Polymerization of Recombinant Hb S-Kempsey (Deoxy-R State) and Hb S-Kansas (Oxy-T State)*

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In order to investigate the role of the R (relaxed) to T (tense) structural transition in facilitating polymerization of deoxy-Hb S, we have engineered and expressed two Hb S variants which destabilize either T state (Hb S-Kempsey, aβ2 Val-6, Asn-99) or R state structures (Hb S-Kansas, aβ2 Val-6, Thr-102). Polymerization of deoxy-Hb S-Kempsey, which shows high oxygen affinity and increased dimer dissociation, required about 2- and 6-fold higher hemoglobin concentrations than deoxy-Hb S for polymerization in low and high phosphate concentrations, and its kinetic pattern of polymerization was biphasic. In contrast, oxy- or CO Hb S-Kansas, which shows low oxygen affinity and increased dimer dissociation, polymerized at a slightly higher critical concentration than that required for polymerization of deoxy-Hb S in both low and high phosphate buffers. Polymerization of oxy- and CO Hb S-Kansas was linear and showed no delay time, which is similar to oversaturated oxy- or CO Hb S. These results suggest that nuclei formation, which occurs during the delay time prior to deoxy-Hb S polymerization, does not occur in T state oxy-Hb S-Kansas, even though the critical concentration for polymerization of T state oxy-Hb S-Kansas is similar to that of T state deoxy-Hb S.

Oxygen binding has a dramatic effect on Hb S polymerization. Deoxygenated Hb S forms gels, while oxygenated Hb S does not (1). The reason for this difference between oxy and deoxy forms of Hb S is the marked change in quaternary conformation of the hemoglobin molecule upon deoxygenation (2, 3). When normal hemoglobin is fully deoxygenated, most of the molecules assume the T structure, which has a relatively low affinity for oxygen and other heme ligands. Conversely, normal oxyhemoglobin exists almost exclusively in the R conformation and has a relatively high affinity for heme ligands such as oxygen. Structural alterations that affect the equilibrium between T and R states are expected to have a marked effect on hemoglobin function as well as Hb S polymerization. X-ray crystallographic studies show that hemoglobin can assume two different quaternary structures: one characteristic of unliganded (T) and the other of liganded (R) hemoglobin (2).

The key to Hb S polymerization is the presence of Val-6 and the formation of a hydrophobic acceptor pocket between E and F helices that occurs in the T state but is absent in the R state (3, 4). However, if a specific amino acid substitution decreases stability of the T structure, then transition to the R state is favored and the molecule exhibits increased oxygen affinity and decreased heme-heme interaction. This has been demonstrated for a number of chemically modified hemoglobins as well as for many hemoglobin variants (3). Hb Kempsey (aβ2 Asn-99) and Hb Kansas (aβ2 Thr-102) are well studied examples (3, 5–17). Hb Kempsey is a δ-chain variant in which Asp-β2 is replaced by Asn (5). In deoxy-Hb A, Asp-β99 normally forms an important hydrogen bond with Tyr-α2 at the aβ interface (6). Upon oxygenation, the two subunits shift in a dovetail fashion, so that the β99-α2 hydrogen bond is broken and another one forms between Asp-β94 and Asn-β102. Hb Kempsey, substitution of Asp with Asn at β99 prevents formation of the former hydrogen bond with Tyr-α2 and therefore decreases stability of ”deoxy” or T state structures. Thus, when this tetramer is fully deoxygenated, it remains partially in the R state.

In contrast, Hb Kansas is a δ-chain variant in which Asn-β102 is replaced by Thr (14). This position is also an important site at the aβ interface, which is involved in the transition between the oxy and deoxy conformation of hemoglobin. Hb Kansas has a low oxygen affinity because the hydrogen bond that usually occurs between Asn-β102 and Asp-α94 in normal Hb A, which functions to stabilize the oxy conformation, is absent, thus shifting the equilibrium toward the deoxy (T) conformation (3, 12, 13, 18).

