Conformational Changes in Hemoglobin S (βE6V) Imposed by Mutation of the βGlu7–βLys132 Salt Bridge and Detected by UV Resonance Raman Spectroscopy*

Laura J. Juszczyk†§§, Christophe Fabel‡¶¶, Veronique Baudín-Creuzat¶¶, Sophie Lesecq-Le Gall§§, Rhoda Elison Hirsch*††, Ronald L. Nagel‡‡, Joel M. Friedman‡§§, and Joséé Pagnier¶¶

From the *Department of Physiology and Biophysics, Albert Einstein College of Medicine, Bronx, New York 10461, the ††Department of Anatomy and Structural Biology, Albert Einstein College of Medicine, Bronx, New York 10461, and the ¶¶INSERM, Unité 473, 84 rue du Général Leclerc, 94276 Le Kremlin-Bicêtre, France

The impact upon molecular structure of an additional point mutation adjacent to the existing E6V mutation in sickle cell hemoglobin was probed spectroscopically. The UV resonance Raman results show that the conformational consequences of mutating the salt bridge pair, βGlu7–βLys132, are dependent on which residue of the pair is modified. The βK132A mutants exhibit the spectroscopic signatures of the R → T state transition in both the “hinge” and “switch” regions of the α,β2 interface. Both singly and doubly mutated hemoglobin (Hb) βE7A exhibits the switch region signature for the R → T quaternary state transition but not the hinge signature. The absence of this hinge region-associated quaternary change is the likely origin of the observed increased oxygen binding affinity for the Hb βE7A mutants. The observed large decrease in the W3 α1β15 band intensity for doubly mutated Hb βE7A is attributed to an enhanced separation in the A helix-E helix tertiary contact of the β subunits. The results for the Hb A βGlu7–βLys132 salt bridge mutants demonstrate that attaining the T state conformation at the hinge region of the α,β2 dimer interface can be achieved through different intraglobin pathways; these pathways are subject to subtle mutagenic manipulation at sites well removed from the dimer interface.

Sickle cell hemoglobin (Hb S, β6(A3) Glu → Val) exhibits the property of anomalous and pathologic self-assembly. DeoxyHb S forms polymers in the erythrocyte, which leads to microvascular blockage, organ damage, and often premature death. Structure-based drug design requires knowledge of optimal polymer disruption sites. The specific interaction in the Hb S polymer involves the steric fit of the mutated hydrophobic β6 donor site in a hydrophobic acceptor site located in an adjacent Hb S tetramer. It is well known that the hydrophobicity and the stereospecificity of the donor site are essential to the initiation of the polymerization.

Lesecq et al. (1, 2) have been investigating whether modification of the polarity close to the β6 site could influence the packing of the donor and acceptor sites, thus modifying the polymerization process. Replacing the hydrophilic Glu β7(A4) residue with a hydrophobic Ala residue resulted in a decreased polymerization of the doubly mutated rHb β6V/β7A. It was postulated (1, 2) that the loss of the normal salt bridge between βGlu7(A4) and βLys132(H10) in the rHb β6V/β7A mutants might lead to an alteration in both the position and the mobility of the A helix, illustrated in Fig. 1. These alterations of the A helix might result in a misfit between the donor and acceptor sites, which could explain the observed diminution in polymerization. It follows from this hypothesis that modifying the other partner of the salt bridge, βLys132(H10), should have similar consequences on polymerization (2).

Visible resonance Raman spectroscopy is very useful in providing detailed information relating to the influence of tertiary and quaternary structure upon specific heme-related vibrational degrees of freedom (3–7). Additionally, UV resonance Raman spectroscopy (UVR) provides information about vibrational modes of aromatic residues within the globin. UVR studies of hemoglobin from several research groups have shown a consistent pattern of tertiary and quaternary structure shifts coupled to spectral changes in specific tyrosine and tryptophan bands (8–17). In particular and most significantly, spectral features have been clearly identified that reflect the key determinants of the quaternary state, specifically in the “hinge” (βTrp37) and “switch” (αTyr45) regions of the α,β2 interface. Band intensities also respond to the packing of the A helix against the E helix and to the integrity of the salt bridge-derived scaffolding that maintains interhelical separations, a feature that is indicative of different tertiary structures within a given quaternary structure. Thus, the UVR technique allows us to couple modifications of both local and global elements of structure with observed functional changes. For example...
While Hb C (β6(A3) Glu → Lys) is yet another naturally occurring mutant of Hb A, which forms crystals in erythrocytes, UVRR spectroscopy, in conjunction with other spectroscopic techniques, has been used to show that the effect of the β6 mutation is communicated to both the A helices and the central cavity where effectors such as inositol hexaphosphate (IHP) bind (18–20).

