# Conformational Changes in FmetHbS Probed with UV Resonance Raman and Fluorescence Spectroscopic Methods

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The tertiary and quaternary structure of HbS has been investigated using UV resonance Raman (UVRR) and fluorescence spectroscopic methods. This well-characterized Hb mutant ($\beta$6 Glu → Val), which forms polymers under deoxygenating conditions, was studied in the tetrameric form as the fluoromet derivative and compared with HbA under the same conditions. The excitation wavelengths employed in this study preferentially probe the Tyr and Trp residues in the protein. Comparison of UVRR and fluorescence difference spectra generated between the T and R states of FmetHbA and FmetHbS are indicative that the $\alpha_1\beta_2$ intersubunit contacts, monitored through the $\beta$37 Trp and the $\alpha$42 Tyr residues, are essentially the same for the two hemoglobins. (The abbreviations used are the following: Hb, hemoglobin; FmetHbA, FmetHbS, the fluoromet derivatives of hemoglobin A and hemoglobin S; UVRR, ultraviolet resonance Raman, IHP, inositol hexaphosphate.) The tertiary conformation of FmetHbS, however, is perturbed relative to that of FmetHbA in both the T and R quaternary states, as shown by an overall increase in the intensity of the Trp modes in the UVRR spectra and a decrease in the fluorescence intensity. These spectral differences are attributed to the $\beta$15 Trp residue, because of the mutation at the $\beta$6 position. The combined spectroscopic studies provide evidence for an altered tertiary structure in HbS where the A-helix is displaced toward the E-helix as monitored by the strength of the $\beta$15 Trp···$\beta$72 Ser H-bond.

## Introduction

In sickle cell hemoglobin (HbS) a single residue mutation in the $\beta$ subunits ($\beta$6 Glu → Val) drives the polymerization of individual tetramers into long fibers. This polymerization process has been studied extensively using a variety of methods including electron microscopy,[1] X-ray crystallography[2] and light scattering;[3] however the mechanism of fiber formation is not fully understood.

An initial step toward understanding the polymerization process is the study of HbA and HbS tetramers. Early optical experiments on HbS have yielded contradictory results; absorption and rotary dispersion experiments[4] did not detect any structural differences between HbA and HbS, while subsequent circular dichroism (CD) measurements only detected differences in the carbon monoxide forms. By inference, these differences in the CD spectra were attributed to the tertiary structure of the $\beta$ chains in HbS, yet direct evidence was lacking.[5] Later experiments, such as the measurement of the p*K* values of surface histidyl residues by NMR spectroscopy,[6] also alluded to a structural perturbation in the amino termini of HbS $\beta$ subunits. More recently, genetically engineered mutants have indicated that A-helix configuration can regulate the polymerization process.[7] Thus, many experimental approaches have implied that the structure of HbS is perturbed and that this perturbation is directly related to its polymerization properties, but the structural details remain undefined. Some clarity has been afforded by the most recent X-ray crystal structure determination of HbS in which it was found that the A-helix in HbS donor $\beta$ subunits is displaced toward the interior of the protein.[8] However, in the crystal, the molecules pack as two strands of HbS molecules that are stabilized by both axial and lateral contacts, which may influence the tertiary structure of an individual tetramer. By the probing of the structure of unassociated HbA and HbS tetramers, our spectroscopic studies address the influence of these axial and lateral contacts on the tertiary structure and directly show that the tertiary structure of individual HbS tetramers is perturbed in both quaternary states.

In this study, the structure of HbS is compared to that of HbA using a combination of UV resonance Raman (UVRR) and fluorescence spectroscopic techniques. Previous studies using UVRR spectroscopy have shown that it is sensitive to changes in both the quaternary[9−12] and tertiary structure[11,13] of the molecule as it undergoes the allosteric transition. To complement our Raman studies, we have also used fluorescence spectroscopy to probe the Trp residues. This technique has been used successfully to characterize the R → T transition[14] and, more recently, has detected tertiary structural differences between HbC ($\beta$E6K) and HbA.[15]

In our laboratory, we have employed the fluoromet derivative of HbS as a functional analogue of HbS. FmetHbS forms fibers upon addition of allosteric effector, and the kinetics of fiber formation as well as the overall morphology of the fibers are comparable to those of deoxy HbS (Yohe, M. E.; Sheffield, K.; Mukerji, I. Manuscript in preparation). The comparison of FmetHbA and FmetHbS tetramers reveals that the quaternary structure with respect to the $\alpha_1\beta_2$ interface is very similar. The tertiary conformation, however, differs markedly as shown by an increase in intensity of Trp modes in the HbS UVRR spectra and a dramatic decrease in HbS Trp fluorescence intensity. These differences are seen in both quaternary states and are attributed to the $\beta$15 Trp residue, which reflects the position of

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the A-helix.[11,13] Thus, the conformational differences detected between HbA and HbS result from a displacement of the A-helix toward the center of the protein in HbS tetramers, which leads to a change in the tertiary structure of the $\beta$ subunits.

