are distinct from those observed for deoxyHb S. Lateral crystal interactions--which are related by a three-fold axis of crystallographic symmetry--are stabilized by hydrophilic and hydrophobic contacts between constituent heterodimers (either $\zeta_1\beta^s_1$ or $\zeta_2\beta^s_2$) *via* symmetry-related determinants (Table 4; Fig. 2A, B). Two of six lateral interactions (mediated by ζ Ser1 8 and ζ Ala64), and five of seven axial interactions (mediated by ζ Lys4, ζ Thr5, ζ Ser12, ζ Tyr89, and ζ Glu120) are unique to T-state CO-Hb $\zeta_2\beta^s_2$, comprising amino-acid residues that are not conserved during $\alpha \rightarrow \zeta$ subunit exchange (Tables 4 and 5). One lateral contact is defined by a novel interaction between the pathological β^s_2 Val6 and a hydrophobic domain comprising ζ_2 Trp14, ζ_2 Ala21, ζ_2 Val63, and ζ_2 Ala64 from the ζ_2 subunit of a neighboring heterotetramer, and is enhanced by a unique hydrogen-bond interaction between β_2 Ser9 and ζ_2 Trp14 (Fig. 2E). These contacts differ from those that occur in Hb $\alpha_2\beta^s_2$, where the pathological β^s_2 Val6 interacts with a hydrophobic domain on the β_1 subunit of an adjacent heterotetramer (β_1 Ala70, β_1 Phe85, β_1 Leu88), and is stabilized by an alternate hydrogen bond between β_1 Thr4 and β_1 Asp73 (Fig. 2F). A second, unique lateral contact region for T-state CO-Hb $\zeta_2\beta^s_2$ comprises ζ_2 Arg56 from one molecule, and three different ζ -chain residues from a second molecule (ζ_2 Glu30, ζ_2 Pro50, and ζ_2 Gly51).

The profound differences in the crystal packing of deoxyHb $\alpha_2\beta^s_2$ and CO-Hb $\zeta_2\beta^s_2$ suggest that--despite their similar T-like quaternary structures--the two heterotetramers are unlikely to assemble into a co-polymer. We investigated this possibility by estimating the relative stabilities of homogeneous crystal deoxyHb $\alpha_2\beta^s_2$ and crystal CO-Hb $\zeta_2\beta^s_2$ from the buried solvent accessible surface area (SASA) calculated for each [30,31]. DeoxyHb $\alpha_2\beta^s_2$ displays a buried SASA value of $2510\text{\AA}^2$, summed from two lateral contacts ($1602\text{\AA}^2$) and one axial contact ($908\text{\AA}^2$) (Fig. 2C, D). CO-Hb $\zeta_2\beta^s_2$, in contrast, displays a buried SASA value nearly twice as large-- $4806\text{\AA}^2$ --reflecting three lateral contacts ($3102\text{\AA}^2$) and one axial contact ($1704\text{\AA}^2$) (Fig. 2A, B). The large combined buried surface area of trimeric Hb $\zeta_2\beta^s_2$, as well as the buried surface area between constituent $\zeta_1\beta^s_1$ and $\zeta_2\beta^s_2$ heterodimers within each Hb $\zeta_2\beta^s_2$ heterotetramer ($2804\text{\AA}^2$), suggest that Hb $\zeta_2\beta^s_2$ is likely to exist in trimeric form in solution [58], and validates the hypothesis that the variant hemoglobin will not co-assemble with deoxyHb S *in vivo*.