Asp-β99 and Asn-β102 in Hb S are located internally at the aβ interface and are not exposed to the surface of tetrameric hemoglobin. These positions are involved in a-b interactions, which lead to formation of tetramers, and are not defined as interaction sites in Hb S polymers. These amino acids do affect the R to T quaternary structural transition of hemoglobin tetramers. In this report, we have studied polymerization of R state deoxy-Hb S and T state oxy or CO Hb S by preparing Hb S-Kempsey (aβ2 Val-6, Asn-99) and Hb S-Kansas (aβ2 Val-6, Thr-102) using a yeast expression system (19) in an attempt to clarify the role that quaternary structural changes associated with T to R state transition play in polymerization of Hb S.

MATERIALS AND METHODS

The plasmid pGS389 Hb S contains the full-length human a- and δ-globin cDNAs under transcriptional control of dual GGAP promoters as well as a partially functional yeast LEU2 gene and the URA3 gene for selection in yeast (19, 20). The plasmid pGS189 δ contains a single GGAP promoter and δ-globin cDNA and was constructed by mutagenesis and subcloning as described previously (19). The basic strategy for site-specific mutagenesis at β99 or β102 involves recombination polymerase chain reaction as described previously (19). Asn-β99 and Thr-
Electrophoretic mobilities of Hb S D β99N and Hb S Np102T. Electrophoretic mobilities of Hb S D β99N (lane 4) and Hb S Np102T (lane 5) were compared with native Hb A (lane 1), Hb S (lane 2), and Hb C (lane 3) following cellulose acetate electrophoresis.

RESULTS

Characterization of Recombinant Hb S Variants Containing Asn-β99 or Thr-β102—Purified recombinant Hb S variants containing Asn-β99 or Thr-β102 (Hb S D β99N and Hb S Np102T, respectively) migrated as single bands following cellulose acetate electrophoresis at pH 8.6 with mobilities intermediate between Hb S and Hb C (Fig. 1). The β99 Hb S variant demonstrated a higher positive surface charge than the β102 Hb S variant and migrated closer to Hb C. It is interesting to note that isoelectric focusing of a recently reported recombinant Hb A variant containing Lys-β99 made using the same yeast expression system showed two bands (26), while both β99 and β102 Hb S variants in our study migrated as single bands on isoelectric focusing. The reason for the existence of two electrophoretic forms for the Hb A variant containing Lys-β99 is not known (26).

Mass spectral analysis of Hb S variants containing Asn-β99 or Thr-β102 showed the expected β-globin chain molecular masses, 15,835.1 and 15,823.8 Da, respectively, which corresponded to expected masses of 15,836 and 15,824 Da, respectively. Absorption spectra of these two Hb S variants were the same as those of native and recombinant Hb S (19). Circular dichroism spectra in the region from 190 to 290 nm for the Hb S variants were also similar to that of native Hb S (Fig. 2), indicating that these substitutions do not significantly affect globin folding and/or all secondary structure of hemoglobin tetramers.

It is known that Hb Kempsey and Hb Kansas have higher and lower oxygen affinity than normal Hb A, respectively (6, 12). Oxygen affinities of Hb S D β99N and Hb S Np102T differed from Hb S as anticipated; the former had higher and the latter lower oxygen affinity than Hb S or Hb A (Fig. 3). P50 values in 0.1 M phosphate buffer, pH 7.0, at 20°C for the Asn-β99 and Thr-β102 Hb S variants were 0.8 and 18, respectively, compared with 6.5 for recombinant Hb S (19). Hill coefficients for both variants were reduced to 1.2 and 1.5 for Hb S D β99N and Hb S Np102T, respectively, compared with 2.6 for Hb S (Table I).

Differences in oxygen affinity for Hb S D β99N and Hb S Np102T compared with Hb S should be correlated with differences in kinetics of dimer formation as has been shown for deoxy-Hb Kempsey and oxy-Hb Kansas (6, 12). Tetramer-dimer dissociation properties of the two Hb S variants were measured by monitoring quenching of haptoglobin fluorescence caused by existing hemoglobin dimers (Table II). Deoxy-Hb S Np102T showed similar dimer formation properties to that of deoxy-Hb S, while deoxy-Hb S D β99N exhibited increased dimer formation. In contrast, oxy-Hb S Np102T showed a slightly increased tendency, while oxy-Hb S D β99N showed a similar tendency for dimer formation as oxy-Hb S.