## Materials and Methods

**Sample Preparation.** The HbA was prepared from fresh human blood following standard procedures.[16] 2,3-Diphosphoglycerate was removed from HbS and HbA by standard methods.[17] HbS was isolated from the blood of homozygous (SS) individuals and was further purified on a DE-52 (Whatman) chromatography column from which it elutes at approximately pH 8.1.[18] Purification of the HbS samples was verified by nondenaturing gel electrophoresis.[17] Both types of Hb were converted to the fluoromet form following the same procedures. Initially, Hb was converted to the met form by reaction with potassium ferricyanide. Upon removal of the resultant ferrocyanide and excess ferricyanide, the Hb was converted to the fluoride derivative by addition of sodium fluoride.[11] For UVRR experiments, the sample concentration was 0.25 mM in a 0.5 M NaF, 25 mM sodium phosphate buffer of pH 6.5. Fluoride ion concentration was maintained at a 500-fold molar excess/heme to ensure that other metHb species such as acid metHb or aquometHb would not be present. The T state was obtained with the addition of a 20-fold molar excess of inositol hexaphosphate (IHP)/tetramer. Formation of the T state was verified by UV−vis absorption spectroscopy.[19] In all UVRR hemoglobin samples, 0.2 M $NaClO_4$ was added as an internal intensity and frequency standard.

**UV Resonance Raman Spectroscopy.** A Nd:YLF pumped Ti:sapphire laser system (Quantronix, New York) was used to generate the excitation wavelengths. Excitation frequencies of 220 and 230 nm were generated by frequency quadrupling the fundamental output of the Ti:sapphire laser with two barium borate (BBO) crystals. The laser was operated at a repetition rate of 1.5 kHz at an average power of 1.3 mW. Spectra were recorded using a 1.25 m monochromator (SPEX 1250M, Instruments, SA, Metuchen, NJ) equipped with a 3600 groove/mm grating. A 10 $cm^{-1}$ spectral slit width was used for both excitation wavelengths. Data were recorded with a SPEX Spectrum-1 liquid nitrogen cooled CCD detection system with an UV-enhanced 1024 × 256 pixel chip (Instruments, SA, Metuchen, NJ). Data were calibrated with acetone and ethanol and are accurate to $\pm 1$ $cm^{-1}$. Samples were contained in a quartz EPR tube and were spun continuously. Samples were cooled continuously with $N_2$ gas that had passed through a dry-ice/2-propanol bath. Acquisition time was 1 h for an individual spectrum, and the spectra show results from the addition of four individual spectra. Data were normalized to unity at the $ClO_4^-$ band at 934 $cm^{-1}$, and all difference spectra were generated using a factor of 1. Data manipulation and analysis were done using the programs Labcalc and Grams/32 (Galactic Industries, NH).

**Fluorescence Spectroscopy.** The fluorescence emission of FmetHbS and FmetHbA was obtained using a Jobin Yvon Spex Fluoromax-2 instrument (Instruments, SA, Metuchen, NJ). All spectra were obtained using a front-face accessory. The slit widths for both excitation and emission were 0.7 mm, giving a band-pass of 3 nm. Samples were maintained at a temperature of 5 °C with a thermostated cell holder for all spectra. The concentration of hemoglobin solutions was 0.155 mM/tetramer. At this concentration and temperature, tetramer dissociation is relatively suppressed and the percentage of dimers is minimal. All samples were in a 25 mM sodium phosphate buffer, pH 6.5, with 0.5 M sodium fluoride. Sample concentrations were verified by absorption measurements at 280 nm, $\epsilon = 28$ $mM^{-1}$ $cm^{-1}$.[19]