DISCUSSION

The principle that the pathobiology of deoxyHb S can be mitigated by α - or β -like subunit exchange is founded upon methodologically independent ultrastructural analyses [5,6,9–12,59,60], as well as *in vitro* and *in vivo* studies of naturally occurring and engineered variant hemoglobins [13,18,22,29,33,34,50,61]. We previously studied the biochemical characteristics of Hb $\zeta_2\beta^s_2$, a heterotetramer that is derived from Hb S by replacing its adult α -globin subunits with developmentally related ζ -globin subunits [23,26]. While this unusual hemoglobin exhibits suboptimal ligand binding properties--high oxygen affinity, decreased cooperativity, weak Bohr effect, and reduced 2,3-DPG allostery [26]--it is

surprisingly effective in mitigating the pathological phenotype of mouse models of sickle-cell disease, even at relatively low levels of expression [23]. Crystallographical studies of Hb $\zeta_2\beta^s_2$ reveal an equally surprising structure in which the heterotetramer maintains a T-like quaternary state, even when fully liganded, accounting for the biochemical properties of Hb $\zeta_2\beta^s_2$ and providing novel insights tertiary and quaternary structural events that are critical to hemoglobin ligand binding in particular, and allosteric function in general [25].

The present study compares two related T-state hemoglobins--deoxyHb $\alpha_2\beta^s_2$ and CO-Hb $\zeta_2\beta^s_2$ to elucidate the several structural bases that underlay the antipolymer characteristics of ζ -substituted heterotetramers. Our results account for the contrasting polymeric activities of the two hemoglobins, including differences in the positionings of corresponding amino acids in each of their globin subunits (Tables 1, 2) and inferred differences in the manner in which the two heterotetramers interact in solution *in vivo* (Fig. 2A–D). While these observations confirm the primacy of the pathological $\beta^s\text{Val6}$ to Hb S polymer assembly, they additionally validate the critical importance of a number of secondary interactions that contribute to the efficiency of this process.

Our initial analyses were surprising in revealing that $\alpha \rightarrow \zeta$ substitution exerts significant long-range effects on the positions of specific residues within the otherwise identical β^s -globin subunits of deoxyHb $\alpha_2\beta^s_2$ and CO-Hb $\zeta_2\beta^s_2$ (Table 1). Both $\beta^s\text{Val6}$ and $\beta^s\text{Thr4}$ are particularly noteworthy in this regard: these residues facilitate polymer-stabilizing intermolecular contacts in deoxyHb $\alpha_2\beta^s_2$, but are repositioned in CO-Hb $\zeta_2\beta^s_2$ to an extent that predicts a significant weakening of these interactions (Fig. 1C). The distinct crystal packings of deoxyHb $\alpha_2\beta^s_2$ and CO-Hb $\zeta_2\beta^s_2$ that we observe indicate that the positional deviations for both $\beta^s\text{Val6}$ and $\beta^s\text{Thr4}$ are biochemically relevant (Fig. 2E, F). Specifically, the displacement of $\beta^s\text{Val6}$ abrogates pathological contact with the $\beta^1\text{Phe85}$ - $\beta^1\text{Leu88}$ hydrophobic pocket (in deoxyHb $\alpha_2\beta^s_2$; Fig. 2F), favoring alternate, novel interactions with $\zeta^2\text{Trp14}$, $\zeta^2\text{Ala21}$, $\zeta^2\text{Val63}$, and $\zeta^2\text{Ala64}$ (in CO-Hb $\zeta_2\beta^s_2$; Fig. 2E); while the repositioning of $\beta^s\text{Thr4}$ ablates critical hydrogen-bond contacts with $\beta^1\text{Asp73}$ (in deoxyHb $\alpha_2\beta^s_2$). In contrast to $\beta^s\text{Val6}$ and $\beta^s\text{Thr4}$, other β^s -chain residues that are implicated in sickle polymer assembly are displaced minimally ($\beta^2\text{Lys8}$, $\beta^1\text{Asn19}$, $\beta^1\text{Lys95}$, and $\beta^1\text{Glu22}$) or not at all ($\beta^1\text{Trp15}$, $\beta^1\text{Lys17}$, $\beta^1\text{Val18}$, $\beta^1\text{Val23}$, $\beta^1\text{Lys66}$, $\beta^2\text{Leu68}$, $\beta^1\text{His116}$, $\beta^1/\beta^2\text{His117}$, $\beta^1/\beta^2\text{Phe118}$, $\beta^2\text{Lys120}$, $\beta^1\text{Glu121}$, $\beta^2\text{Glu122}$), and are therefore likely to subserve polymer-stabilizing effects that are indifferent to $\alpha \rightarrow \zeta$ exchange. These data are consistent with the known importance of $\beta^s\text{Val6}$ to sickle pathobiology, and additionally emphasize the critical contribution of secondary non-pathological contacts to sickle polymer assembly.

