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

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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 αβ2 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 αβ2 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,1 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 βE6V/E7A. It was postulated (1, 2) that the loss of the normal salt bridge between βGlu7(A4) and βLys132(H10) in the rHb βE7A 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 (UVRR) provides information about vibrational modes of aromatic residues within the globin. UVRR 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” (αTyr42) regions of the αβ2 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 UVRR technique allows us to couple modifications of both local and global elements of structure with observed functional changes. For example, in Hb S polymerization, the helical motion of the A helix is limited by the rigidity of the β6 donor site, which is tightly packed in the quaternary polymer. Replacing Glu7(A4) with Ala10(H10) in rHb βE6V/E7A decreases the polymerization, but this decrease is not seen in doubly mutated rHb βE6V/E7A. The UVRR results suggested that the decreased polymerization of the doubly mutated rHb might be due to a lack of a proper interhelical separation of the A helix.

The UV resonance Raman results are consistent with these ideas: 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 αβ2 interface. Both singly and doubly mutated hemoglobin (Hb) βE7A exhibit 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 αβ2 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.

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example, 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, βK132A, 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 IHP. The UVRR data are averages of these four independent measurements. The measurements for the CO-ligated Hb/E6V/K132A with and without IHP were accumulated rather than 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.

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 pATPrcl1FXβ-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 cyanohemin and the partner α-subunits prepared from wild type Hb A, to form the tetrameric Hb αβ2, to form the tetrameric Hb A, αβ2 (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 Hapes at pH 7.35. 0.4 mM 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 accumulated before and after exposure to the UV laser beam. 2) If absorption changes were noted, the sequential UVRR acquisitions were examined. 3) Acquisitions that showed substantial 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. 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 for evidence of band changes. 3) Acquisitions that showed substantial 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 1530–1670 cm⁻¹ frequency window, and the intensity was normalized at the W3 band (1558 cm⁻¹) for each set of spectra in Figs. 2 and 3. The software
program Grams/32 AI, version 6.00 (Galactic Industries Corp., Salem, NH) was used to determine the Y8a and W3 α14β15 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 αβ 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 (deoxygenated) is accompanied by the R → T state transition. The conformational changes of the aromatic residues in the interface following the R → T state transition yield two major UVRR spectral changes. These are a ~2 cm⁻¹ increase in frequency of the Y8a band from 1548 cm⁻¹ to 1517 cm⁻¹ and an intensity increase of ~37% in the W3 shoulder (β37) at 1549 cm⁻¹ (7, 9). Several investigators have used these UVRR spectral changes to characterize the effect of site mutation on hemoglobin (6, 7, 9, 10, 12–15, 17, 20, 25–28).

Thus, the association of these small spectral changes with specific changes in hemoglobin structure and ligation state is both well documented and consistent. The UVRR spectra shown in Figs. 2 and 3 have been truncated to highlight the 1530–1650 cm⁻¹ frequency window. This spectral window contains the conformation-sensitive W3 and Y8a reporter bands.

The W3 band at 1558 cm⁻¹ has two contributions (9, 13, 27, 29). The central feature that peaks at 1558 cm⁻¹ is derived from the two A helix tryptophans (α14 and β15), whereas the R → T state sensitive shoulder at 1550 cm⁻¹ originates from βTrp37. Intensity changes in the central peak of the W3 band at 1558 cm⁻¹ have been shown to originate from variations in the hydrogen bond between the A helix tryptophans and their hydrogen-bonding partners on the E helix (25). Increased and decreased intensities correlate with increases and decreases in hydrogen bond strength, respectively. Modulation of the hydrogen bond strength is attributed to changes in the distance between the two helices.

The intensity for the ~1550 cm⁻¹ shoulder on the W3 band increases as the hinge region of the αβ interface undergoes an R → T state transition, reflecting the changes in the hydrogen bonding pattern of βTrp37 with αAsp54. Thus, the W3 band provides information on both the functionally important hinge region of the αβ interface and the packing of the A and E helices. The tyrosine-derived Y8a band at 1548 cm⁻¹ shows a ~1–2 cm⁻¹ shift to higher frequency when liganded R-state Hb A is converted to the deoxy T state (9, 29). This frequency shift originates primarily from αTyr42 (13, 17, 28) in the switch region of the αβ interface. The intensity of this band has been correlated with the integrity of the scaffolding supporting the H helices.