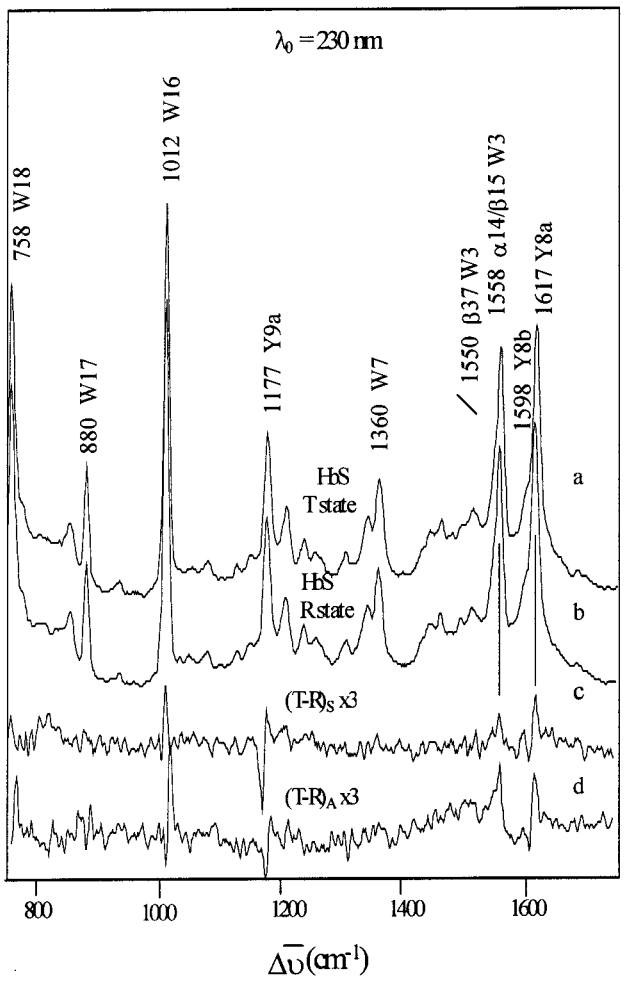


**Figure 1.** Comparison of FmetHbA and FmetHbS using 230 nm excitation. UVRR spectra of (a) FmetHbS + IHP, (b) FmetHbS, (c) [(FmetHbS + IHP)−FmetHbS], and (d) [(FmetHbA + IHP)−FmetHbA]. Difference spectra are multiplied by a *y*-scale factor of 3. Hb concentrations were 0.25 mM/tetramer in 25 mM sodium phosphate buffer, pH 6.5. A 20-fold molar excess of IHP/tetramer was added to induce formation of the T state.

## Results

**UV Resonance Raman Spectroscopy.** *230 nm Excitation.* UVRR spectra obtained with an excitation wavelength of 230 nm are shown in Figure 1. This excitation wavelength mainly enhances the vibrational modes of the Trp and Tyr residues. The vibrational modes have been previously assigned, and we use the numbering convention of Harada and Takeuchi,[20] in which the letter of the mode indicates the residue type from which it originates. The difference spectra of HbS and HbA are strikingly similar (Figure 1 parts c and d) and closely resemble UVRR difference spectra previously reported for HbA[10] and FmetHbA.[11,12,21] The main features are the Y8b and Y8a modes at 1584 and 1603 $cm^{-1}$, respectively, which have been previously assigned to the $\alpha$42 Tyr.[10] This residue forms a H-bond across the $\alpha_1\beta_2$ interface with $\beta$99 Asp in the T state, giving rise to the features observed in the difference spectrum. Other prominent bands include the Trp W16 and W3 modes at 1010 and 1550 $cm^{-1}$, respectively. In earlier studies using the mutant Hb Rothschild (W$\beta$37R), the shoulder at 1550 $cm^{-1}$ was

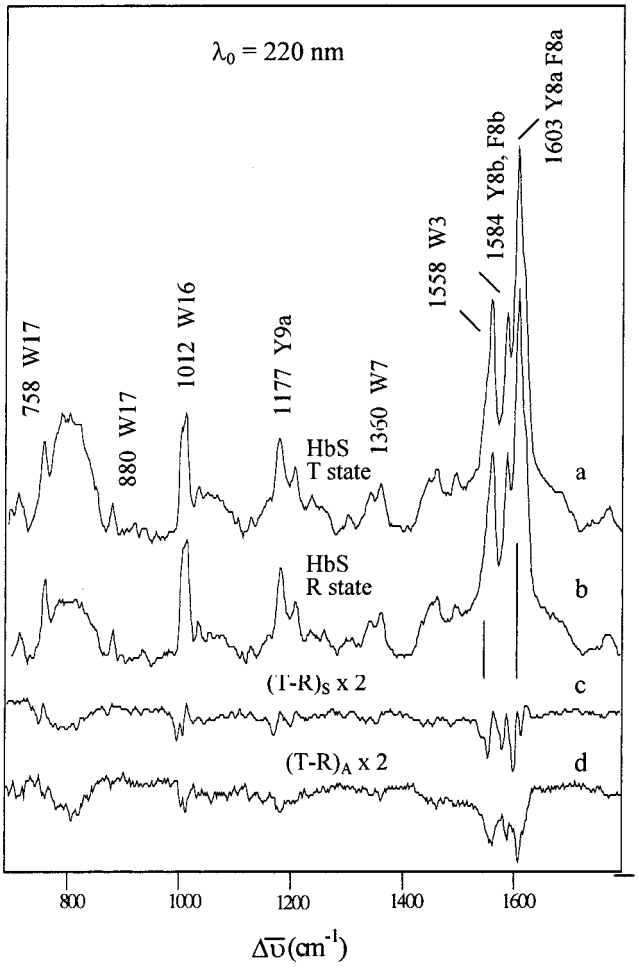


**Figure 2.** Comparison of FmetHbA and FmetHbS using 220 nm excitation. UVRR spectra of (a) FmetHbS + IHP, (b) FmetHbS, (c) [(FmetHbS + IHP)−FmetHbS], and (d) [(FmetHbA + IHP)−FmetHbA]. Difference spectra are multiplied by a *y*-scale factor of 2. Experimental conditions are the same as those in in Figure 1.