In this study, UVRR spectroscopy is utilized to probe the conformational consequences of disrupting the salt bridge between βGlu7(A4) and βLys132(H10) through examination of the tryptophan W3 and tyrosine Y8 bands, which are reporter bands for Hb tertiary and quaternary structure. The results for singly and doubly mutated recombinant Hbs βE6V, βE7A, βE6V/E7A, and βE6V/K132A are compared with those for both wild type and recombinant Hb A (Hb A and rHb A, respectively). The deoxygenated and CO derivatives of each Hb species are examined, as is the CO derivative in the presence of IHP. The UVRR results reveal the impact of the βGlu7–βLys132 salt bridge mutations on the positioning of the A helix and on the functioning of the quaternary β37 hinge, which suggest a molecular explanation for the macroscopic changes in polymerization and ligand binding for these mutants.

MATERIALS AND METHODS

Preparation of the Recombinant Hbs—The βE6V, βE7A, and βK132A mutations were introduced into the β-globin cDNA by site-directed mutagenesis using synthetic primers (Genset, France). The mutated β-globin subunits were produced as fusion proteins in Escherichia coli, using the expression vector pATPrclIFXβ-globin (21). After extraction and purification, the fusion proteins were cleaved by digestion with bovine coagulation factor Xa (22). The presence of the mutation(s) was confirmed by reverse-phase high performance liquid chromatography of the tryptic digests and amino acid analysis of the abnormal peptides. The purified β-subunits were folded in the presence of cyanohemins and the partner α-subunits prepared from wild type Hb A, to form the tetrameric Hb $\alpha_2\beta_2$ (21, 23). The folded recombinant tetrameric Hbs were purified by preparative isoelectrofocusing on Ultrodex dextran gel using Ampholine (Amersham Biosciences, Uppsala, Sweden). Electrophoretic studies included electrophoresis on cellulose acetate and analytical isoelectrofocusing of the recombinant Hbs.

UVRR Spectroscopy—The Hb samples were all at a concentration of 0.5 mM heme in 50 mM Hepes at pH 7.35. 0.4 M sodium selenate was added as an internal standard, yielding a UVRR band at 834 cm$^{-1}$. Where applicable, the IHP was 0.75 mM or six times the Hb tetrameric concentration. The data were collected on samples chilled to 10 ± 4 °C to minimize photodamage. An argon laser system, described elsewhere (20), was used to generate the excitation wavelength of 228.9 nm with an incident laser power of 1.8 mW. Four 3-min acquisitions were averaged; the error bars are thus not shown in Fig. 4 for these species. The data frequency scale was calibrated with indene and toluene and is accurate to ± 1 cm$^{-1}$. The issue of spectral reproducibility was addressed in the following manner: 1) The absorption spectra were collected before and after exposure to the UV laser beam. 2) If absorption changes were noted, the sequential UVRR acquisitions were examined collected before and after exposure to the UV laser beam. 3) If absorption changes were rejected and not included in the final UVRR average spectra. That is, separate acquisitions that were consistent in peak intensity and frequency as well as band shape were included in the averaged spectra. Spectral data were truncated to a 1530–1650 cm$^{-1}$ frequency window, and the intensity was normalized at the W3 band ($\sim$1558 cm$^{-1}$) for each set of spectra in Figs. 2 and 3. The software...
program Grama/32 AI, version 6.00 (Galactic Industries Corp., Salem, NH) was used to determine the Y8α and W3α14β15 peak heights used in Table I (full width at half-maximum) using the subroutine, Peakstat, and for curve fits to the W3 band, from which band heights for the β37 shoulder at ~1548 cm⁻¹ were determined. All W3 bands were fit to two curves of 0.7 Lorentzian/0.3 Gaussian band shape as the vibrational signature for the α14 and β15 Trps is coincidental and cannot be resolved; the second curve is for the β37 Trp. All of the band intensities given in Table I and Fig. 4 were normalized against the selenate 834 cm⁻¹ peak. The numerical errors listed in Table I and the error bars shown in Fig. 4 were determined from the normalized peak intensities of the independent component acquisitions used for each averaged UVRR spectrum as measured by the aforementioned Peakstat subroutine. Common spectral processing techniques include smoothing to improve the signal-to-noise ratio (24); spectral smoothing, however, was not employed here.