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UVRR Spectroscopy of Hb S Salt Bridge Mutants

Fig. 2. W3 (~1558 cm⁻¹) and Y8 (~1615 cm⁻¹) UVRR bands for hemoglobin A (top panel, set a, wild type; set b, recombinant) and hemoglobin S (bottom panel, set a, wild type; set b, recombinant). Solid line, deoxygenated Hb; broken line, CO ligated Hb; dotted and dashed line, CO ligated Hb in the presence of IHP. The spectra have been normalized to the W3 peak.

The intensity of the UVRR spectra in each set in Figs. 2 and 3 have been normalized at the W3 peak to clarify the differences in the β37 shoulder at 1548 cm⁻¹. Both wild type Hb A and Hb A (Fig. 2, top panel, sets a and b, respectively) yield the well documented Y8a frequency increase (2.9 and 1.8 cm⁻¹, 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 Y8a 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 Y8a 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 Y8a band intensity 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,
and Table I). The W3 β37 band intensity increase for deoxy wild type Hb S is comparable (0.45) with that obtained for either species of deoxy Hb A (0.43–0.44), whereas the intensity increase obtained for deoxy rHb S is higher (0.53) (Table I). Conversely, ligation, which corresponds to the T → R state transition, results in intensity increases for both the W3 and Y8 bands of wild type Hb S (0.19 and 0.26, respectively; Table I) and for those of rHb S (0.13 for W3 and 0.17 for Y8a; Fig. 4 and Table I). Addition of IHP to wild type COHb yields little, if any, decrease in the W3 band intensity (Fig. 4a and Table I) and an unchanged Y8 band intensity (Fig. 4b and Table I). For CO rHb S, the addition of IHP results in an increase in the Y8a band (Fig. 4b and Table I).

rHb βE7A

The characteristic Y8a frequency upshift accompanying the R → T state transition is also manifest by rHb βE7A (Fig. 3, top panel, set a, and Table I). The usual W3 β37 intensity increase, however, is not found in the UVRR results for this rHb mutant (Fig. 3, top panel, set a, and Table I). The W3 α14β15 and Y8a band intensities for deoxy rHb βE7A are similar in magnitude to those of wild type deoxyHb A (Fig. 2, top panel, set a), whereas ligation to rHb βE7A yields a small decrease in the W3 α14β15 band intensity and no change in the Y8a band intensity (Fig. 4 and Table I). The addition of IHP to CO rHb βE7A leads to intensity increases in the Y8a and W3 β37 bands: a 0.36 increase in Y8a relative to the Y8a band of deoxy rHbβE7A (Table I) and a 0.16 increase in the W3 β37 band, comparable with the 0.11 band increase found in the results for wild type COHb A + IHP (Table I). Under the same conditions, the W3 α14β15 band intensity has merely been restored to its deoxy value (Fig. 4a and Table I).

rHb βE6V/E7A

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 deoxyHb A (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 αTyr(15)(C7) during the R → T state transition. This upshift has been shown to originate largely from βAsp3(9) hydrogen bond donation to αTyr42(C7) upon Hb ligation (9, 13, 30). Similarly, the W3 β37
band intensity increase, associated with the R → T state transition hinge motion at the α1β2 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 β37 band intensity increase has been associated with changes in the hydrogen bond between βTrp15(C3) and αAsp94(G1).

The addition of IHP to the CO derivatives results in small to moderate intensity increases for the W3 β37 band, but addition of IHP to the liganded derivative does not result in the Y8a band to partially upshift toward the T state value (data not shown).