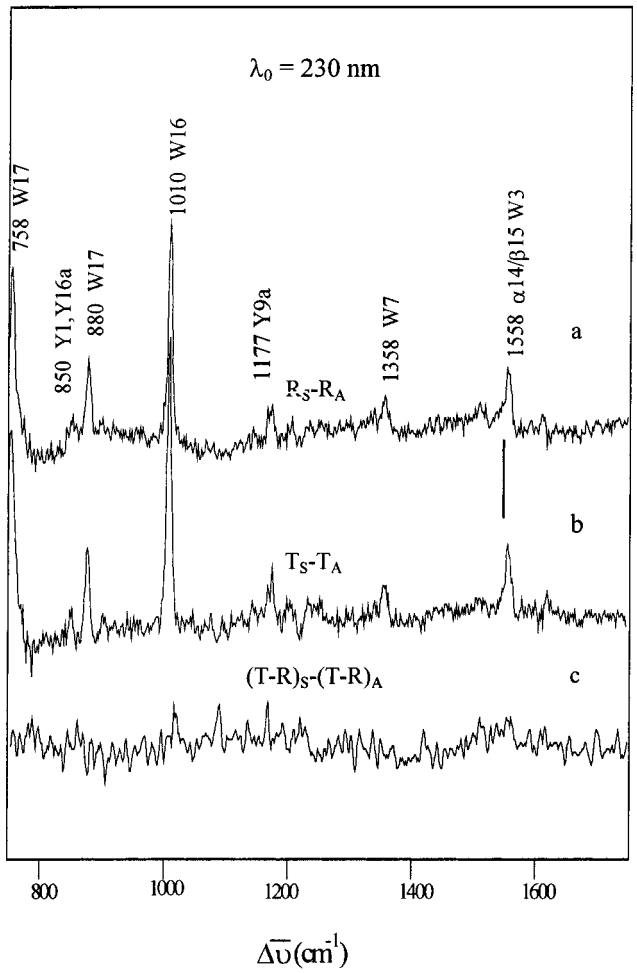


**Figure 3.** Comparison of the T and R states of FmetHbS and FmetHbA: (a) FmetHbS−FmetHbA in the T state; (b) FmetHbS−FmetHbA in the R state; (c) double difference spectrum generated from the T−R difference spectra of FmetHbS and FmetHbA. For all spectra shown, the excitation wavelength was 230 nm.

assigned to the $\beta$37 Trp.[10] The presence of the 1550 $cm^{-1}$ band in the difference spectrum confirms that FmetHbS forms the $\alpha$94 Asp−$\beta$37 Trp H-bond upon addition of IHP. A peak at 1558 $cm^{-1}$ is also observed, which is not seen in unmodified HbA. This Trp band results from stronger H-bonding at the interior ($\alpha$14/$\beta$15) Trp residues and arises from the presence of the fluoride ligands in the T state.[11] The strong similarities between the UVRR T−R difference spectra of FmetHbS and FmetHbA (Figure 1 parts c and d) demonstrate that FmetHbS forms the intersubunit contacts characteristic of the T state upon addition of IHP. Furthermore, the overall R- and T-state conformations of FmetHbS strongly resemble those of FmetHbA to the extent that the tertiary structural perturbation observed in the FmetHbA difference spectrum, the peak at 1558 $cm^{-1}$, is also observed in the FmetHbS difference spectrum.

*220 nm Excitation.* Figure 2 depicts UVRR spectra of FmetHbS in the T and R states (Figure 2 parts a and b) obtained with an excitation wavelength of 220 nm. The observed vibrational modes arise from the Phe, Tyr, and Trp residues in the protein. This excitation wavelength selectively enhances Phe residues relative to the Tyr and Trp residues.[9,22,23] The most dominant bands in the spectrum at 1603, 1584, and 1004 $cm^{-1}$ arise predominately from Phe residues with some contributions from Tyr and Trp residues. The T−R difference spectra (Figure 2 parts c and d) reported for HbS and HbA in which all of the spectral features are negative, as has been previously observed are remarkably similar.[11,12,24] These negative features result because the aromatic residues experience a more hydrophobic environment and form stronger H-bonds in the T state, which leads to a red-shift in the excitation profile.[10] The 220 nm excited spectra confirm the results obtained with 230 nm excitation, namely that T−R UVRR difference spectra of FmetHbS and FmetHbA are directly comparable in both the frequency and intensity of spectral bands. Small differences that are observed result from differences in signal-to-noise. This strong similarity is indicative that the overall quaternary conformations for both hemoglobins are the same.