RESULTS AND DISCUSSION

Hb A

The changes in Hb globin structure that accompany ligation at the heme can be followed by UVRR spectroscopy because of the critical sites occupied by several of the UV-resonating Trp and Tyr residues. Two of these are highly responsive to quaternary structural changes: βTrp37 and αTyr42 are located in the hinge and switch regions of the α1β2 dimer interface, respectively. In addition, there is a tryptophan on the A helix of both the α (α14) and β (β15) subunits that provides a UVRR signature for the status of the packing distance between the A and E helices (25). For human wild type Hb A, the change in ferrous heme ligation state from fully ligated to fully deligated (deoxy) is accompanied by the R → T state quaternary structure transition. The conformation-sensitive W3 and Y8α reporter bands.

The intensity for the W3 band (Fig. 4a and Table I), whereas the Y8α band intensity increases for ligated wild type Hb A only (Fig. 4b and Table I). The W3 β37 band intensity increase (0.43 and 0.44, respectively; Table I), and the W3 β37 band intensity increase (0.43 and 0.44, respectively; Table I) associated with the R → T state transition, as discussed above. The spectral difference between the W3 β37 bands for the two hemoglobin ligation states is 1 order of magnitude above the noise level of the constituent spectral acquisitions. For both species of Hb A, ligation results in an increase of intensity for the W3 α14β15 band (Fig. 4a and Table I), whereas the Y8α band intensity increases for ligated wild type Hb A only (Fig. 4b and Table I). Addition of a 5-fold excess of the effector, IHP, to ligated forms of both Hb As results in an enhancement of the W3 β37 band intensity, which is indicative of a more T state-like hinge region, but apparently has little or no effect on the Y8α band position, which remains R state like (Fig. 2, top panel, sets a and b, and Table I). The addition of IHP has a large enhancement effect on the Y8α band intensities of both ligated Hb As (Fig. 4b and Table I) and on the W3 α14β15 band intensity of wild type Hb A (Fig. 4a and Table I).

Hb S (βE6V)

The upshift in the Y8 band frequency and the W3 β37 band intensity increase accompanying the R → T state transition for both Hb As are also found in the UVRR results for wild type Hb S and rHb S (Fig. 2, bottom panel, sets a and b, respectively,
The UVRR results for rHb βE6V/E7A following the R → T state transition are similar to those for rHb βE7A; the Y8a frequency upshift occurs, but the W3 β37 band intensity increase does not (Fig. 3, top panel, set b, and Table I). This double mutation results in the lowest deoxy W3 α14β15 and Y8a band intensities observed for this set of Hbs (Fig. 4). Ligation substantially increases the intensities of both bands (Fig. 4 and Table I). Addition of IHP to the ligated species has a small effect on the intensity of the W3 α14β15 band (Fig. 4a) but results in a substantially enhanced W3 β37 band intensity (Fig. 3, top panel, set b, and Table I). The intensity of the Y8a band is minimally influenced by the addition of IHP to CO rHb βE6V/E7A (Fig. 4b and Table I).

rHb βK132A

The K132A mutation did not affect the Y8 band shift associated with the R → T state transition (Fig. 3, bottom panel, set a, and Table I). The W3 β37 band intensity increase associated with this transition, however, was greater than that seen for wild type HbA (Fig. 3, bottom panel, set a, and Table I). Both the W3 α14β15 and Y8a band intensities for deoxy rHb K132A are higher than those for wild type deoxyHbA (Fig. 4). Ligation to rHb K132A further enhances both of these bands, and the addition of IHP leads to additional band intensity increases, with a nearly 2-fold Y8a band increase for CO rHb K132A + IHP over that of wild type deoxyHbA (Fig. 4 and Table I). In contrast to the results for the ligated rHb mutants discussed above, the addition of IHP to CO rHb K132A does not result in an increase in the W3 β37 band (Fig. 3, bottom panel, set b, and Table I).