Unlike the β^s -globin chains--which are identical in Hbs $\alpha_2\beta^s_2$ and $\zeta_2\beta^s_2$ --the α - and ζ -globin chains differ at 57 of 141 amino-acid positions (Fig. 1A). This situation raises the possibility that the antipolymer activities of CO-Hb $\zeta_2\beta^s_2$ may be effected both by spatial displacement of specific residues, as well as by differences in their identities. The principle that nonconservative amino-acid substitutions in the α -like subunit can mitigate biochemical and clinical characteristics of SCD has been firmly established by studies of recombinant and naturally occurring variant α -globins both *in vitro* and *in vivo* [18–22,29,49,50]. Our analyses suggest that three nonconservative substitutions-- $\alpha\text{His20} \rightarrow \zeta\text{Gln}$, $\alpha\text{Asn68} \rightarrow \zeta\text{Asp}$, and $\alpha\text{Asn78} \rightarrow \zeta\text{Gly}$ --may be particularly important to the antipolymer activities of CO-Hb

$\zeta_2\beta^s_2$ (Table 2), a model that is consistent with naturally occurring mutations that alter the kinetics and/or thermodynamics of Hb S polymer assembly *in vitro*, or mitigate the severity of the SCD condition *in vivo* [21,22,29,45–48]. The antipolymer characteristics of CO-Hb $\zeta_2\beta^s_2$ are likely to be additionally enhanced--though perhaps to a lesser extent--by conserved residues whose repositioning is predicted to weaken specific polymer-stabilizing hydrogen-bond interactions, as observed for ζ Pro114 (Table 2). While it is difficult to apportion antipolymer activity among residues that change position and those that change identity, the data suggest that the movement of β^s -chain residues, and the substitution of α -like-chain residues, are fundamental to this process.

Our own analyses of deoxyHb $\alpha_2\beta^s_2$ suggest several amino acids at the periphery of the polymer interface that have not previously been recognized as determinants of deoxyHb S fiber stability, but may in fact facilitate polymer assembly (Table 3). Early ultrastructural studies incompletely describe the precise topology of the polymer interface region [8,15,49,57], and do not account for long-range structural perturbations resulting from peripheral amino-acid substitutions [56]. For example, incorporation of variant α Memphis (α Glu23→Gln) or α Anantharaj (α Lys11→Glu) into heterotetrameric Hb S significantly alters the thermodynamics of hemoglobin fiber assembly, despite the fact that neither α Glu23 nor α Lys11 participates in a crystal contact [7,14,22,28,29]. The present study expands the catalogue of polymer determinants to include a lateral hydrogen-bond interaction between the side chains of α 2His50 and β 1Asn80 that is weakened by a non-conservative α His50→ ζ Pro50 substitution; and a long-range axial hydrogen-bond contact between α 2Tyr24 and β 1Glu22 that is abrogated by the α Tyr24→ ζ Ile24 substitution. A full accounting for the antipolymer effect of both of these nonconservative amino-acid changes will require future functional and structural studies.