*Comparison of the T and R States.* In Figure 3 the T and R states of FmetHbA and FmetHbS are compared using UVRR difference spectroscopy. In the 230 nm excited difference spectra, $T_S-T_A$ (Figure 3a) and $R_S-R_A$ (Figure 3b), an increase in intensity of all Trp modes is observed in both difference spectra. In the 220 nm excited spectra (data not shown) these same Trp modes are observed to decrease in intensity. From these observations, it is inferred that the HbS Trp residues experience stronger H-bonding and a more hydrophobic environment relative to HbA Trp residues. This results in a decrease in Raman intensity with 220 nm excitation but an intensity increase with 230 nm excitation. The frequency and intensity of the W7 mode, which are diagnostic of Trp environment, also reflect the increase in hydrophobicity.[21] The observed intensity at 1358 $cm^{-1}$ and the absence of a peak at 1340 $cm^{-1}$ in the

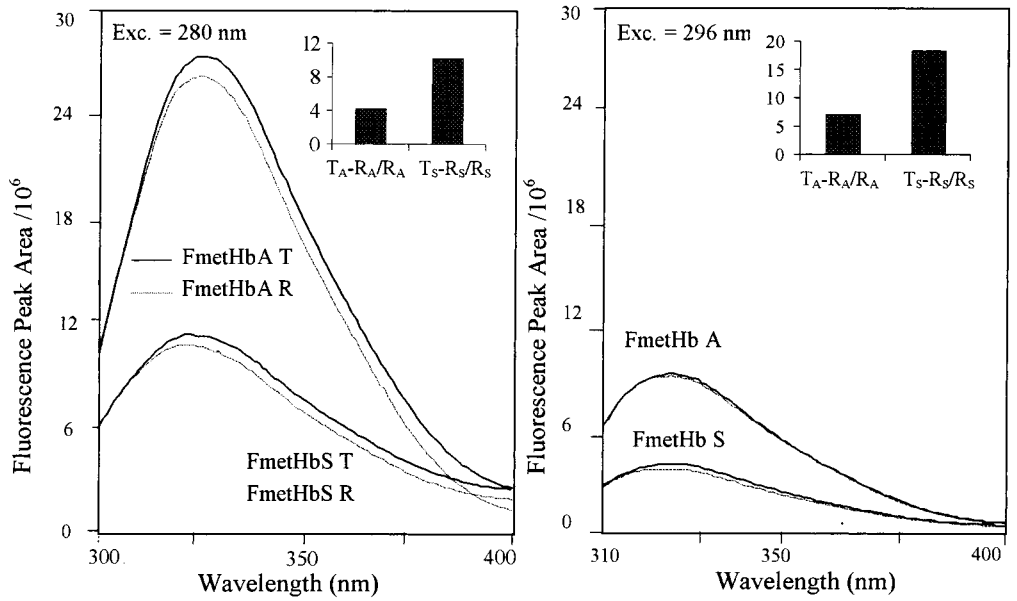


**Figure 4.** Comparison of the fluorescence intensities of FmetHbA in the T (—) and R state (- - -) with that of FmetHbS in the T (—) and R state (- - -). Excitation wavelength is 280 nm (left) and 296 nm (right). The inset shows the percent changes in relative peak area upon conversion from the R to the T state relative to the R state in FmetHbA and FmetHbS. The hemoglobin solutions were 0.155 mM/tetramer in 25 mM sodium phosphate buffer, pH 6.5. T state formation was induced with the addition of a 20-fold molar excess of IHP/tetramer.

difference spectra are strongly indicative that the Trp environment in FmetHbS is more hydrophobic than in FmetHbA.

The relative difference in the $\chi^{2,1}$ angle of the $\alpha$14, $\beta$15, and $\beta$37 Trp residues leads to heterogeneity in the W3 mode such that the $\beta$37 W3 mode occurs at 1550 $cm^{-1}$.[10,25] In the difference spectra (Figure 3 parts a and b) the W3 mode is observed at 1558 $cm^{-1}$ and therefore can be attributed to the interior Trp residues ($\alpha$14 and $\beta$15), which are not distinguishable by UVRR. We suggest that the intensity increase of the Trp modes in the 230 nm excited difference spectra results from the relative motion of the A-helix toward the interior of the protein. This leads to stronger H-bonds between the interior Trp residues and their partners on the E-helix ($\alpha$67 Thr/$\beta$72 Ser, respectively) and a more hydrophobic environment.