Polymerization Properties of Recombinant Hb S Variants Containing Asn-β99 or Thr-β102—Polymerization properties of the deoxy forms of the two Hb S variants were studied in...
Polymerization of Hb S in Deoxy-R and Oxy-T States

TABLE I
Oxygen binding properties of Hb S Dj99N and Hb S Nj102T

| Hb S         | Hb S Dj99N | Hb S Nj102T |
|--------------|------------|-------------|
| P50 (mM)     | 6.9        | 0.8         | 18          |
| nmax (%)     | 2.6        | 1.2         | 1.5         |

TABLE II
Quenching of haptoglobin fluorescence by Hb S Dj99N and Hb S Nj102T dimers

| Fluorescence quenching | Hb S | Hb S Dj99N | Hb S Nj102T |
|------------------------|------|------------|-------------|
| Deoxy                  | 10   | 43         | 12          |
| Oxy                    | 43   | 44         | 53          |

vitro by the temperature jump method employing 1.8 M phosphate buffer, pH 7.4, at 30 °C (23). Polymerization of deoxy-Hb S is characterized by a delay time prior to polymer formation whose length depends on hemoglobin concentration: the lower the concentration, the longer the delay time (23). Deoxy (T) structure of Hb Kempsey (α2β2Asn-99) is destabilized by the Asn for Asp substitution at Dj99, which results in Hb Kempsey remaining partially in the R state upon deoxygenation (3, 6). Deoxy-Hb S Dj99N was deoxygenated in the presence of more than 100-fold excess Na2S2O4 after exposure to nitrogen (23). The optical absorption spectrum of deoxygenated Hb S Dj99N was characteristic of hemoglobin in the deoxy form. Deoxy-Hb S Dj99N required about a 6-fold higher hemoglobin concentration than deoxy-Hb S for polymerization. At higher concentrations (more than 10-fold that of deoxy-Hb S), deoxy-Hb S Dj99N polymerization occurred without a delay time by a linear and not a nucleation-controlled mechanism (Fig. 4). At lower hemoglobin concentrations (<330 mg/dl), kinetics of polymerization of deoxy-Hb S Dj99N were biphasic, occurred without a delay time, reached a plateau, and then continued following sigmoidal kinetics (Fig. 4). Furthermore, the time required to reach the plateau of the second phase of polymer formation was longer than that of the first. Logarithmic plots of the initiation time for the second phase of polymerization for deoxy-Hb S Dj99N versus hemoglobin concentration showed a straight line (Fig. 5), and polymerization was reversed after exposure to CO or by lowering the temperature to 0 °C. We also performed gelation studies of deoxy-Hb S Dj99N in 0.1 M phosphate buffer in order to assess polymerization properties near more physiological conditions. Under these conditions, deoxy-Hb S Dj99N required about a 2-fold higher (47 g/dl) concentration than deoxy-Hb S for polymerization (Table III).

In contrast to Hb Kempsey, the deoxy form of Hb S Nj102T (Hb S-Kansas) polymerized like deoxy-Hb S (Fig. 6); however, a small amount of detectable polymers formed prior to the major phase of polymerization. Logarithmic plots of delay time versus hemoglobin concentration for the major phase of deoxy-Hb S Nj102T polymerization showed a straight line slightly shifted to the right of the line for deoxy-Hb S (Fig. 5). It is known that the oxy structure is destabilized in Hb Kansas, which favors the low oxygen affinity T (deoxygen) state (3, 12). Polymerization of liganded oxy- and CO-Hb S-Kansas occurred at a slightly higher concentration than that required for deoxy-Hb S polymerization; however, polymer formation was not accompanied by a delay time prior to polymerization, and oxy-Hb S Nj102T polymerized linearly like oxy- and CO-Hb S (27) (Fig. 7). A hemoglobin concentration 20 times less than that for oxy-Hb S was, however, required to initiate polymerization. The CO form of Hb S Nj102T also polymerized linearly like the oxy form (27). Polymerization of oxy, CO, and deoxy-Hb S Nj102T was reversed by decreasing the temperature to 0 °C; however, when deoxy-Hb S Nj102T polymers were exposed to CO, about 60% of the polymers depolymerized (Fig. 8) in contrast to complete depolymerization for deoxy-Hb S polymers (19). It is noteworthy that polymerization of oxy-Hb S Nj102T in 1.8 M phosphate buffer was not affected by addition of inositol hexaphosphate, even though organic phosphates are known to stabilize the T state of liganded Hb Kansas (18).