rHb βE6V/K132A

The UVRR results for the R → T state transition of rHb βE6V/K132A are similar to those for rHb K132A (Fig. 3, bottom panel, and Table I). Generally, the W3 α14β15 and Y8a band intensities for deoxy rHb βE6V/K132A are greater than those observed for wild type deoxyHbA (Fig. 4). Ligation, however, has little effect on either the W3 α14β15 band intensity or on the intensity of the Y8a band (Fig. 4). The addition of IHP enhances both the W3 α14β15 and Y8a bands, but the Y8a band for ligated rHb βE6V/K132A is reduced relative to that for ligated rHb K132A (Fig. 4b). As for CO rHb K132A, the addition of IHP does not enhance the intensity of the W3 β37 band (Fig. 3, bottom panel, set b, and Table I).

Two categories of UVRR spectral differences are observed in comparing the different Hb derivatives. One set is associated with R → T state differences and is comprised of a Y8a band shift and a W3 β37 intensity increase. The former change is observed when comparing the deoxy and CO derivatives for all the Hb species examined in the present study, whereas the latter is observed for all species except the E7A mutants. The second set of spectroscopic changes consists of intensity changes in the W3 α14β15 and Y8a UVRR bands when comparing both the different derivatives (ligation state) of a given Hb and the same derivative from different Hbs.

None of the Hb S-related mutations discussed here eliminate the Y8 band 1.5–3 cm⁻¹ upshift (Figs. 2 and 3 and Table I) associated with the switch motion of the αTyr³⁵(C7) during the R → T state transition. This upshift has been shown to originate largely from βAsp⁵⁰(G9) hydrogen bond donation to αTyr³⁵(C7) upon Hb ligation (9, 13, 30). Similarly, the W3 β37
band intensity increase, associated with the R \rightarrow T state transition hinge motion at the \alpha_1/\beta_2 interface (9), is seen in all the deoxy versus CO UVRR comparisons for the Hbs discussed here except for those with the E7A mutation (Figs. 2 and 3 and Table I). The W3 \beta37 band intensity increase has been associated with changes in the hydrogen bond between \beta Trp37/C3 and \alpha Asp94 (G1).

The addition of IHP to the CO derivatives results in small to moderate intensity increases for the W3 \beta37 shoulder for the Hb A, the Hb S species, and the E7A mutants but not for the K132A mutants (Figs. 2 and 3 and Table I). This intensity increase is in the direction of the R \rightarrow T state transition-associated change. Several features are worthy of additional comment. It is indeed intriguing that for the E7A mutants, deoxygenation does not induce the typical R \rightarrow T state transition-associated intensity increase in the W3 \beta37 band, but addition of IHP to the liganded derivative does. This observation indicates that there are clearly multiple intraglobin pathways for inducing changes at the hinge region of the dimer interface. The concept of multiple pathways is further substantiated by the results from the E132A mutants, which exhibit the reverse effect in that deligation but not the addition of IHP induces the R \rightarrow T state transition-associated changes in the hinge sensitive W3 \beta37 shoulder. The results also show that changes in the hinge and switch regions are necessarily coupled because under the present conditions the addition of IHP does not result in the Y8a, R \rightarrow T state-associated frequency shift. At the significantly lower pH of 6.3, IHP addition to the CO derivatives of HbA, rHb \beta6E, and rHb E7A also induces the Y8 band to partially upshift toward the T state value (data not shown).

R \rightarrow T state Transition-associated Spectral Changes

Effect of the \beta E7A Mutation—The rHb \beta E7A mutation replaces a negatively charged residue with one that is noncharged and aliphatic, resulting in the loss of the salt bridge with the positively charged residue, \beta Lys132. Based on the W3 \beta37 UVRR results presented here, it appears that the disruptive effect of the uncompensated \beta Lys132 charge on the hinge region of the \alpha_1/\beta_2 interface is equivalent to the loss of the R \rightarrow T state transition (Fig. 3, top panel). One possible communication pathway for this disruption to the \beta Trp37 hinge region is through \beta Glu151, which is adjacent to the \beta salt bridge partner, \beta Lys132, and is noncovalently linked to the interfacial \alpha His152 (31). This disruption of the quaternary contacts within the hinge region of the T state \alpha_1/\beta_2 interface is consistent with the increase in oxygen affinity of the rHb E7A mutants reported by Lesecq et al. (1). Disruption of the hinge via mutagenic manipulation of \beta37 has been shown to enhance the ligand binding properties of the T state and reduce proximal strain at the heme (32, 33).