In Figure 3c, the quaternary difference spectra $(T-R)_S$ and $(T-R)_A$ generated with 230 nm excitation are compared. The resultant difference spectrum is relatively featureless, which is suggestive that the conformational changes induced by the allosteric transition are the same for FmetHbS and FmetHbA. A featureless difference spectrum is also observed if the 220 nm excited difference spectra are compared (data not shown). This implies that the change in the Trp environment detected for FmetHbS occurs to approximately the same magnitude within each quaternary state, as shown by the similarity of the $T_S-T_A$ (Figure 3a) and $R_S-R_A$ (Figure 3b) difference spectra, and that this perturbation does not affect the allosteric transition.

**Fluorescence Spectroscopy.** The fluorescence emission of FmetHbA and FmetHbS was monitored with and without IHP using the excitation wavelengths 280 nm (Figure 4, left) and 296 nm (Figure 4, right). Excitation at 280 nm reflects contributions from both Tyr and Trp residues, whereas 296 nm excitation is used to distinguish the contribution of the Trp residues alone without any fluorescence energy transfer from the Tyr residues. We employ 280 nm excitation because the signal is 3 times more intense and small changes in fluorescence intensity can be more reliably determined. At both excitation wavelengths, the fluorescence intensity of FmetHbS is substantially diminished relative to that of FmetHbA in the T and the R states (Figure 4). When 280 nm excitation is used, FmetHbS

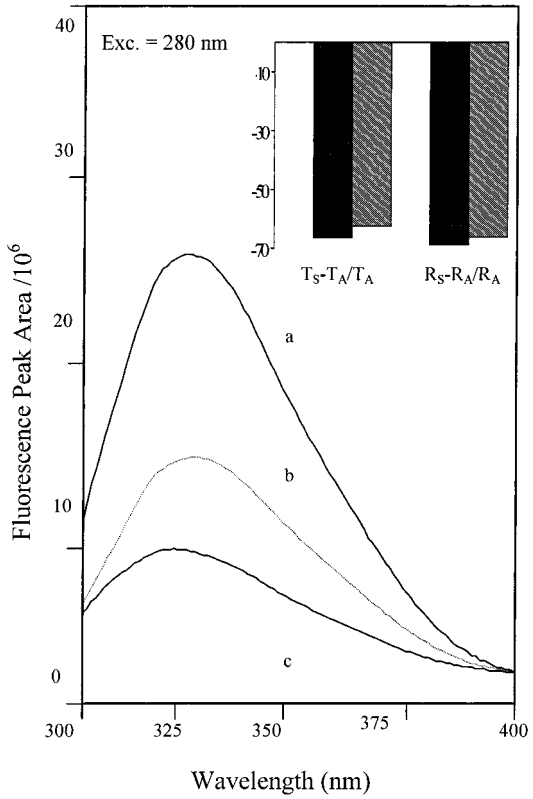


**Figure 5.** Comparison of the fluorescence intensities of FmetHbA and FmetHbS in the R state. 280 nm excited fluorescence spectra of (a) FmetHbA and (c) FmetHbS and (b) the FmetHbA − FmetHbS difference spectrum. The inset shows the percent change in the fluorescence peak area of FmetHbS relative to FmetHbA for both the T and R states. The results from 280 nm (black) and 296 (grey) nm excited spectra are compared in the inset. Experimental conditions are the same as those in Figure 4.

exhibits a 68.6% decrease in fluorescence intensity relative to FmetHbA in the R state and a 66.4% decrease in fluorescence intensity for the T state (Figure 5). The two types of Hb were maintained at the same concentration, and the absorbance at

**TABLE 1: Change in Fluorescence Peak Area between FmetHbA and FmetHbS in the T and R States**

| peak areas | 280 nm excitation[a] Δ (peak area) | 296 nm excitation[b] Δ (peak area) |
|---|---|---|
| $T_A-R_A/R_A$[c] | 0.042 ± 0.009 | 0.071 ± 0.024 |
| $T_S-R_S/R_S$[d] | 0.102 ± 0.013 | 0.185 ± 0.024 |
| $R_S-R_A/R_A$ | −0.686 ± 0.007 | −0.661 ± 0.004 |
| $T_S-T_A/T_A$ | −0.664 ± 0.004 | −0.625 ± 0.012 |

[a] Integration of peak areas was performed from 300−400 nm. [b] Integration of peak areas was performed from 310−400 nm. [c] $T_A$ = FmetHbA with IHP; $R_A$ = FmetHbA. [d] $T_S$ = FmetHbS with IHP; $R_S$ = FmetHbS.