We also evaluated gelation and polymerization of CO Hb S Nj102T at room temperature in low phosphate concentration (0.1 M) buffer, pH 7.0, under more physiological conditions. CO Hb S Nj102T formed gels at a concentration of 25.7 g/dl compared with 24 g/dl for deoxy-Hb S and 24.5 g/dl for deoxy-Hb S Nj102T (Table I). CO Hb S Nj102T polymerized linearly without a delay time in 0.1 M phosphate buffer, pH 7.0, at 30 °C (Fig. 9), which is similar to results in 1.8 M phosphate buffer (Fig. 7). Solubility after polymerization of CO Hb S Nj102T in 0.1 M phosphate buffer, pH 7.0, at 30 °C was 19.2 versus 17 g/dl for deoxy-Hb S. Depolymerization of CO Hb S Nj102T in low phosphate buffer was also observed upon lowering the temperature to 0 °C.

Total polymer formed in 1.8 M phosphate buffer as a function of hemoglobin concentration was also determined in order to...
evaluate effects of the Asn-β99 and Thr-β102 substitutions on the critical concentrations required for polymerization. Critical concentration depends on deoxyhemoglobin solubility: the higher the solubility, the higher the concentration required for polymerization. Polymer formation for the two variants increased linearly with increases in initial hemoglobin concentration (Fig. 10). Critical concentration for polymer formation was then determined by extrapolation of the lines to zero turbidity (19). Values for deoxy-Hb SNb102T, CO Hb SNb102T, and deoxy-Hb SDβ99N were 1.2-, 1.6-, and 6.3-fold higher, respectively, than that for deoxy-Hb S.

**DISCUSSION**

The functional properties of normal Hb A and polymerization of Hb S depend on transition of their three-dimensional conformation, which accompanies the addition and removal of oxygen. X-ray crystallographic studies show that hemoglobin tetramers exist in equilibrium between two quaternary conformations: R and T (2, 11). The change from T to the R conformation involves a well defined series of structural changes, including rupture of the salt bridges that stabilize the T conformation and rotation of the β-chains relative to the α-chains (3, 11). Intramolecular “movements” also occur during conformational isomerization at the αβ1 interface. Structural alterations that affect the equilibrium between R and T states are expected to have marked effects on hemoglobin function and Hb S polymerization. Thus, if a specific amino acid substitution decreases T structure stability, then transition to the R state would be expected to occur at an earlier stage in ligation, and this hemoglobin would exhibit increased oxygen affinity and decreased heme-heme interaction. In contrast, substitutions that decrease R structure stability result in tetramers with decreased oxygen affinity and increased heme-heme interaction.

These results have been observed for a number of hemoglobin variants. Both Hb Kempsey and Hb Kansas, which have amino acid substitutions at the αβ1 interface, are well studied examples (5–18). In most respects, deoxy-Hb Kempsey with high oxygen affinity bears more resemblance to oxyhemoglobin A than to deoxyhemoglobin A and is partially in the R conformation (6–8). Recombinant Hb S-Kempsey exhibited high oxygen affinity with low cooperativity. Polymerization of deoxy-Hb S-Kempsey occurred at higher concentrations than...
These results suggest that Hb S-Kempsey is not completely linear as well as nucleation-controlled mechanisms (19, 27). Concentration. Turbidity at the plateau of the polymerization curves in 1.8 mM phosphate buffer at various hemoglobin concentrations was measured at 700 nm. □, ■, and □ refer to native deoxy-Hb S, deoxy-Hb S Dj99N, deoxy-Hb S Nβ102T, and oxy-Hb S Nβ102T, respectively.

deoxy-Hb S, and kinetic studies suggested polymerization by linear as well as nucleation-controlled mechanisms (19, 27). These results suggest that Hb S-Kempsey is not completely converted to the R state upon deoxygenation. Asp-β99 is located in the interior of Hb S, but yet the change from Asp-β99 to Asn in Hb S-Kempsey inhibits polymerization, no doubt by affecting the quaternary structure. These results indicate that inhibition and/or acceleration of polymerization of Hb S is not always controlled by residues at direct interaction sites of deoxy-Hb S polymers. We are now attempting to define effects of this substitution at the allosteric interface of Hb S on polymer and crystal structure.