Effect of the \beta K132A Mutation—The effect of eliminating the \beta7–\beta132 salt bridge by replacing positively charged Lys132 with uncharged, aliphatic Ala is to enhance the R \rightarrow T transition state-associated W3 \beta37 band intensity increase via \alpha vs \alpha that for wild type Hb A (Table I). Thus, the loss of charge at \beta132 creates a “hyper” T state hinge signature. This result, in association with those from the \beta E7A mutant, indicates that uncompensated charge at the \beta132 site destabilizes the T state hinge, whereas loss of charge at this site enhances the T state hinge, and the function of the salt bridge is to modulate the T state hinge by offsetting the charge at \beta132.

Y8a and W3 a14b15 Band Intensity Changes—The relative intensities of the W3 and Y8 bands appear to follow a pattern upon comparing derivatives of a given Hb. Although there are several exceptions (Fig. 4), the intensity of the W3 and Y8 bands generally increases in the sequence of deoxy:CO:CO + IHP.

Doubly Mutated rHb \beta E6V/E7A—As shown in Fig. 4 and Table I, the UVRR spectra obtained from deoxy rHb \beta E6V/E7A show the most pronounced intensity decrease for both the W3 a14b15 and Y8 bands. Building on the findings of Spiro and co-workers (9, 12, 13, 25), this intensity decrease in the W3 a14b15 peak can be explained by a separation of the A helix.

### Table I

|                       | \(\Delta \theta_{Y8a}^0\) | \(\Delta \theta_{Y8a}^0\) | \(\Delta \theta_{W337}^0\) | \(\Delta I_{W337}\) |
|-----------------------|--------------------------|--------------------------|--------------------------|------------------|
| Wild type Hb A deoxy  | 2.9                      | 0.38 (0.37/0.39)         | 0.19 (0.18/0.20)         | 0.43 (0.38/0.5)  |
| CO                    |                          | 0.68 (0.64/0.77)         | 0.45 (0.44/0.46)         | 0.11 (0.11/0.12) |
| CO + IHP              | -0.2                     | 0.01 (0.01/0.01)         | 0.13 (0.13/0.14)         | 0.44 (0.42/0.45) |
| rHb A deoxy           | 1.8                      | 0.36 (0.34/0.37)         | 0.17 (0.16/0.18)         | 0.22 (0.21/0.23) |
| CO                    |                          | 0.27 (0.26/0.29)         | 0.19 (0.17/0.22)         | 0.45 (0.45/0.46) |
| CO + IHP              | 0.4                      | 0.26 (0.24/0.28)         | 0.11 (0.11/0.11)         | 0.38 (0.37/0.41) |
| Wild type Hb S deoxy  | 1.8                      | 0.35 (0.34/0.36)         | 0.17 (0.16/0.18)         | 0.82 (0.22/0.02) |
| CO                    |                          | 0.17 (0.15/0.18)         | 0.13 (0.12/0.14)         | 0.53 (0.50/0.57) |
| CO + IHP              | 0.2                      | 0.35 (0.34/0.37)         | 0.07 (0.06/0.07)         | 0.16 (0.15/0.19) |
| rHb E7A deoxy         | 2.1                      | 0.41 (0.39/0.44)         | 0.26 (0.23/0.22)         | 0.09 (0.08/0.10) |
| CO                    | 0.15 (0.14/0.15)         | 0.57 (0.54/0.60)         | 0.27 (0.24/0.29)         | 0.36 (0.35/0.37) |
| CO + IHP              | 0.1                      | 0.25 (0.24/0.26)         | 0.26 (0.25/0.27)         | 0.50 (0.49/0.52) |
| rHb E6V/E7A K132A deoxy| 1.8                      | 0.47 (0.42/0.54)         | 0.34 (0.29/0.38)         | 0.69 (0.60/0.63) |
| CO                    | 0.2                      | 0.08                     | -0.02                    | 0.49 (0.45/0.51) |
| CO + IHP              | 0.1                      | 0.33                     | 0.21                     | 0.06             |
from the E helix of the β chains caused by the combined change in the polarity and hydrophobicity of both the β6 and β7 residues, as illustrated in Fig. 1. It is not clear whether changes in hydration induce slight changes in the α helix itself. The α14 and β15 tryptophan residues are not distinguishable by UVRR spectroscopy, but given that the mutations studied here are located on the β chain, all of the differences observed on the W3 α14β15 main band can be reasonably assigned to βTrp15 (14). βTrp15 is an A helix residue situated in the crevice formed by the A and E helix. It normally forms a hydrogen bond with βSer72 of the E helix in both the deoxy and oxy structure (Fig. 1, top panel). A decrease in the intensity of the 1558-cm⁻¹ band reveals a weakening of this hydrogen bond. In a study of C terminally deleted Hb A, Wang and Spiro (7) observed intensity increases in the W3 α14β15 (A12) peak of both deoxy and ligated Hb A, which they attribute to a collapse of the A helix toward the E helix. This collapse is purported to originate from the loss of the C-terminal anchor of the H helix. The plausible description above, this proposed bond weakening should re-