280 nm was the same; therefore, the observed intensity change results from differences in the proteins themselves and not from any differences in protein concentration or absorbance. Similar decreases in fluorescence intensity are observed with 296 nm excitation, 66.1% for the R state and 62.5% for the T state (data not shown). The differences in fluorescence intensity are comparable at the two excitation wavelengths, indicating that the differences arise from the Trp residues themselves and not from any changes in the Tyr → Trp energy transfer pathway.

The change in fluorescence intensity upon conversion from the R to the T state was also examined. For both FmetHbA and FmetHbS, the T-state emission is more intense relative to that of the R state. When 280 nm excitation is used, FmetHbA with IHP (T state) exhibits a 4.2% increase in intensity relative to FmetHbA without IHP (R state) and for FmetHbS an increase of 10.2% is observed in the T state (Figure 4). When Trp residues are selectively excited, the fluorescence intensity in the T state is increased by 7.1% in FmetHbA, while for FmetHbS the fluorescence intensity is increased by 18.5% (Figure 4). The increase in fluorescence intensity is primarily attributed to the $\beta$37 Trp residue,[14,26] which forms an H-bond across the $\alpha_1\beta_2$ interface in the T state. At both excitation wavelengths, the increase in intensity upon forming the T state is greater for FmetHbS than for FmetHbA. However, these T−R differences are small (∼10%) relative to the dramatic difference in fluorescence intensity observed when the same quaternary state of HbA and HbS are compared (∼65%) (Table 1). Thus, the fluorescence data are in good agreement with UV Raman data in which large differences are observed between the tertiary structures of HbS and HbA but not between the quaternary structures.

Trp fluorescence intensity in different hemoglobins is known to be sensitive to ligation state and to the presence of any mutations.[14,15,26] In previous fluorescence studies, it was observed that deoxy HbA exhibits a 22% increase in fluorescence intensity relative to oxy HbA; however, metHbA with IHP exhibited a 5% decrease in fluorescence intensity relative to metHbA without IHP.[14] The current study indicates that the fluorescence behavior of the FmetHb derivatives resembles that of deoxyHb more than that of metHb and further verifies that FmetHbS represents a good functional analogue of HbS.

## Discussion

**Quaternary Structural Changes of FmetHbS and FmetHbA.** Excitation wavelengths of 230 and 220 nm have been used to probe the quaternary structural changes of FmetHbA and FmetHbS using UVRR spectroscopy. The T−R difference spectra of FmetHbA and FmetHbS exhibit spectral features characteristic of the R to T transition[10,11] and are strikingly similar to previously reported T−R UVRR spectra of FmetHbA.[11,20] The observed difference features were previously assigned to the formation of important intersubunit H-bonds between the $\beta$37 Trp···$\alpha$94 Asp and $\alpha$42 Tyr···$\beta$99Asp residues.[10] The similarities of the difference spectra imply that the same quaternary contacts are being formed in FmetHbA and FmetHbS. Moreover, the absence of any spectral features in the difference spectrum generated between the FmetHbA T−R and FmetHbS T−R difference spectra is suggestive that the strength of the intersubunit hydrogen bonds is approximately the same for both hemoglobins.

The change in fluorescence intensity between the T and R states for FmetHbS and FmetHbA is approximately the same and is consistent with UVRR spectral observations. The majority of the fluorescence observed in Hb has been previously assigned to the $\beta$37 Trp residue,[14,26] and the difference in fluorescence intensity between the T and R states has been attributed to changes occurring at that residue. The lack of any significant differences in the comparison of HbA and HbS T−R fluorescence and UVRR spectra is indicative that the quaternary structural contacts at the $\alpha_1\beta_2$ interface are the same. This finding is consistent with the X-ray crystal structure determinations of HbS,[2,8,27] which found no large conformational difference between HbS and HbA. It is also in good agreement with previous NMR studies that found the $\alpha_1\beta_1$ and $\alpha_1\beta_2$ subunit interfaces to be the same in HbA and HbS, as inferred from the chemical shifts of surface-exposed His residues.[28] Thus, the quaternary structures of HbS and HbA, as investigated by UVRR and fluorescence spectroscopy, are largely similar, and the similarities in quaternary structure are retained in the fluoromet derivative.