Finally, our data addresses the possibility that α → ζ exchange impacts the rate of deoxyHb assembly, rather than the stability of the polymer *per se*. As this rate is dependent upon the 30th–50th power of the Hb reactant concentration, the exclusion of even very small quantities of nonreactant T-state Hb $\zeta_2\beta^s_2$ would be expected to have a disproportionately large effect on the rate of deoxyHb S assembly [8]. A related mechanism appears to underlie the antipolymer activity of Hb F ($\alpha_2\gamma_2$), which recombines with Hb S into asymmetrical heterotetramers of the form $\alpha_2\gamma\beta^s$ that are excluded from the polymer, thereby slowing the kinetics of its assembly [33]. The unique crystal packing and high calculated buried SASA value for crystal CO-Hb $\zeta_2\beta^s_2$ suggests that the ζ -substituted Hb S favors a highly stable trimer-of-heterotetramer arrangement in solution, and strongly disfavors its participation in other structures, including double-stranded deoxyHb S polymers. The non-pathological trimeric structure of Hb $\zeta_2\beta^s_2$ arises from changes in amino-acid content and/or position that are effected by α → ζ exchange, and that abolish critical inter-molecular interactions required to stabilize the classical deoxyHb S double-strand polymer. Collectively, these data account for the significant antipolymer activity of ζ -substituted Hb S, and recommend investigation of therapeutic approaches to SCD that are based upon α -globin subunit exchange.

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- Liganded Hb $\zeta_2\beta^s_2$ displays antipolymer properties despite a Tense-like structure
- The pathological $\beta^s\text{Val6}$ is displaced, weakening a critical intermolecular contact
- Four α -chain polymer contact site residues are similarly displaced or substituted
- Liganded Hb $\zeta_2\beta^s_2$ exhibits a unique trimer-of-heterotetramers crystal packing

A

α VLSPADKTNVKAAWGKVGHAAGEYGAELERMFLSFPTTKTYFPHFDLSHGSAQVKGHGK 60
 ζ SLTKTERTIIVSMWAKISTQADTIGTETLERLFLSHPQTKTYFPHFDLHPGSAQLRAHGS 60

 α KVADALTNAVAHVDDMPNALSALSDLHAHKLRVDPVNFKLLSHCLLVTLAAHLPAEFTPA 120
 ζ KVVAAVGDAVKSIIDIGGALSGLSELHAYILRVDPVNFKLLSHCLLVTLAAARFPADFTAE 120

 α VHASLDKFLASVSTVLTSKYR 141
 ζ AHAAWDKFLSVSSVLTEKYR 141

B **C**

Figure 1. Structural comparison of developmentally related α - and ζ -globin subunits
 (A) Primary sequences of human α - and ζ -globin chains. Identical and non-identical amino acids are indicated in red and in black, respectively. (B) Ribbon diagrams of superposed α - and ζ -globin subunits from deoxyHb $\alpha_2\beta^s_2$ (magenta) and CO-Hb $\zeta_2\beta^s_2$ (yellow). Labeled α -chain residues (and corresponding ζ -chain residues, in parentheses) are discussed in detail in the text. (C) Ribbon diagram illustrating the position of β^s_2 Val6 in Hb $\zeta_2\beta^s_2$ (yellow)--relative to its position in Hb $\alpha_2\beta^s_2$ (magenta)--weakening its interaction with a hydrophobic acceptor pocket comprising β_1 Ala70, β_1 Phe85, and β_1 Leu88 (blue). The figure also illustrates a repositioning of β^s_2 Thr4 in Hb $\zeta_2\beta^s_2$, moderating a hydrogen-bond interaction with β_1 Asp73 that is critical for deoxyHb polymer assembly.