Hb Kansas contains Thr instead of Asp at β102, which is also a site at the α3β2 interface (3, 12). However, in contrast to Hb Kempsey, Hb Kansas has decreased oxygen affinity, which is due in part to a relatively unstable R structure, thereby shifting the equilibrium to that of the T structure. Our results with Hb S-Kansas showed low oxygen affinity, and liganded forms had decreased solubility, therefore facilitating polymerization much like deoxy-Hb S. The critical concentrations for polymerization of liganded forms of Hb S-Kansas were similar to the deoxy form of Hb S-Kansas. Facilitation of polymerization of liganded hemoglobin can be explained by shifting conformational equilibrium to favor the T state like Hb Kansas. However, the kinetics of polymerization of liganded Hb S-Kansas were different from those of deoxy-Hb S and deoxy-Hb S-Kansas, which were accompanied with a delay time prior to polymerization. These results suggest that the surface structure of liganded Hb S-Kansas resembles that of the T structure of Hb A or Hb S. The lack of nucleation during polymerization of oxy or CO Hb S-Kansas, even though the T-state is favored, may be caused by insufficient protein-protein interactions that are required to form deoxy-Hb S or deoxy-Hb S-Kansas nuclei.

There are seven known natural hemoglobin variants with substitutions for Asp-β99 (5, 6, 26, 28–34). Recently, recombinant Hb A containing Asp-β99 → Lys and a double mutant (Asp-β99 → Asn and Tyr-α42 → Asp), which were produced in yeast and Escherichia coli expression systems, respectively, were characterized (26, 35). All these variants show increased oxygen affinity with reduced cooperativity. There are also three known natural hemoglobin variants with substitutions at β102, and recombinant Hb A Djβ102A made in yeast was also recently reported (36–38). These variants show decreased oxygen affinity with reduced cooperativity. Functional properties of hemoglobin variants with substitutions at β99 and β102, which involve the α3β2 contact, have been attributed to increased dissociation of the variant tetrameric hemoglobin dimers. Deoxy-Hb S-Kempsey and oxy-Hb S-Kansas show increased dissociation to dimers like Hb Kempsey and Hb Kansas (6, 12). Recent studies of recombinant Hb A Dj99N and Hb A Nβ102A indicated that dimerization properties of these variants depend on hemoglobin concentration: the higher the concentration, the lower the tetramer-dimer dissociation constant of hemoglobin (26, 38). Although polymerization properties of dimeric forms of Hb S are not known, the effect of dimerization on polymerization of Hb S-Kansas and Hb S-Kempsey should be negligible, since hemoglobin concentrations used for polymerization in both high and low phosphate buffers (50–100 μM and 1.5–6 mM, respectively) are too high to favor dimer formation. Polymerization properties of oxy- or CO Hb S-Kansas and deoxy-Hb S-Kempsey in low and high phosphate buffers can therefore be attributed to the quaternary and tertiary structural differences from deoxy-Hb S rather than dimerization differences. It is interesting to note that deoxy-Hb Kansas crystallizes like deoxy-Hb A and that exposure of these crystals to CO results in formation of two new crystal forms that are not identical to crystals formed upon deoxygenation (15). In contrast, deoxy-Hb A crystals dissolve upon exposure to CO. These results are similar to those of exposure of deoxy-Hb S-Kansas polymers to CO. Polymers of CO Hb S-Kansas may also remain in the T state conformation, which may not favor CO-induced depolymerization. Structural analyses of these Hb S variants are now required to further our understanding of the relationship between quaternary structure, hemoglobin function, and polymerization of Hb S.

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