βTrp15(A12)–βSer72(E16) hydrogen bond, which is suggestive of a displacement of the A helix away from the E helix.

In the present study, the deoxy rHb βE6V/E7A Y8a band is 16% less intense than that observed for deoxy rHb S (Fig. 4b and Table I). Y8a band intensity changes can reasonably be attributed to either or both of the two penultimate tyrosine residues in the Hb molecule, αTyr146 and βTyr145. Both residues are integral parts of the scaffolding linking the H and A helices. The Y8a intensity changes observed in the present study are likely to originate from βTyr145. βTyr145(HC2) occupies the pocket made by the H and F helices in deoxy Hb A and deoxy Hb S and forms a hydrogen bond with βVal89(FG5), which contributes to the scaffolding of the A helix by the H helix. The decrease in the deoxy rHb βE6V/E7A Y8a band intensity (Fig. 4b) is postulated to arise from a weakening of the βTyr145–βVal89 hydrogen bond through a shift in the H helix.

**Single Mutants, rHb βE7A and βK132A and Doubly Mutated rHb βE6V/K132A:** Involvement and Role of the Salt Bridge β7–β132—The W3 and Y8a bands intensities for deoxy rHb βE7A are similar to those of deoxy rHb S (Fig 4 and Table I). No weakening of the hydrogen bond involving βTrp15(A12) and βSer72(E16) can be inferred. This result suggests that the absence of the salt bridge between βGlu7(A4) and βTyr132(H10) per se does not influence the separation of the A and E helices and the A and H helices.

This claim is supported by the results from mutants rHb βK132A and rHb βE6V/K132A. The W3 and Y8a bands for the deoxy derivative of these rHbs exhibit increased intensities compared with that for deoxy rHb S (Fig 4 and Table I). In these mutants, the β7 (A4)–β132 (H10) salt bridge is also absent, whereas the βTrp15(A12)–βSer72(E16) hydrogen bond is apparently strengthened, showing a tighter packing of the A helix against the E helix as follows from the discussion above. The other ligation state derivatives of these mutants show either no additional change or a further increase in the W3 and Y8a bands intensities (Fig. 4 and Table I). It appears that complete loss of the charge at position β132 results in a loss of the A-H scaffolding with a concomitant decrease in the spacing between the A and E helices. It follows that the β7–β132 salt bridge modulates the charge at the β132 residue in a way that allows for the appropriate degree of interhelical scaffolding and proper behavior of the hinge region at the dimer interface.

**Importance of the Hydrogen Bond Involving βTyr145 and βVal89—**In a study of βTyr145 mutants, Ishimori et al. (34) showed that the presence of the phenolic side chain in the H-F pocket is a contributing factor to T state stability, whereas the hydrogen bond strength between βTyr145(HC2) and βVal89(FG5) is a modulating factor for the extent of proximal strain within the T state. Loss of the hydrogen bond is associated with a decrease in proximal strain as reflected in an increase in the frequency of the iron-proximal histidine-stretching mode for the deoxy derivative of the βY145F mutant. An increase by Togi and co-workers (35) showed that this hydrogen bond is also important in stabilizing the βTyr145(HC2)–βVal89(FG5) hydrogen bond because of the aforementioned modifications. Based on the β145 mutant studies described above, this proposed bond weakening should re-
sult in a higher oxygen affinity for Hb, which has been observed for singly and doubly mutated Hb ε72As (1).