**Tertiary Structural Changes of FmetHbS and FmetHbA.** The tertiary structures of FmetHbS and FmetHbA were examined by comparing the UVRR and fluorescence T- and R-state spectra. The main features observed in the $T_S-T_A$ and $R_S-R_A$ difference spectra (Figure 3) result from the Trp residues ($\alpha$14, $\beta$15, and $\beta$37) in the protein and can be assigned to the Trp $\alpha$14 and $\beta$15 residues because of the frequency of the W3 mode. In the UVRR difference spectra ($T_S-T_A$ or $R_S-R_A$), the W3 mode occurs at 1558 $cm^{-1}$ with no shoulder at 1550 $cm^{-1}$, indicating that the origin of the Raman signal is the Trp $\alpha$14 and $\beta$15 residues, which are not distinguishable by Raman spectroscopy. These residues stabilize the tertiary structure of the individual subunits by forming H-bonds with the Thr $\alpha$67 and Ser $\beta$72 residues located in the E-helix. The increase in intensity of Trp modes in FmetHbS is consistent with an alteration of tertiary structure within both quaternary states and is attributed to stronger H-bonding in HbS, possibly resulting from closer proximity of the A- and E-helices. We suggest that Trp $\beta$15 is responsible for this difference because of its location in the same helix as the $\beta$6 (Glu → Val) mutation.

A large decrease in fluorescence intensity is observed in HbS T- and R-state spectra relative to those of HbA (see Table 1). The dramatic change in fluorescence intensity observed could result from altered Tyr → Trp and Trp → heme energy-transfer pathways. The fact that approximately the same change in fluorescence intensity is observed with both the 280 and 296 nm excitation wavelength demonstrates that the difference arises from only the Trp residues. The source of the decreased fluorescence intensity could be altered energy-transfer pathways between Trp residues and the heme; however, the similarity of the heme environments of HbA and HbS as determined by X-ray crystallography argues against this interpretation.[8] We cannot rule out the possibility that the presence of the fluoride ligand could perturb the local heme environment to a greater extent in HbS relative to HbA. Nevertheless, the decrease in fluorescence intensity, in conjunction with the UVRR results, is strongly

indicative that the spectral differences arise in part from a change in environment and H-bonding of Trp residues. The frequency of the W3 mode in the $T_S-T_A$ and $R_S-R_A$ UVRR difference spectra further allows these differences to be unambiguously assigned to the α14 and $\beta$15 Trp residues and by inference to the $\beta$15 residue.

This residue assignment is well supported by the most recent X-ray crystal structure determination of HbS, which detected a displacement of A-helices toward the interior of the protein in donor $\beta_S$ subunits relative to HbA.[8] In a HbS tetramer $\beta$ subunits can either act as acceptor pockets for mutant Val residues or they can donate the mutant residue to the hydrophobic interaction. In the X-ray crystal structure the tertiary structures of the donor and acceptor $\beta_S$ subunits differ; in donor $\beta_S$ subunits, the A-helix is displaced toward the protein interior but not in acceptor $\beta_S$ or α subunits.[8] In the UVRR difference spectra, the relative contributions from the two types of $\beta$ subunits cannot be distinguished, as the intensity of the signal depends not only on number of residues contributing to the signal but also on the strength of the interaction. Nevertheless, the current spectroscopic results confirm the findings of the X-ray crystal structure determination and further extend them in terms of both the quaternary state and aggregation state of the protein. In this study, A-helix displacement is observed in the T and the R states and the extent of the displacement appears to be the same in both states. In addition, these studies directly show that the A-helix position is altered in individual, unassociated tetramers and does not result from the association of tetramers into the strands observed in the X-ray crystal structure.

Interestingly, in a previous study of HbC ($\beta$6 Glu → Lys) an increase in Trp fluorescence of approximately 10% relative to that of HbA was observed in the oxy state but not in the deoxy state.[15] The authors attributed this difference in spectral behavior to the $\beta$15 Trp because of the mutation at the $\beta$6 position. HbC differs from HbS in that it does not polymerize but shows a greater propensity toward crystallization under oxygenating conditions.[15] In oxy HbC, the increase in fluorescence intensity and the relative decrease in Raman intensity of Trp modes was attributed to a weakening of the $\beta$15 Trp···$\beta$72 Ser H-bond caused by a displacement of the A-helix away from the E-helix.[15] In HbS, the opposite effect is observed; the fluorescence intensity decreases and the Trp Raman intensity increases, indicating a strengthening of the $\beta$15 Trp···$\beta$72 Ser hydrogen bond. In addition, the fluorescence intensity change is more dramatic in HbS than in HbC relative to HbA (−68% vs +10% in the R state). Thus, we suggest that in HbS, in contrast to in HbC, the A-helix is displaced toward the interior of the protein and potentially is displaced to a greater extent. We note that the relative flexibility of the A-helix and its ability to be displaced toward the interior or the exterior of the protein appears to be an important factor in determining the ability of an Hb $\beta$6 mutant protein to aggregate into higher order structures. These results argue that not just the mutation but also the local structure of the $\beta$ subunits and particularly the A-helix is important for the polymerization process. Future experiments will focus on a study of the A-helix position in HbS fibers.

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