Figure 2. Crystal packing of deoxyHb $\alpha_2\beta^S_2$ and CO-Hb $\zeta_2\beta^S_2$
(A) Schematic depiction of CO-Hb $\zeta_2\beta^S_2$ heterotetramers that organize into a lateral trimer (molecules 1–3; magenta, green and grey, respectively). Molecule 3 interacts with an axially located heterotetramer (molecule 4; brown) that is constituent to a separate trimer-of-heterotetramers (molecules 4–6; brown). Molecules 1 and 2 similarly interact with axially located trimers-of-heterotetramers (not shown). The diagram illustrates a novel intermolecular interaction between the pathological $\beta^S\text{Val6}$ (red) and a novel hydrophobic ζ -globin pocket comprising ζTrp14 (yellow), ζAla21 , ζVal63 , and ζAla64 (to preserve

clarity, only $\zeta 2\text{Trp}14$ is shown). The diagram also illustrates a second novel intermolecular contact between $\zeta 2\text{Arg}56$ and three other ζ -globin residues ($\zeta 2\text{Glu}30$, $\zeta 2\text{Pro}50$, and $\zeta 2\text{Gly}51$; to preserve clarity, only $\zeta 2\text{Pro}50$ is shown). (B) Ribbon figure of the crystal packing of CO-Hb $\zeta 2\beta^s$, as depicted and described in panel A. (C) Schematic depiction of the deoxyHb $\alpha 2\beta^s$ molecules that organize into a double-strand fibril (magenta, blue). The diagram illustrates the known interaction between the pathological $\beta^s 2\text{Val}6$ (red) and a hydrophobic pocket comprising $\beta 1\text{Ala}70$, $\beta 1\text{Phe}85$, and $\beta 1\text{Leu}88$ (yellow). The building block of the double strand comprises one axial interaction (between molecules 2 and 3), and two lateral interactions (between molecules 2 and 1; and molecules 3 and 4). (D) Ribbon figure of the crystal packing of deoxyHb $\alpha 2\beta^s$, as depicted and described in panel C. (E) The pathological $\beta^s 2\text{Val}6$ from one molecule of CO-Hb $\zeta 2\beta^s$ (grey) interacts with a novel hydrophobic pocket comprising $\zeta 2\text{Trp}14$, $\zeta 2\text{Ala}21$, $\zeta 2\text{Val}63$, and $\zeta 2\text{Ala}64$ from the $\zeta 2$ subunit of an adjacent molecule (magenta). This interaction is stabilized by a hydrogen-bond contact between $\beta 2\text{Ser}9$ and $\zeta 2\text{Trp}14$; and by a water-mediated interaction between the amide nitrogen of $\beta 2\text{Val}6$ and the amide oxygen of $\zeta 2\text{Ala}64$. (F) The pathological $\beta^s 2\text{Val}6$ from deoxyHb $\alpha 2\beta^s$ in one strand (magenta) interacts with a different hydrophobic pocket formed by $\beta 1\text{Ala}70$, $\beta 1\text{Phe}85$, and $\beta 1\text{Leu}88$ from the $\beta 1$ subunit of a heterotetramer positioned in the adjacent polymeric strand (blue). This interaction is stabilized by a hydrogen-bond contact between $\beta 2\text{Thr}4$ and $\beta 1\text{Asp}73$, as well as by water-mediated interactions (not shown).

Table 1Displacement of corresponding β^S -chain residues in deoxyHb $\alpha_2\beta^S_2$ and CO-Hb $\zeta_2\beta^S_2$

Residue	Proposed Contact ^a	Displacement (Å) ^{b,c}
β Thr4	$\beta 2$ lateral	2.2
β Val6	$\beta 2$ lateral	1.9
β Lys8	$\beta 2$ lateral	1.5
β Trp15	$\beta 1$ axial	0.3
β Gly16	$\beta 1$ axial	<0.2
β Lys17	$\beta 1$ axial	<0.2
β Val18	$\beta 1$ axial	0.7
β Asn19	$\beta 1$ axial	1.4
β Glu22	$\beta 1$ axial	1.0
β Val23	$\beta 1$ axial	<0.2
β Glu43	No contact	0.7
β Lys66	$\beta 1$ lateral	0.4
β Leu68	$\beta 2$ inter-DS	0.8
β Asp73	$\beta 1$ lateral	0.8
β Asn80	$\beta 1$ lateral	0.4
β Gly83	$\beta 1$ lateral	0.7
β Phe85	$\beta 1$ lateral	0.5
β Thr87	$\beta 1$ lateral	0.8
β Leu88	$\beta 1$ lateral	0.7
β Lys95	$\beta 1$ lateral	0.9
β His116	$\beta 2$ axial	<0.2
β His117	$\beta 1$ axial, $\beta 2$ axial	<0.2, 0.4
β Phe118	$\beta 1$ axial, $\beta 2$ axial	0.3, <0.2
β Gly119	$\beta 1$ axial, $\beta 2$ axial	<0.2, <0.2
β Lys120	$\beta 2$ axial	<0.2
β Glu121	$\beta 1$ axial	<0.2
β Glu122	$\beta 2$ axial	<0.2
β Ala142	No contact	<0.2