**Conclusion**

The spectroscopic results in this study show that the consequences of disrupting the salt bridge between βGlu and βLys is a function of which contributing residue is altered. The salt bridge clearly does not function simply as a taut spring linking two helices together, which when disrupted results in an automatic separation of the spring-linked elements. The salt bridge appears to modulate the effect of the charge of βLys. Complete loss of the charge, as occurs in the βK132A mutants, results in spectroscopic signatures for a collapse of the scaffolding supporting the A and H helices leading to a more “compressed” overall structure. The resulting structure is still capable of undergoing the full range of R → T state transition-associated hinge and switch motions as reflected in the UVRR spectrum. There are also spectroscopic indications that the charge at β342 affects the stability of the T state hinge region of the αβ dimer interface. Both the single and double βE7A mutants, where the charge on β342 is fully unshielded, fail to show the hinge region-associated W3 β37 band intensity increase for the R → T state transition that is seen for all the other mutants, including βK132A. The absence of the R → T state change in the hinge region is a likely factor in the increased oxygen binding affinity observed for the Rb βE7A mutants. Thus, too little shielding of the charge at βLys results in an altered T state hinge region of the αβ dimer interface, whereas a complete neutralization of the charge results in a compaction of the overall structure. The βGlu/βLys salt bridge appears to play a role in supporting the appropriate charge balance that in turn maintains the A helix-H helix scaffolding and the proper T state hinge.

The UVRR spectrum from the double mutant Hb βE6V/E7A indicates an enhanced separation in the A helix-E helix tertiary contact, as reflected in a weakening of the hydrogen bond between βTrp and βSer. This finding supports the idea that a change in the A helix packing is responsible for the observed decrease in Hb βE6V/E7A polymerization (1, 2). It is not clear whether the weakening of the hydrogen bond between βTrp and βSer is purely the result of the local perturbation on the A helix or the combined result of the A helix mutations with the unshielded charge at βLys. The double mutant, Hb βE6V/E7A, also shows a substantially decreased Y8a band intensity, attributed to an increased separation between the H helix and the F helix. The combined effect is suggestive of a global expansion or loosening of the tertiary structure.

The spectroscopic changes occurring upon addition of IHP to the CO-saturated derivatives of all the species examined is consistent with IHP inducing a general tightening of the overall globin structure. This tightening is reflected in spectroscopic signatures of a strengthened hydrogen bonding between the A and E helices and the H and F helices. At pH 7.35, addition of IHP to the CO saturated derivatives does not perturb the switch region of the αβ dimer interface but does induce T state character into the hinge region of all but the βK132A mutants. Thus, the βE7A mutation eliminates the deligation-induced R → T state hinge transition but not the IHP-induced effect on the hinge, whereas the βK132A mutation eliminates the IHP effect but maintains (or even enhances) the deligation-induced change. It follows that attaining the T state conformation at the hinge region of the αβ dimer interface can be achieved through different pathways and that these pathways are subject to subtle mutagenic manipulation at sites well removed from the interface.