R → T state Transition-associated Spectral Changes

Effect of the βE7A Mutation—The rHb βE7A mutation replaces a negatively charged residue with one that is non-charged and aliphatic, resulting in the loss of the salt bridge with the positively charged residue, βLys132. Based on the W3 β37 UVRR results presented here, it appears that the disruptive effect of the uncompensated βLys132 charge on the hinge region of the α1β2 interface is equivalent to the loss of the R → T state transition (Fig. 3, top panel). One possible communication pathway for this disruption to the βTrp15 hinge region is through βGln131, which is adjacent to the β salt bridge partner, βLys132, and is noncovalently linked to the interfacial αHis130 (31). This disruption of the quaternary contacts within the hinge region of the T state α1β2 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 β37 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 βK132A Mutation—The effect of eliminating the β7–β132 salt bridge by replacing positively charged Lys132 with uncharged, aliphatic Ala is to enhance the R → T transition state-associated W3 β37 band intensity increase vis-à-vis that for wild type Hb A (Table I). Thus, the loss of charge at β132 creates a “hyper” T state hinge signature. This result, in association with those from the βE7A mutant, indicates that uncompensated charge at the β132 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 β132.

Y8a and W3 a14β15 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 + IHP.

Doubly Mutated rHb β6E7VA—As shown in Fig. 4 and Table I, the UVRR spectra obtained from deoxy rHb β6E7VA show the most pronounced intensity decrease for both the W3 a14β15 and Y8 bands. Building on the findings of Spiro and co-workers (9, 12, 13, 25), this intensity decrease in the W3 a14β15 peak can be explained by a separation of the A helix
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 α53 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 claim was made that the H helix acts as a scaffold for the A helix, which keeps the A helix separated from the E helix (Fig. 1). A weakening of the scaffolding through changes in the hydrogen bonds and salt bridges of the H helix allows the A helix to pack more tightly against the E helix, thus allowing for a stronger βTrp15(A12)→βSer72(E16) hydrogen bond. Hirsch et al. (19) observed a decreased intensity of the W3 α14β15 band of Hb C, where β glutamic acid is replaced by a lysine residue. These authors demonstrated a weakening of the 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/βE7A 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, αTyr145 and βTyr145 (34). 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 (34). 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 βVal8(FG5), which contributes to the scaffolding of the A helix by the H helix. The decrease in the deoxy rHb βE6V/βE7A Y8a band intensity (Fig. 4b) is postulated to arise from a weakening of the βTyr145→βVal8 hydrogen bond through a shift in the H helix.

Single Mutants, rHb βE7A and βK132A and Doubly Mutated rHb βE6V/βK132A: 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 βLys132(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/βK132A. 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 β Trp15(A12) 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 βVal8—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 βVal8(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. This result supports the hypothesis that the hydrogen bond is also important in stabilizing the R→T transition state. Loss of the hydrogen bond increased the energy of the transition state and thereby slowed the R→T state transition.
sult in a higher oxygen affinity for Hb, which has been observed for singly and doubly mutated Hb βE7As (1).

**Conclusion**

The spectroscopic results in this study show that the consequences of disrupting the salt bridge between βGlu<sup>6</sup> and βLys<sup>132</sup> 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<sup>132</sup>. Complete loss of the charge, as occurs in the βK132A mutants, results in spectroscopic signatures for a collapse of the scaffold supporting the A and H helices leading to a more “compressed” overall structure. The results of the charge on β132 effects the stability of the T state hinge region of the αβ<sub>d</sub> dimer interface. Both the single and double βE7A mutants, where the charge on β132 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 rHb βE7A mutants. Thus, too little shielding of the charge at βLys<sup>132</sup> results in an altered T state hinge region of the αβ<sub>d</sub> dimer interface, whereas a complete neutralization of the charge results in a compaction of the overall structure. The βGlu<sup>6</sup>–βLys<sup>132</sup> 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<sup>15</sup> and βSer<sup>72</sup>. 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<sup>15</sup> and βSer<sup>72</sup> 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<sup>132</sup>. 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<sub>d</sub> 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<sub>d</sub> 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.

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Conformational Changes in Hemoglobin S (βE6V) Imposed by Mutation of the βGlu
7−βLys132 Salt Bridge and Detected by UV Resonance Raman Spectroscopy
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