^aCompiled from references 7, 9, 14, and 22.^bThe rmsd for C α atoms of residues 3–144 is 0.8Å for β^S_1 v β^S_1 , and 0.8Å for β^S_2 v β^S_2 .^cSome residues (e.g., His117) are involved in polymer contacts in both symmetry-related β^S_1 and β^S_2 subunits.

Table 2Displacement of corresponding α - and ζ -chain residues in deoxyHb $\alpha_2\beta^s_2$ and CO-Hb $\zeta_2\beta^s_2$

Residue	Proposed Contacts ^a	Corresponding ζ -globin Residue	Displacement ($\text{\AA}$) ^{b,c}
α Asp6	$\alpha 1$ inter-DS	Glu	0.8
α Lys11	No contact	Val	0.2
α Lys16	$\alpha 2$ axial	Lys	1.0
α His20	$\alpha 2$ axial	Gln	0.9
α Glu23	No contact	Thr	1.3
α Asp47	$\alpha 1$ inter-DS, $\alpha 2$ inter-DS	Asp	0.9, 0.8
α Ser49	$\alpha 2$ lateral	His	1.7
α Gln54	$\alpha 1$ inter-DS, $\alpha 2$ inter-DS	Gln	<0.2, <0.2
α Asn68	$\alpha 1$ inter-DS, $\alpha 2$ inter-DS	Asp	0.7, 0.7
α Asp75	$\alpha 1$ inter-DS, $\alpha 2$ inter-DS	Asp	0.2, 0.5
α Asn78	$\alpha 1$ inter-DS, $\alpha 2$ inter-DS	Gly	0.8, 0.6
α Leu113	$\alpha 2$ axial	Phe	<0.2
α Pro114	$\alpha 1$ axial, $\alpha 2$ axial	Pro	1.2, 1.3
α Ala115	$\alpha 1$ axial, $\alpha 2$ axial	Ala	0.9, 1.1
α Glu116	$\alpha 2$ axial	Asp	<0.2

^aCompiled from references 7, 9, 14, and 22.^bThe rmsd for Ca atoms of residues 3–138 is 0.8 $\text{\AA}$ for $\alpha 1$ v $\zeta 1$, and 0.8 $\text{\AA}$ for $\alpha 2$ v $\zeta 2$.^cSome residues (e.g., Asp47) are involved in polymer contacts in both symmetry-related $\alpha 1$ and $\alpha 2$ subunits.

Table 3

Characteristics of corresponding α - and ζ -chain surface residues that neighbor known polymer contact sites

DeoxyHb $\alpha_2\beta_2$	Proposed Contact Site	CO-Hb $\zeta_2\beta_2$	Displacement (Å) ^a
α Ala12	$\alpha 2$ axial	Ser	<0.2
α Gly18	$\alpha 2$ axial	Ser	1.7
α Ala19	$\alpha 2$ axial	Thr	1.9
α Gly22	$\alpha 2$ axial	Asp	1.9
α Glu23	$\alpha 2$ axial	Thr	1.3
α Tyr24	$\alpha 2$ axial	Ile	<0.2
α Ala26	$\alpha 2$ axial	His	1.0
α His50	$\alpha 2$ lateral	Pro	2.1
α Ala71	$\alpha 1$ inter-DS	Lys	<0.2
α His72	$\alpha 1$ inter-DS	Ser	0.2

^aThe rmsd for C α atoms of residues 3–138 is 0.8Å for $\alpha 1$ v $\zeta 1$, and 0.8Å for $\alpha 2$ v $\zeta 2$.