**REFERENCES**

1. Lesecq, S., Baudin, V., Kister, J., Marden, M., Poyart, C., and Pagnier, J. (1996) *J. Biol. Chem.* 271, 17211–17214
2. Lesecq, S., Baudin, V., Kister, J., Poyart, C., and Pagnier, J. (1997) *J. Biol. Chem.* 272, 15242–15246
3. Ather, S. (1981) *Methods Enzymol.* 76, 371–413
4. Rousseau, D. L., and Friedman, J. M. (1988) in *Biological Applications of Raman Spectroscopy* (Spiro, T. G., ed) Vol. III, pp. 153–215, Wiley & Sons, New York
5. Kitagawa, T. (1988) in *Biological Application of Raman Spectroscopy* (Spiro, T. G., ed), Vol. III, pp. 97–112, Wiley & Sons, New York
6. Jayaraman, V., Rodgers, K. R., Mukerji, I., and Siro, T. G. (1995) *Science* 269, 1843–1848
7. Wang, D., and Siro, T. G. (1998) *Biochemistry* 37, 9940–9951
8. Kitagawa, T. (1992) *Prog. Biophys. Mol. Biol.* 58, 1–18
9. Rodgers, K., Su, S., Subramaniam, S., and Siro, T. (1992) *J. Am. Chem. Soc.* 114, 3971–3975
10. Jayaraman, V., Rodgers, K. R., Mukerji, I., and Siro, T. G. (1993) *Biochemistry* 32, 4547–4551
11. Mukerji, I., and Siro, T. G. (1994) *Biochemistry* 33, 13132–13139
12. Rodgers, K. R., and Siro, T. G. (1994) *Science* 265, 1067–1069
13. Hu, X., and Siro, T. G. (1997) *Biochemistry* 36, 15701–15712
14. Sokolov, L., and Mukerji, I. (1998) *J. Phys. Chem. B* 102, 8314–8319
15. Juszczak, L., and Friedman, J. (1999) *J. Biol. Chem.* 274, 30357–30360
16. Nagami, S., Nagai, M., Truneshige, A., Yonetani, T., and Kitagawa, T. (1999) *Biochemistry* 38, 9659–9666
17. Nagai, M., Wajcman, H., Lahary, A., Nakatsukasa, T., Nagutomo, S., and Kitagawa, T. (1999) *Biochemistry* 38, 1243–1251
18. Hirsch, R., Juszczak, L., Fataliev, N., Friedman, J., and Nagel, R. (1999) *J. Biol. Chem.* 274, 13777–13782
19. Hirsch, R. E. L., Miyata, T., Higashihara, G. V., Huang, S., Friedman, J. M., and Nagel, R. L. (1996) *J. Biol. Chem.* 271, 372–375
20. Juszczak, L., Hirsch, R., Nagel, R., and Friedman, J. (1996) *J. Raman Spectrosc.* 29, 963–968
21. Bikoreou, M., Baudin, V., Marden, M., Lacahe, N., Bohn, B., Kister, J., Schaad, O., Dumoulin, A., Edelstein, S., Poyart, C., and Pagnier, J. (1992) *Protein Sci.* 1, 145–150
22. Nagai, M., and Togher, H. (1984) *Nature* 309, 810–812
23. Nagai, K., Perutz, M., and Poyart, C. (1985) *Proc. Natl. Acad. Sci. U. S. A.* 82, 7232–7235
24. Wang, Y., and Van Wart, H. (1993) *Methods Enzymol.* 226, 319–373
25. Wang, D., Zhao, X., Shen, T.-J., Hu, C., and Siro, T. (1999) *J. Am. Chem. Soc.* 121, 11197–11203
26. Jayaraman, V., and Siro, T. G. (1995) *Biochemistry* 34, 4511–4515
27. Nagai, M., Kaminaka, S., Ohya, Y., Nagai, M., Mizutani, Y., and Kitagawa, T. (1995) *J. Biol. Chem.* 270, 1636–1642
28. Huang, S., Peterson, E. S., Hu, C., and Friedman, J. M. (1997) *Biochemistry* 36, 6197–6206
29. Su, C., Park, Y. D., Liu, G., and Siro, T. G. (1989) *J. Am. Chem. Soc.* 111, 3457–3459
30. Huang, J., Juszczak, L. J., Peterson, E. S., Shannon, C. F., Yang, M., Huang, S., Vidugiris, G. V. A., and Friedman, J. M. (1999) *Biochemistry* 38, 4514–4525
31. Chang, C., Simplaceana, V., and Ho, C. (2002) *Biochemistry* 41, 5644–5655
32. Kwiatkowski, I. D., Hui, H. L., Wierzb, A., Noble, R. W., Walder, R. Y., Peterson, E., Silgar, S., and Sanders, K. (1997) *Biochemistry* 36, 4325–4335
33. Peterson, E. S., and Friedman, J. M. (1998) *Biochemistry* 37, 4346–4357
34. Ishihomri, K., Imai, M., Miyazaki, K., Kitagawa, T., Wada, Y., Morimoto, H., and Morishima, I. (1992) *Biochemistry* 31, 3256–3264
35. Togi, A., Ishihomri, K., Una, M., Konno, T., Morishima, I., Miyazaki, G., and Imai, K. (1993) *Biochemistry* 32, 10165–10169