Table 4Intermolecular lateral interactions between CO-Hb $\zeta_2\beta^S_2$ heterotetramers^a

Molecule 1	Molecule 2	Molecule3	Distance (Å)
β_2 Val6 (CG2)	ζ_2 Trp14 (CZ2)		3.5
β_2 Val6 (CG1)	ζ_2 Ala21 (CB)		4.0
β_2 Val6 (CG2)	ζ_2 Ala63 (CG1)		4.9
β_2 Val6 (CG2)	ζ_2 Val64 (CA)		3.9
β_2 Ser9 (OG)	ζ_2 Trp14 (NE1)		2.9
β_2 Ser9 (O)	ζ_2 Ser18 (OG)		3.5
β_2 Ala10 (N)	ζ_2 Ser18 (OG)		3.2
ζ_2 Glu30 (OE2)	ζ_2 Arg56 (NH2)		3.8
ζ_2 Pro50 (O)	ζ_2 Arg56 (NH1)		3.9
ζ_2 Gly51 (N)	ζ_2 Arg56 (NH1)		3.6
	β_2 Val6 (CG2)	ζ_2 Trp14 (CZ2)	3.7
	β_2 Val6 (CG1)	ζ_2 Ala21 (CB)	3.8
	β_2 Val6 (CG2)	ζ_2 Ala63 (CG1)	5.0
	β_2 Val6 (CG2)	ζ_2 Val64 (CA)	3.8
	β_2 Ser9 (O)	ζ_2 Ser18 (OG)	3.4
	β_2 Ser9 (OG)	ζ_2 Trp14 (NE1)	2.9
	β_2 Ala10 (N)	ζ_2 Ser18 (OG)	3.2
	ζ_2 Glu30 (OE2)	ζ_2 Arg56 (NH1)	4.9
	ζ_2 Pro50 (O)	ζ_2 Arg56 (NE)	3.6
	ζ_2 Gly51 (N)	ζ_2 Arg56 (NH1)	3.7

^a Selected interactions. Symmetrical interactions exist between molecules 2 and 3.

Table 5Intermolecular axial interactions between CO-Hb $\zeta_2\beta^S_2$ heterotetramers^a

Molecule 1	Molecule 2	Distance (Å)
ζ_1 Tyr89 (OH)	β_1 Asp47 (N)	3.6
ζ_1 Tyr89 (OH)	β_1 Ala53 (O)	3.2
ζ_1 Arg92 (NH1)	β_1 Asp47 (OD1)	3.6
ζ_2 Thr5 (OG1)	ζ_1 Thr5 (OG1)	3.5
ζ_2 Thr5 (OG1)	ζ_1 Glu120 (OE1)	3.4
ζ_2 Thr8 (OG1)	ζ_1 Thr5 (OG1)	3.4
ζ_2 Ser12 (OG)	ζ_1 Lys4 (OG1)	3.8
β_2 Arg40 (NH2)	β_1 Asp43 (O)	3.6
β_2 Arg40 (NH2)	β_1 Gly46 (N)	3.4
β_2 Arg40 (NH2)	β_1 Gly46 (O)	3.2
β_2 Glu43 (OE2)	β_1 Glu43 (OE1)	2.8
β_2 Gly46 (O)	β_1 Arg40 (NH2)	3.6
β_2 Asp47 (N)	ζ_2 Tyr89 (O)	2.8
β_2 Asp47 (OD2)	β_1 Trp37 (NE1)	3.3

^a Selected interactions.